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Mutations in the *rpmBG* Operon of *Escherichia coli* That Affect Ribosome Assembly

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The *rpmBG* operon of *Escherichia coli* codes for ribosomal proteins L28 and L33. Two strains with mutations in the operon are AM81, whose ribosomes lack protein L28, and AM90, whose ribosomes are without protein L33. Neither strain showed major defects in ribosome assembly. However, when the mutations were transferred to other strains of *E. coli*, ribosome synthesis was greatly perturbed and precursor ribonucleoproteins accumulated. In the new backgrounds, the mutation in *rpmB* was complemented by synthesis of protein L28 from a plasmid; the *rpmG* mutation was not complemented by protein L33 because synthesis of protein L28 from the upstream *rpmB* gene was also greatly reduced. The results suggest that protein L33, in contrast to protein L28, has at best a minor role in ribosome assembly and function.

The work reported herein was done to resolve a paradox noted during a study (6) on ribosome assembly by strains of *Escherichia coli* with mutations in the *rpmBG* operon. This operon codes for two proteins, L28 and L33, from the larger (50S) ribosomal subunit. Reconstitution in vitro has provided a map showing the interdependence of ribosomal proteins in the assembly of this subunit. A general feature of the map is that in nearly all cases, including that of proteins L28 and L33, the L proteins specified by an operon interact with each other (11). A recent model shows these two proteins as near-neighbors in the 50S subunit (23). These factors suggest that proteins L28 and L33 may have related roles in ribosome synthesis and that strains of *E. coli* with mutations in the *rpmBG* operon should be defective in ribosome assembly.

One such strain is TP28, which makes proteins L28 and L33 at about half their normal rates (2) due to a single base change (G to A) in the *rpmB* Shine-Dalgarno sequence in the leader region of the mRNA (6). As a consequence, strain TP28 oversynthesizes RNA and accumulates an abnormal precursor to 50S ribosomal subunits. These precursor (47S) particles lack L28 and L33 and contain substoichiometric amounts of three or four other proteins. The 70S ribosomes of strain TP28 have a full complement of ribosomal proteins, including L28 and L33, but about half the peptidyltransferase activity of that of the 70S ribosomes from their parent strain and so synthesize proteins poorly (21). Abnormal assembly may generate ribosomes that are conformationally altered.

Other strains with defects in the *rpmBG* operon originated differently. Dabbs (7) isolated an erythromycin-dependent mutant, strain AM, and showed that the dependency was readily suppressed by spontaneous mutations that resulted in failure to make one protein (or sometimes two) from a set of about 15 ribosomal proteins. Among the erythromycin-independent revertants with proteins missing from ribosomes were strain AM81, without protein L28, strain AM90, without protein L33, and strain AM108, without either protein. Total cell proteins from strains AM90 and AM108 did not cross-react with antibodies raised against the missing proteins; strain AM81 was not tested (9). We (6) found that strain AM108 has the mobile element IS*1* inserted into the *rpmB* coding sequence, while strain AM90 has the element IS*3* inserted into *rpmG*. However, in both strains, ribosome synthesis was little different from normal, with no accumulation of ribosome precursors akin to the 47S particles of strain TP28. Thus, synthesis of proteins L28 and L33 at half-normal rates apparently unbalanced ribosome assembly much more than failure to make these proteins. One possibility (6) was that in strains AM90 and AM108, other defects inherited from strain AM had an effect on ribosome assembly. These defects include mutations conferring erythromycin dependence and resistance. When the *rpmBG* mutation in strain TP28 was transferred to other strains, the latter acquired a phenotype similar to that of strain TP28 (1, 3). Here, we describe experiments that put the defective *rpmBG* operons from strains AM81 and AM90 in other genetic backgrounds. The strains thus generated had different phenotypes from those of strains AM81 and AM90. Their properties are described.

MATERIALS AND METHODS

Strains and plasmids. The strains of *E. coli* used were the following: (i) JM107 [*endA1 thi gyrA96 hsdR17 relA1 supE44* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)]; (ii) CP78 (*thr-1 leuB6 his-65 argH46 thi-1 ara-13 gal-3 malA1 xyl-7 mtl-2 tonA2*; from B. Bachmann); (iii) BW322 (*relA1 rfa-210*::Tn*10*, *pyrE70 spoT1*? *thi-1*; from B. Bachmann); (iv) A19 (Hfr *relA1 spoT1 rna-19 metB1*; from B. Bachmann), the parent of the erythromycin-dependent strain AM; (v) AM81 and AM90 (7) (from E. R. Dabbs; revertants of strain AM to erythromycin independence); (vi) BM19, BM81, and BM90 (see Results), which have the wild-type *rpmBG* operon (from strain A19) or mutant alleles from strains AM81 and AM90 in the background of strain JM107; and (vii) TP28 (6).

Plasmids pKKL28 (5) and pKKL33 have the genes for proteins L28 and L33 (amplified by PCR), respectively, inserted between the *Eco*RI and *Pst*I sites of plasmid pKK223-3 (Pharmacia). The latter has a *tac* promoter positioned so that, in the derivatives, transcription of L28 (or L33) mRNA is induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in hosts (such as strain JM107) that are *lacI*^{q}. Translation is from a ribosome-binding site provided by the vector that is 10 nucleotides upstream from the translational start codon. Plasmid manipulations were done by standard procedures (20). Transformed strains were grown in M medium. Synthesis of protein L28 from pKKL28 was induced by 1 mM IPTG. Less IPTG (0.03 mM) was required for an equivalent level of synthesis (estimated visually on gels) of protein L33 from plasmid pKKL33. Synthesis of L33 but not L28 could be detected in the absence of inducer. These facets were not explored but may reflect different stabilities of the two mRNAs.

Plasmids for sequencing were prepared with a QIAprep Spin Plasmid kit (from Qiagen). PCR products were excised from agarose gels and purified with a Quiex II extraction kit. Sequencing reactions were performed with Sequenase version 2.0 (U.S. Biochemical Corp.).

