Ribosomal assembly defective mutants of Escherichia coli

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SUMMARY

Two conditional mutants affected in a 30S ribosomal subunit component are described. One of them is cold sensitive and shows alterations in the assembly process of both ribosomal subunits at 20° C. The other one is thermosensitive and only the assembly of the 30S subunit is blocked at 42°.

INTRODUCTION

The genetic and biochemical analysis of bacterial ribosomal mutants have already provided helpful informations on the structure and function of ribosomes (1-4).

The study of ribosome assembly defective mutants has been particularly useful to confirm the stepwise ordered character of the assembly process (5, 6, 7). Many of these mutants are conditional on temperature to the extent that they cannot assemble complete subunits at 20°, while they grow normally at 42°. Moreover some of them are characterized by their inability to assemble both 30S and 50S subunits because of a defect in one 30S subunit protein (6).

In an attempt to isolate new ribosome assembly defective mutants, E.coli strains which carry a strAD allele and in which the dependence on streptomycin is supressed by an extragenic mutation. have been screened for sensitivity both to low temperatures (20°) and to high temperatures (42°). The present communication describes some of the biochemical properties of two temperature conditional

strains. One is cold sensitive and is rather similar to mutants already described. Preliminary experiments on the second strain which is thermosensitive have been already described (9).

MATERIAL AND METHODS

1) Selection of mutants

Mutants were screened for resistance to streptomycin or spectinomycin on L plates supplemented with the antibiotic and for temperature sensitivity on L plates.

TAM plates used for selection of transductants (table 1) are minimum plates supplemented with Tryptophan Assay Medium (DIFCO) (10 g/l).

Strain 209 is a spontaneous streptomycin dependent mutant isolated from strain K 10 (Hfr Cavalli). A single colony was inoculated in L medium supplemented with glucose (0.2 %) and streptomycin (500 µg/ml). 10^7 to 10^8 cells from an overnight culture were plated on L plates and incubated at 34°. Spontaneous revertants, no longer dependent on streptomycin, were checked for resistance to streptomycin (15 µg/ml or 40 µg/ml) and growth at 20°, 34° and 42°.

2) Growth of cells

Cells are grown in BT medium (1,3 % Bacto Tryptone Difco ; 0,7 % NaCl). The permissive temperatures are respectively 42° and 30° for 217 (sud_1) and 219 (sud_2) ; the non-permissive temperatures are 20° and 42°.

3) Biosynthesis of proteins and RNAs

To follow the biosynthesis of proteins or RNAs ^{14}C-arginine or ^{14}C-alanine (1 µC/3 µM/ml) or ^{14}C-uracil (1 µC/2 µM/ml) were added to 10 ml of an exponentially growing culture. At the appropriate time the culture flasks were transfered to the non-permissive temperature. ^{14}C-aminoacide incorporation into hot acid insoluble precipitate and ^{14}C-uracil incorporation into cold acid insoluble precipitate were measured on 0.1 ml aliquots.

4) Labelling of cells

A 25 ml culture was grown up to 3.10^7 cells/ml in the presence of 30 µCi of ^{14}C-uracil (48 mCi/mM). Cells were harvested and washed once with BT Medium supplemented with 200 µg/ml of ^{12}C-uracil. Cells were resuspended in 25 ml of BT uracil medium and incubated at the permissive temperature for 15 min. Growth resumed almost immediately. 25 ml of BT uracil medium equilibrated at the non-permissive temperature were then added and the culture transfered at this temperature. After 15 min. 2 mCi of carrier free $^{32}PO_4^{3-}$ were added and the incubation was prolonged for 100 min. Cells were harvested on ice in the presence of 50 mg (wet weight) of carrier exponential Escherichia coli RNase I^-_{10} cells and 500 mg of KH_2PO_4.