Media. D broth contained, per liter, 10 g of tryptone, 10 g of yeast extract, 5 g of glucose, 3 g of KH_2PO_4, 7 g of K_2HPO_4, 500 mg of trisodium citrate · $3H_2O$, and 200 mg of $MgSO_4 \cdot 7H_2O$. L broth (used for transductions) contained, per

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liter, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 g of glucose, and 30 mg of L-cysteine · HCl. M medium had the inorganic constituents of D broth plus, per liter, 2 g of glucose, 1 g of $(NH_4)_2SO_4$, 40 mg each of 19 L-amino acids (the set of 20 without proline), 8 mg of adenine, 8 mg of uracil, and 4 ml of a vitamin supplement. The latter contained, per liter, 320 mg of nicotinamide, 160 mg of Na pantothenate, 160 mg of thiamine, 32 mg of biotin, 32 mg of cyanocobalamin, and 0.3 mg of folic acid. Cultures were usually inoculated to an A_{450} of ca. 0.05. Media were supplemented, as appropriate, with 50 μg of ampicillin ml^{-1} or 20 μg of tetracycline ml^{-1} and solidified with 2% agar.

Transductions. Transductions used a clear-plaque mutant of bacteriophage P1 and were done as described previously (3).

Preparation of extracts and density gradient centrifugation. Bacteria were usually grown to an A_{450} of ca. 0.5 in 10 to 50 ml of D broth, sometimes with [^{14}C]uracil (0.1 μCi ml^{-1}) and/or [^{3}H]uracil (1.0 μCi ml^{-1}) added. Cultures were harvested, washed with 0.5 volume of THM buffer (10 mM Tris-HCl [pH 7.4], 10 mM magnesium acetate), resuspended in 1 ml of THMKSp buffer (THM buffer containing 100 mM KCl and 1 mM spermidine-HCl), and broken in a French pressure cell. Portions of extracts (usually 100 μl) were layered onto 15 to 30% sucrose gradients made in THMKSp buffer and centrifuged at 4°C, usually for 2.5 h at 50,000 rpm in a Beckman SW 55Ti rotor. Gradients were collected through an Isco density gradient fractionator. For one experiment, RNA was phenol-extracted from gradient fractions as described previously (17).

Electrophoresis of ribosomal proteins. For labelling during continuous growth, organisms were grown for two generations to an A_{450} of 0.5 in M medium with the lysine concentration reduced to 5 μg ml^{-1} and containing either 0.5 μCi of [^{3}H]lysine ml^{-1} or 0.05 μCi of [^{14}C]lysine ml^{-1}. For pulse-chase experiments, carrier-free [^{3}H]lysine (0.5 μCi ml^{-1}) was added at an A_{450} of 0.4 to cultures growing exponentially in M medium without lysine; after times given in the text, the addition of nonradioactive lysine (final concentration, 1 mg ml^{-1}) followed. Samples (10 ml) taken at intervals were harvested onto 10 g of crushed ice had washed in TMKSH buffer (10 mM Tris-HCl [pH 7.4], 15 mM magnesium acetate, 60 mM KCl, and 7 mM β-mercaptoethanol). A sample, resuspended in 1 ml of TMKSH buffer, was then mixed with one from 10 ml of a reference culture which had been labelled for two generations with 0.1 μCi of [^{14}C]lysine ml^{-1}. The mixed cells were broken, and carrier (nonradioactive) ribosomes from strain BM19 were added before proteins were extracted with acetic acid as described previously (2). Protein samples were analyzed by two-dimensional polyacrylamide gel electrophoresis in a system that combined the basic first dimension described by Madjar et al. (15) with the second dimension described by Kenny et al. (13). This combination was the best found for resolving proteins L28 and L33 from their neighbors. The radioactivities in protein spots were measured by two-channel counting, and their ratios were normalized (10); for protein *i*, the ratio *Ai* equals (^{3}H/^{14}C in protein *i*) divided by (^{3}H/^{14}C in total ribosomal protein).

For N-terminal sequencing of protein L28* (see Results), a gel was electroblotted onto a ProBlott membrane in a Bio-Rad Trans-Blot electrophoretic transfer cell. The spot in the position of L28* was excised for sequencing for 20 cycles on an Applied Biosystems 494A Procise protein sequencer.

RESULTS

Phenotypes of strains AM81 and AM90. Strains AM81 and AM90 carry mutations in the *rpmBG* operon that suppress the erythromycin dependence of their parent strain, AM; the strains were resistant to erythromycin. In D broth at 37°C, typical mean generation times were 65 min for strain AM81, 60 min for strain AM90, and 20 min for strain A19 (the parent of strain AM). The strains were grown in D broth, and extracts were centrifuged through sucrose gradients. The sedimentation profile of strain A19 (Fig. 1a) shows that native 30S and 50S ribosomal subunits were present in small quantities relative to 70S ribosomes; the profiles from strains AM81 and AM90 (Fig. 1b and c) are similar to that of strain A19, although, as expected for slow-growing strains, the proportion of ribosomal subunits was somewhat increased. By contrast, the profile from strain TP28 (Fig. 1d) shows three ribonucleoprotein components present in similar amounts. One is a mixture of 30S ribosomal subunits and an unresolved precursor to them (17), the second comprises 47S particles (see the introduction), and the third comprises 70S ribosomes. The areas under the peaks are characteristic of this mutant and, because 47S particles sediment more slowly than 50S subunits, the spacings are rather different from that in, for example, strain A19.

Transfer of *rpmBG* mutations to other backgrounds. The transfer of the mutations in the *rpmBG* operons of strains

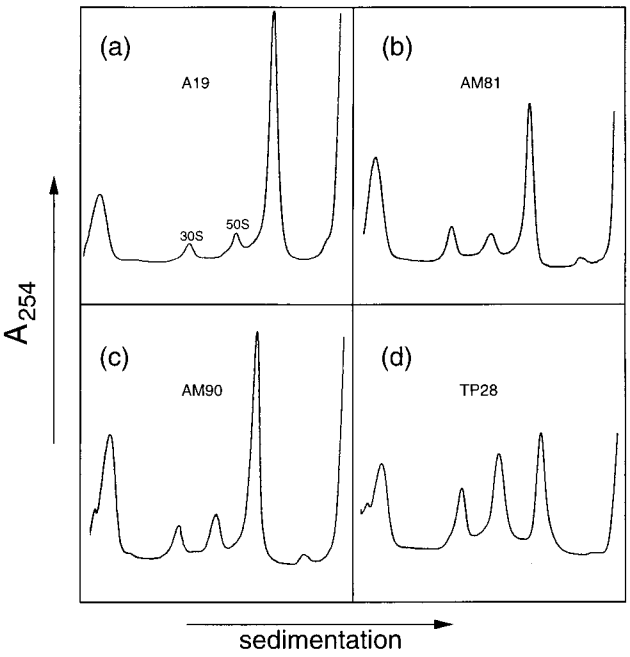

FIG. 1. Sedimentation profiles of ribosomal particles from wild-type and mutant strains. Extracts were from strains A19 (a), AM81 (b), AM90 (c), and TP28 (d).

AM81 and AM90 to other strains was attempted. For this, *rfa pyrE* derivatives of strains CP78 and JM107 were made by growth of bacteriophage P1 on strain BW322 (*rfa*::Tn*10 pyrE*) followed by transductions that selected tetracycline resistance. *rfa* and *pyrE* were 20 to 40% linked and are on opposite sides of *rpmBG*. Bacteriophage P1 grown on strain AM81 (or AM90) was then used to infect typical *rfa pyrE* derivatives of strains CP78 and JM107. We reasoned that most transductants that were PyrE^{+} (the marker selected for on M agar without uracil) and tetracycline sensitive (assessed on L agar) were likely also to have inherited the ribosome mutation between the markers. Moreover, if the latter affected ribosome assembly in these new backgrounds and so slowed growth, transductants with the mutation would form small colonies.

With bacteriophage grown on strains AM81 and AM90, these expectations were realized (Table 1). After 2 days, PyrE^{+} transductants were of two colony sizes, large (L; about 3 mm in diameter) and small (s; about 1 mm in diameter). This distinction was maintained when colonies were restreaked onto either solidified D broth or M medium. With bacteriophage P1 grown on strain AM81, 47% of transductants of strain CP78 were tetracycline sensitive as were 62% of transductants of strain JM107. Nearly all (92% for strain CP78, 97% for strain JM107) tetracycline-sensitive transductants grew slowly. Thus, s can be treated as an allelic marker which is 68% (strain CP78) or 67% (strain JM107) linked to *pyrE*. These values are consistent with a gene order *rfa*::Tn*10 s*/*L pyrE* and with the transfer of the *rpmBG* mutation (3). Results with bacteriophage P1 grown on strain AM90 were similar (Table 1); nearly all (93% in strain CP78) or all (strain JM107) tetracycline-sensitive transductants gave small colonies. The small variants from transductions with bacteriophage grown on strain AM90 were more prone to throw off faster-growing variants than those from strain AM81. In all transductions, tetracycline-resistant small colonies grew slightly less well than tetracycline-sensitive small colonies. The

TABLE 1. Transduction of ribosome mutations[a]

Recipient strain	Donor strain	No. of recombinants that were:				Total no. of recombinants	% Cotransduction	
		L Tet^r	L Tet^s	s Tet^r	s Tet^s		Tet^s recombinants	s recombinants
CP78 *rfa*::Tn*10 pyrE*	A19	38	27	0	0	65	41	0
	AM81	30	4	27	46	107	47	68
	AM90	74	6	29	74	183	44	56
JM107 *rfa*::Tn*10 pyrE*	A19	36	21	0	0	57	37	0
	AM81	32	2	7	62	103	62	67
	AM90	50	0	15	132	197	67	75

[a] PyrE$^+$ was the selected marker. L (large) and s (small) refer to colony sizes, as explained in the text.

distinction between small and large was nevertheless always clear.

Sedimentation profiles of transductants. A selection of transductants was studied further. Six PyrE$^+$ derivatives (one L Tet^s, one L Tet^r, two s Tet^s, and two s Tet^r) from the transduction with strain AM81 as the donor and JM107 *rfa pyrE* as the recipient were grown in D broth. Extracts were made and centrifuged through sucrose gradients. The L variants had mean generation times of 28 min (Tet^s) and 32 min (Tet^r); their sedimentation profiles were very similar to that of strain A19 (Fig. 1a). As expected, s variants grew more slowly. Mean generation times were 64 and 68 min for Tet^s transductants and 72 and 86 min for Tet^r transductants. The four sedimentation profiles were similar to each other and to that from strain TP28 (Fig. 1d), with few 70S ribosomes and a major component sedimenting in the approximate position of 47S particles. Examples are shown in Fig. 2a and b. A similar set of PyrE$^+$ isolates was chosen from the transduction with strain AM90 as the donor and JM107 *rfa pyrE* as the recipient. One L Tet^r variant had a generation time of 26 min and gave a wild-type sedimentation profile (data not shown). Three s Tet^s isolates grew slowly (mean generation times, ca. 105 min), and two Tet^r isolates grew more slowly still (ca. 115 min). The five sedimentation profiles were similar to each other (examples are shown in Fig. 2c and d) to those of s isolates from the cross involving strain AM81, and to the profile given by strain TP28.