5) Preparation and analysis of cell extracts

The cells were washed once in 10 ml of the appropriate buffer : either TAM_1 (10 mM Tris-HCl pH 7.8 ; 30 mM NH_4Cl ; magnesium acetate 10 mM ; 6 mM β -mercapto-ethanol), or TAM_{II} (identical to TAM_I except that the magnesium acetate concentration is reduced to 0.3 mM). After centrifugation, the pellet was resuspended in 1.5 ml of the appropriate buffer and the cells disrupted in a French pressure cell at 10.000 psi in the presence of 20 µg/ml of RNase free DNase.

Unbroken cells and debris were eliminated at 30.000 g for 20 min. 1 ml of the supernatant was layered on a 5,20 % sucrose gradient prepared either in TAM_I or in TAM_{II} buffer. The gradients prepared in TAM_{II} buffer were run for 15 hours at 24,000 rpm ; those prepared in TAM_I were run for 15 hours at 21,500 rpm in a SW27 Spinco rotor. Gradients were collected in 1 ml fractions on an ISCO D gradient fractionator. 0.1 ml aliquots were diluted in 10 ml of Bray's scintillation fluid (11) and counted in a Tri-Carb 3380 Packard spectrometer.

6) Association of particles into 70S ribosomes

The ability of particles to associate with normal ribosomal subunits was tested under the conditions described by Kikuchi and Monier (12). Particles were incubated at 37° C for 60 min. in TAM_I

buffer in the presence of an excess of unlabelled subunits prepared from E.coli RNase I^-_{10}. After incubation the mixture was layered on a 5.20 % sucrose gradient prepared in the TAM_I buffer. Centrifugation was at 20,000 rpm for 15 hrs in a SW27 Spinco rotor. The gradients were fractionated as before and the total fractions were used to measure radioactivities.

7) RNA extraction and analysis

RNA extractions were performed by phenol-m cresol deproteinization as previously described (13). Agarose-polyacrylamide gel electrophoresis was done according to Dahlberg, Dingman and Peacock (14). 1 mm slices of the gel were cut, incubated overnight in concentrated ammonia and counted in 10 ml of Bray's fluid.

RESULTS

Three strains isolated as described in Material and Methods were purified and characterized as resistant to low concentrations of streptomycin and as cold sensitive (strain 217), thermosensitive (strain 219) and neither cold sensitive nor thermosensitive (strain 220). The presence of the strAD mutation of strain 209 was checked in the three strains by P_1 transduction of this allele into strain K10. Therefore the suppression of dependance was attributed to secondary mutations, sud_1 in 217, sud_2 in 219 and sud_3 in 220. The resistance to streptomycin is, at least, partially conserved since high concentrations of streptomycin have only inhibitory effects.

In order to localize sud mutations with respect to the spc locus we constructed by P_1 transduction strain 348, a strAD derivative of strain 345 (aroE24 spc R400). Strain 348 was used as a recipient in three transductions with P_1 lysates grown on strains 217 and 220 at 37° and strain 219 at 30°. Results in table I show that sud mutations are cotransducible with aroE or spc. Their loci appear to be localized between the aroE and spc loci.

TABLE I

Cotransduction between sud and spc mutations

Genetic constitution of the donnor strains : $aroE^+$ sud^- spc^+ strAD

Genetic constitution of the recipient strains : $aroE_{24}$ sud^+ $spcR_{400}$ strAD

Experiment	Transductants		sud-spc Cotransduction (%)
	Genetic constitution	Number	
A	$aroE^+$ sud_1 $spcR_{400}$ strAD	9	
	$aroE^+$ sud_1 spc^+ strAD	926	99.1
B	$aroE^+$ sud_2 $spcR_{400}$ strAD	17	
	$aroE^+$ sud_2 spc^+ strAD	394	96.0
C	$aroE^+$ sud_3 $spcR_{400}$ strAD	29	
	$aroE^+$ sud_3 spc^+ strAD	3137	99.1

The recipient (strain 348) is grown in L medium complemented with glucose (0.2 %) and streptomycin (500 µg/ml). Transductants are selected on TAM plates at 37° (experiments A and C) or at 30° (experiment B).