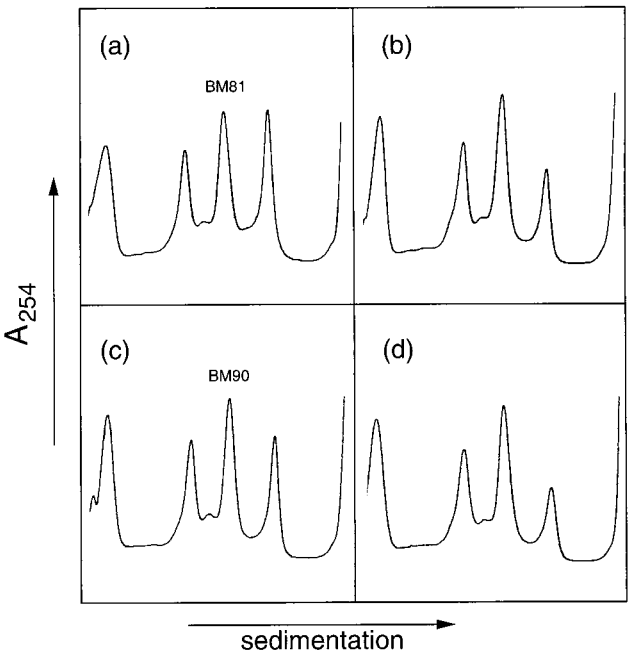

FIG. 2. Sedimentation profiles of ribosomal particles from transductants of strain JM107 *rfa pyrE*. Extracts were from an s Tet^s variant (strain BM81) with strain AM81 as the donor (a), an s Tet^r variant with strain AM81 as the donor (b), an s Tet^s variant (strain BM90) with strain AM90 as the donor (c), and an s Tet^r variant with strain AM90 as donor (d).

A similar selection of transductants derived from strain CP78 *rfa pyrE* was screened. The results were the same as those described above. L isolates grew quickly and gave normal sedimentation profiles; s isolates grew more slowly and, irrespective of tetracycline resistance or sensitivity, accumulated ribonucleoproteins as shown in Fig. 2. Derivatives of strain JM107 had some practical advantages for future work. Two that were selected were strains BM81 and BM90; these were PyrE$^+$ s Tet^s clones from transductions with bacteriophage grown on strains AM81 and AM90, respectively, as the donor. (Their sedimentation profiles are shown in Fig. 2a and c.) A variety of their properties were compared either with those of strain JM107 or with those of a PyrE$^+$ Tet^s strain (BM19) chosen from the progeny of a transduction of JM107 *rfa pyrE* with bacteriophage grown on strain A19. No differences between strains JM107 and BM19 were noted with respect to the properties described below.

The 47S particles of strains BM81 and BM90. Sedimentation profiles suggested that strains BM81 and BM90 accumulated material with a sedimentation coefficient somewhat lower than that of native 50S ribosomal subunits. Confirmation of this was sought. Strains JM107 and BM81 were grown in D broth with, respectively, [^{14}C]uracil or [^{3}H]uracil added. Mixed extracts were sedimented through a sucrose gradient. The profile (Fig. 3a) showed that the 30S and 70S materials of the two strains cosedimented but that the ribonucleoprotein component in strain BM81 did not sediment as far as the 50S ribosomal subunits of strain JM107 with a sedimentation coefficient of ca. 47S. Mixed extracts from strains BM90 and JM107 were sedimented with the same result (Fig. 3b).

The 47S particles of strains BM81 and BM90 could be ribosome precursor particles (as in strain TP28) or ribosomal subunits with altered sedimentation properties. Extracts were sedimented through sucrose gradients, and fractions containing 47S particles and 30S material were pooled separately. RNA extracted from the pools was sedimented, with RNA from wild-type cells used as a reference. As in strain TP28 (17), the RNA from 47S particles was 23S rRNA while only 16S rRNA was extracted from 30S material. The metabolic status of the particles was then examined. For this, strain BM81 was labelled with both [^{3}H]uracil and [^{14}C]uracil during growth for four generations in D broth. Organisms collected by centrifugation were resuspended in D broth with only [^{3}H]uracil added (at the same specific radioactivity as that before resuspension). When growth resumed, there was no loss of ^{14}C radioactivity

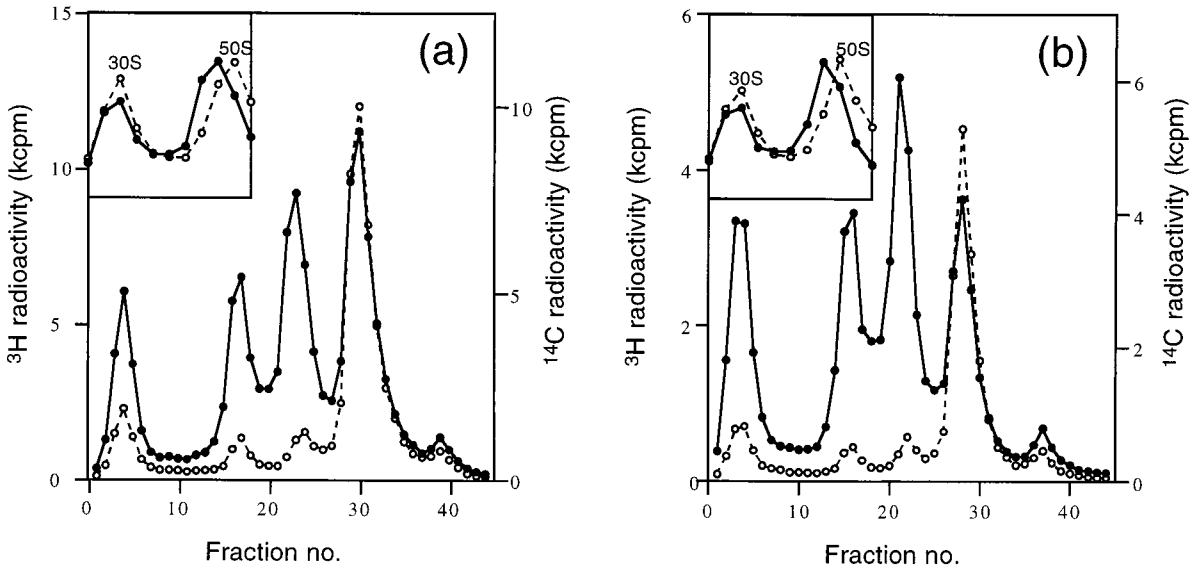

FIG. 3. Comparison of sedimentation profiles of strains JM107, BM81, and BM90. (a) Mixtures of extracts were prepared from strain JM107 grown in D broth with [^{14}C]uracil and from strain BM81 grown with [^{3}H]uracil. (b) The mixture was from strain JM107 grown with [^{14}C]uracil and from strain BM90 grown with [^{3}H]uracil. In the insets, the scales of radioactivity have been adjusted to emphasize the difference in sedimentation properties of 47S particles (strains BM81 and BM90) and 50S subunits (strain JM107). Symbols: ○, ^{14}C radioactivity; ●, ^{3}H radioactivity.

that was insoluble at 0°C in 5% trichloroacetic acid. Extracts made from samples taken at intervals were centrifuged through sucrose gradients. Immediately after resuspension of strain BM81, the profiles of ^{3}H- and ^{14}C-labelled material were identical. The ^{3}H profile did not change significantly during subsequent growth but there was a marked loss of ^{14}C-material from both 30S and 47S regions of the gradient and an increase in that of 70S ribosomes. This change was rapid and complete within about a generation (Fig. 4a). With strain BM90, the results were very similar (Fig. 4b). If the ^{14}C-material had been in native ribosomal subunits, these would have exchanged with the subunits of 70S ribosomes during continued growth and the distribution of radioactivity would not have altered. The rapid redistributions without loss of ^{14}C radioactivity suggest that the 47S (and 30S) material in strains BM81 and BM90 comprises ribosome precursor particles but do not completely preclude schemes in which the material is degraded and ribosomes are made from fragments.

Strain BM81. (i) The nature of the suppressor mutation. The type of mutation in strain BM81 (and AM81) was established by isolation and amplification of *rpmB* DNA, followed by sequencing of both strands. Sequences from the two strains were the same. Comparison with wild types (strains A19 and BM19) showed that strain BM81 had a single G deleted from the 63rd codon of the L28 sequence (Fig. 5). A predicted consequence of this frameshift is translation beyond the normal stop codon and termination at the second and third codons within *rpmG* to give a protein 9 amino acids longer than L28 (molecular weight of 9,914 rather than 8,857) but with a similar isoelectric point.

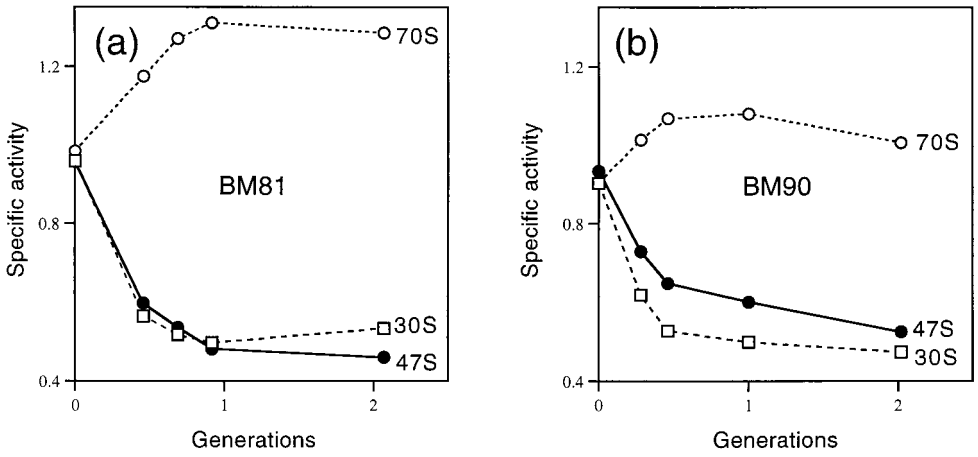

FIG. 4. Metabolic fate of ribonucleoproteins in strains BM81 (a) and BM90 (b). Strain BM81 or BM90 was grown from an A_{450} of 0.025 to 0.4 in D broth containing [^{3}H]uracil and [^{14}C]uracil and then harvested, washed, and resuspended in 4 volumes of D broth with only [^{3}H]uracil added. Samples taken at intervals were sedimented through sucrose gradients, and fractions were collected for precipitation in 5% trichloroacetic acid and measurement (6) of specific activity of ribonucleoproteins (^{14}C/^{3}H ratio in the peak for each component divided by ^{14}C/^{3}H ratio in the tRNA at the top of gradients).

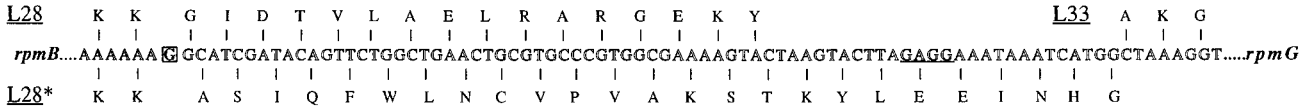

FIG. 5. Frameshift mutation in strain BM81. The sequence change is shown, and the potential consequences are indicated. The Shine-Dalgarno sequence for protein L33 is underlined.

An altered L28 (termed L28*) in strain BM81 was therefore sought. Proteins were extracted from strain BM81 and electrophoresed in two-dimensional gels to display ribosomal proteins. After staining, protein L28 was not visible but there was a faint extra spot in a position consistent with the higher molecular weight of L28*. This anomalous spot was more clearly visible (Fig. 6a) when proteins from 70S ribosomes were displayed; protein L28 was again absent. Subsequently, proteins from 70S ribosomes were electrophoresed. Protein L28* was transferred by electroblotting, and the first 20 amino acids at the N-terminal end of the protein were determined. The sequence was SRVCQVTGKRPVTGNRSHA. . .. This is identical to that of wild-type protein L28.

(ii) Complementation. Plasmid pKKL28 was transformed into strain BM81 with selection for ampicillin resistance. A typical clone was grown in M medium. This medium lacks proline to help maintain the sex factor (with *lacI*^{q}) in strain JM107. In medium without IPTG, the mean generation time of the transformant was 70 min. With IPTG, growth was faster (mean generation time, 42 min); induction of synthesis of L28 also changed the sedimentation profile from one showing accumulation of 30S material and 47S particles (Fig. 7a) to one characteristic of wild-type organisms in this medium (Fig. 7b). The mean generation time of strain BM19 in M medium was also 42 min; growth was not affected by transformation with pKKL28 and induction of L28 synthesis.

Proteins were extracted from 70S ribosomes of strain BM81 that had been transformed with pKKL28 and induced with IPTG. After electrophoresis and staining, protein L28 was present but L28* was not visible (Fig. 6b). A likely reason is that wild-type protein L28 from the plasmid outcompeted protein L28* for incorporation into ribosomes; this is discussed further below.

Synthesis of protein L28 from plasmid pKKL28 was measured in transformants of strain BM19 and BM81. Cultures were grown for two generations in M medium with [^{3}H]lysine added and in the absence and presence of IPTG. The cells were mixed with those from (untransformed) strain BM19 grown similarly but with [^{14}C]lysine. Proteins were then extracted from the mixtures. After electrophoresis, ribosomal protein spots were excised and the ratios of radioactivities in them were converted to *Ai* values which compare the molar ratios of proteins in the two strains (see Materials and Methods). (Protein L28* could not be quantitated because it is absent from the reference strain, BM19). Protein L28 had an *Ai* value of 1.3 in transformed strain BM81 induced with IPTG; without IPTG, the value (0.3) was at background levels. In both induced and uninduced organisms, *Ai* values for other ribosomal proteins (including L33) were close to unity (Fig. 8a and b). In the transformant of strain BM19, the molar ratios of

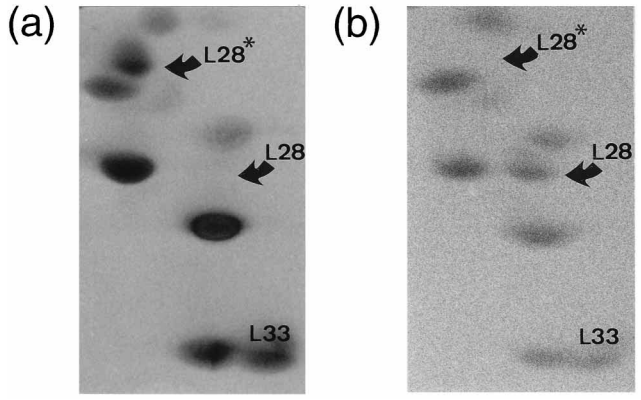

FIG. 6. Two-dimensional electrophoresis of proteins from the 70S ribosomes of strain BM81 (a) and strain BM81 transformed with plasmid pKKL28 and grown with IPTG present (b). The significant portions of the gels are shown.

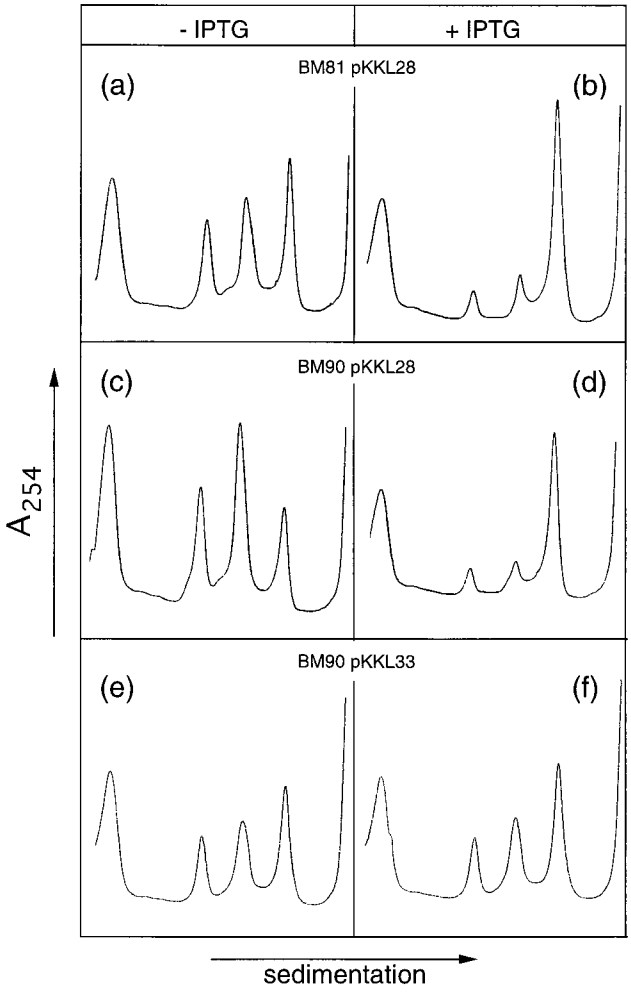

FIG. 7. Strains BM81 transformed with plasmid pKKL28 (a and b), BM90 transformed with plasmid pKKL28 (c and d), and BM90 transformed with plasmid pKKL33 (e and f) were grown for four generations in portions of M medium without (a, c, and e) and with (b, d, and f) IPTG. Extracts were centrifuged through sucrose gradients.

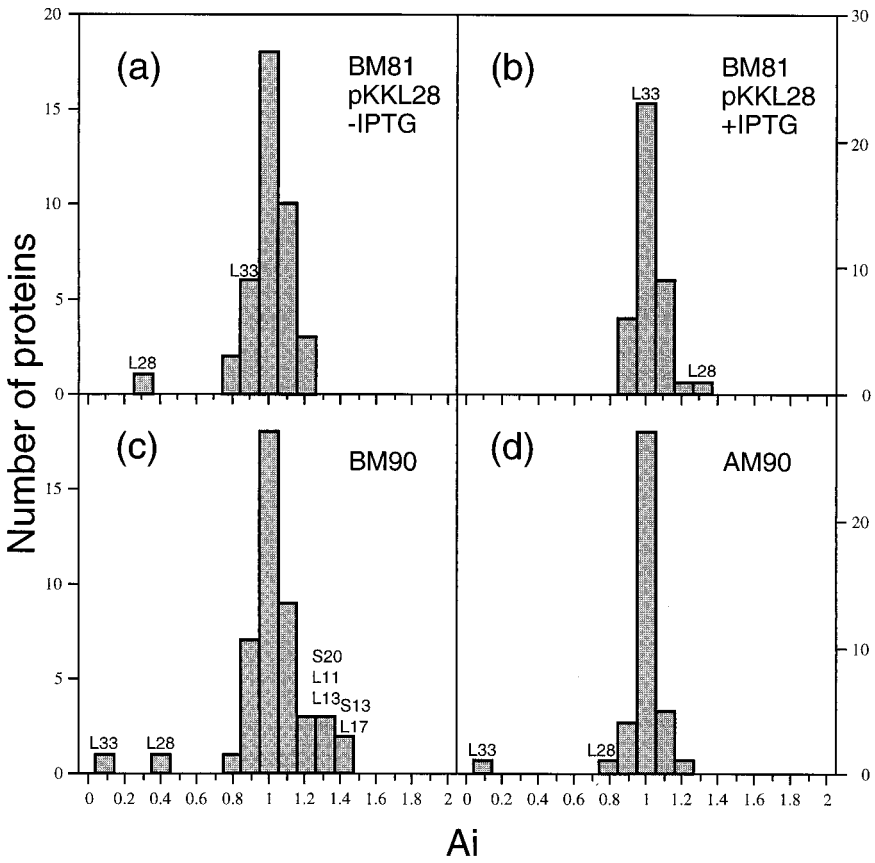

FIG. 8. Ribosomal protein contents. The histograms show *Ai* values for the ribosomal proteins from strain BM81 with plasmid pKKL28 grown without IPTG (a), strain BM81 with plasmid pKKL28, induced with IPTG (b), strain BM90 (c), and strain AM90 (d).

all ribosomal proteins were close to unity except that the *Ai* value for protein L28 was 1.70 after induction. Induced synthesis of L28 from pKKL28 thus contributed close to a molar equivalent of the protein in both derivatives.

Strain BM90. (i) Complementation. Strain BM90 was transformed with plasmid pKKL33, with selection for ampicillin resistance. A typical clone grew slowly, with a mean generation time of about 130 min; the sedimentation profile showed accumulation of 30S material and 47S particles. Growth with IPTG present was still slow (mean generation time, 110 min), and the profile was very similar to that without inducer (Fig. 7e and f). Strain BM90 was also transformed with plasmid pKKL28. A typical transformant grew slowly (mean generation time, 140 min) and had a sedimentation profile similar to that of strain BM90. Surprisingly, however, when synthesis of protein L28 was induced, the culture now grew rapidly (mean generation time, 42 min) and extracts had sedimentation profiles similar to that of the wild type (Fig. 7c and d). Thus, provision of the protein (L33) presumed missing from strain BM90 was without significant effect on growth or ribosome assembly, whereas protein L28, presumed to be synthesized, restored the parental phenotype. This anomaly was resolved by examination of the ribosomal proteins in strain BM90.

(ii) Ribosomal proteins in strain BM90. Proteins extracted from strain BM90 were electrophoresed to display ribosomal proteins. After staining, no protein L33 was visible, as expected. However, although protein L28 was present, staining was less intense than that in equivalent experiments with strain AM90, A19, or BM19. This suspected deficiency of protein L28 was quantitated by mixing cells of strain BM90 grown for two generations in M medium with [^{3}H]lysine added with those from strain BM19 grown similarly but with [^{14}C]lysine. Proteins were extracted for electrophoresis. Four experiments, averaged (Fig. 8c), gave a background *Ai* value of 0.1 for protein L33 and one of only 0.4 for protein L28. The latter was therefore also significantly underrepresented. The *Ai* values for most other proteins were within the normal range (0.8 to 1.2), although values for five proteins (S13, S20, L11, L13, and L17) were higher. Similar experiments were done with strain AM90, which carries the same ribosome mutation as that of strain BM90 (Fig. 8d). Protein L33 was again absent. The synthesis of ribosomal proteins was better balanced in AM90 than in strain BM90 in that more proteins had *Ai* values within the normal range. The value (0.8) for protein L28 was double that in strain BM90; this confirmed previous measurements (6).

(iii) Synthesis and stability of protein L28. The low content of protein L28 in strain BM90 could be the result of L28 being undersynthesized and/or unstable in the absence of protein L33. These possibilities were investigated. Strain BM90 growing in M medium was pulse-labelled with carrier-free [^{3}H]lysine for 2 min and then chased for 1 min with excess nonradioactive lysine. A sample was then taken, and other samples were taken at 20, 50, and 100 min; each was mixed with a second culture of strain BM90 labelled with [^{14}C]lysine during growth for two generations. Proteins were extracted from the mixtures for electrophoresis. The *Ai* values in the first (3-min) sample show rates of synthesis of individual proteins relative to

those of bulk ribosomal proteins; later values follow the stability of the newly made proteins. Because the comparison was between cultures of strain BM90 with similarly low contents of protein L28, the *Ai* value for this protein after a long chase should be close to 1.0. For protein L28, the *Ai* values were 1.05 (at 3 min), 1.2 (20 min), 1.1 (50 min), and 1.1 (100 min). These values showed that the protein was stable and gave no evidence for more rapid initial synthesis than was indicated by steady-state levels. In another experiment, protein L28 made during a shorter (30-s) pulse was stable during a subsequent chase of up to 4 min.

The experiments described above showed that protein L28 was present in 1.5- to 2-fold excess in strain BM19 transformed with pKKL28 and induced with IPTG. Such a culture was pulse-labelled for 1 min and then chased with excess lysine. The ^{3}H-proteins were extracted and mixed with those from strain BM19 grown for 2 generations with [^{14}C]lysine. In the sample taken at 1.5 min, protein L28 had an *Ai* value of 1.65. Subsequent values were 1.5 (at 2.5 min), 1.55 (5 min), and 1.5 (30 min). Thus, excess protein L28 was stable. In the four samples, the *Ai* values for protein L33 were 1.15, 1.2, 1.1, and 1.1, respectively. Synthesis (and stability) of protein L33 was thus not affected by oversynthesis of L28 from the plasmid.

DISCUSSION

The mutations in the *rpmBG* operon that suppress erythromycin dependence give different phenotypes in different backgrounds. Strains AM81 and AM90 grow slowly. However, ribosome assembly is little affected. Ribosomal subunits are present in small quantities relative to 70S ribosomes; a more detailed examination of assembly in strain AM90 (6) showed only minor differences from strain A19. However, in the backgrounds of either strain JM107 or strain CP78, the phenotypes change very considerably. There is accumulation of 30S material and 47S particles that are largely converted into 70S ribosomes during continued growth.

We think that the derivatives of strains CP78 and JM107 show the normal consequences of the ribosome mutations that arose in strains AM81 and AM90. First, the phenotypes produced are very similar in the stringent strain CP78 and the relaxed strain JM107 (and in a third strain, HVT45.2 [3], to which the *rpmBG* mutations have also been transferred). Second, an at least superficially similar accumulation of ribosome precursors occurs in strain TP28; this accumulation persists when the mutation is transferred to other strains (1, 3). Third, the *rpmBG* mutations in strains AM81 and AM90 act to suppress the antibiotic dependence of strain AM and thus clearly interact with mutations carried by the latter. Strain AM is both erythromycin dependent and erythromycin resistant. The dependency results from a mutation in *rpoB* coding for the β subunit of RNA polymerase (16). The separate resistance mutation has not been mapped but is likely to be in *rplD* (the classic *eryA* locus) because a more acidic ribosomal protein L4 is present in strain AM (22). Some mutations in *rplD* affect ribosome assembly (4). Interactions between two mutations in *rplD* and *rpmBG* might well rectify defects in assembly caused by one. Similar considerations may also apply to other revertants of strain AM to erythromycin independence. Except for a mutation in *rplX* that results in the absence of protein L24 (12), the mutants show no obvious assembly defects (8).

A more subtle effect of genetic background on ribosome assembly is shown by strains AM90 and BM90. In strain AM90, the *rpmBG* mutation leads to loss of protein L33 and has little effect on the synthesis of protein L28. Strain BM90, with the same mutation, was complemented by plasmid-coded protein L28 because not only was protein L33 absent but protein L28 was substoichiometric, with an *Ai* value of 0.4. The results suggest that this was due to reduced synthesis rather than instability of newly made protein L28. Ribosomal proteins made in stoichiometric excess during unbalanced synthesis are often degraded (2, 6), but in strain BM19 carrying pKKL28, excess protein L28 is stable. The mutation in strains AM90 and BM90 that prevents synthesis of protein L33 is the insertion of the mobile element IS*3* into *rpmG*. The *rpmBG* mRNA is now likely to specify protein L28 and an unstable N-terminal fragment of protein L33 (6). Perhaps the 3′ end of the mRNA is now more accessible than usual to nucleolytic cleavage and then, by events similar to those described for retroregulation in the *spc* operon (18), subject to the action of nucleases. This effect might be less marked in the background of strain AM90.

Strain BM90, transformed with plasmid pKKL28 and induced to synthesize protein L28, has a sedimentation profile and mean generation time indistinguishable from those of the wild type. The ability to grow and assemble ribosomes normally in the absence of protein L33 suggests that L33, in contrast to protein L28, has only a minor role in ribosome assembly and function.

The mutation in strain AM81 (and so also in strain BM81) is a frameshift and therefore differs from those in strains AM90 and AM108, where insertion of IS*3* and IS*1* is responsible (6), and from that in the other sequenced example, strain AM109, where an ochre mutation stops synthesis of ribosomal protein L24 (19). The sequence from strain AM81 predicts synthesis of an altered protein L28 (L28∗) that is 9 amino acids longer than wild-type L28. Electrophoresis of the proteins from the 70S ribosomes of strain AM81 showed that L28 was missing; an unusual protein at the position expected for L28∗ had an N-terminal amino acid sequence identical to that of L28. Thus, L28∗ is made and incorporated into completed ribosomes. The impression imparted by stained gels (e.g., Fig. 6a) is of stoichiometric incorporation, but this was not measured directly.

Synthesis of the proteins of the *rpmBG* operon is translationally coupled (6) as is usual in ribosomal protein operons (14). Translation of L28∗ terminates at the second or third codon within *rpmG*, 14 nucleotides beyond the internal Shine-Dalgarno sequence. Initiation of translation of *rpmG* in strain AM81 may thus involve a 3′-to-5′ adjustment of the position of the ribosome. This seems not to affect the efficiency of coupling. In strain AM81, protein L33 has a normal stoichiometry; pulse-labelling experiments not reported here showed that rates of synthesis of L33 were similar in strains AM81 and BM19.

Since strain BM81 grows exponentially, ribosomes with L28∗ incorporated are at least partially functional. However, these ribosomes assemble with difficulty and with accumulation of precursor ribonucleoproteins. In strain TP28 (see the introduction), where rates of synthesis of proteins L28 and L33 are halved, ribosome assembly is perturbed and 47S particles that lack both L28 and L33 accumulate. Synthesis in strain AM81 may be similarly rerouted when L28 is replaced by a new form that binds less well during ribosome assembly. Preliminary measurements have shown that 47S particles from strain BM81 contain no L28 and little or no L28∗. Complementation by wild-type L28 restores the maximum growth rate and a normal ribosome content; L28∗ is no longer present in 70S ribosomes. These changes are most simply explained by an ability of wild-type L28 to participate in assembly more readily than L28∗. Another, less preferred, possibility is that protein L28 regulates the *rpmBG* operon by binding weakly within the leader region of the mRNA to inhibit translation. Wild-type L28, made from a plasmid, could then inhibit chromosomal synthe-

sis of L28*. However, protein L33 has a normal stoichiometry in whole cells irrespective of whether protein L28 is overproduced (synthesis induced from pKKL28 in strains BM19 and BM81), synthesized normally (strain BM19 with pKKL28, not induced), or altered (strain BM81 with pKKL28, not induced). If translational feedback regulates synthesis from the *rpmBG* operon, it is therefore unlikely to be by protein L28. A difficulty is that we previously argued (6) that the normal synthesis and stability of protein L28 in strain AM90, which lacks protein L33, make protein L33 also unlikely to be directly involved in regulation. The complexities of the phenotypes of strains AM90 and BM90 described above make this latter conclusion rather less certain. A possibility for which we have some (unpublished) evidence is that proteins L28 and L33 cooperate to repress translation; this is being explored.

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