The ram mutation, which has a suppressor effect on streptomycin dependance (16) and which affects the structure of the 30S subunit protein S4 (17) was localized between aroE and spc and is 98 % cotransducible with spc (10). In strain 219, protein S4 was found to be abnormal (18), in agreement with the idea that mutation sud_2 alters protein S4. Although no direct evidence is available, it is very likely that mutations sud_1 and sud_3 also affect protein S4.

All the transductants which received either sud_1, sud_2 or sud_3 are at the same time streptomycin independant and resistant to low concentrations of this antibiotic. The transductants which received sud_1 (table 1, part A) or sud_2 (table 1, part B) are cold sensitive or thermosensitive respectively. Therefore sud_1, sud_2 and sud_3 which are spontaneous mutations, can be considered as unique mutational events which affect at the same time different aspects of the phenotype.

An alteration of a ribosomal component may alter either the function or the assembly of the subunits. The first criterion for a ribosomal assembly mutant is the persistence of the functions of preexisting ribosomes when the cells are shifted from the permissive to the non-permissive temperature. The incorporation of a ^{14}C-aminoacid into hot acid-insoluble material and of ^{14}C-uracil in cold acid-insoluble form are not stopped in strains 217 and 219 under these conditions (Fig. 1, A and B). This observation prompted us to study the assembly of ribosomal particles at the non-permissive temperature in these two strains.

I - STUDY OF STRAIN 217

a) Analysis of cell extracts

This strain does not grow at 20°. Several authors have already described mutants of this kind (5, 19). Nashimoto and Nomura (6) more recently characterized several cold sensitive strains, one group of which is similar to strain 217 ; therefore we examined the pattern of particles accumulated at the non-permissive temperature.

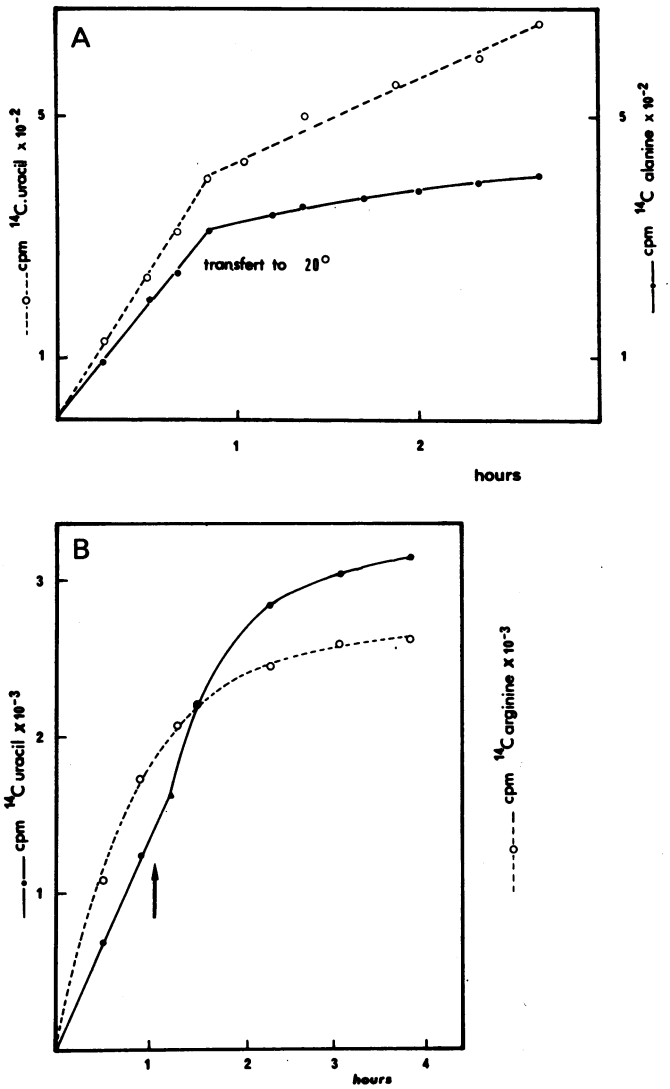

Figure 1 Incorporation of ^{14}C-aminoacids and ^{14}C-uracil by strain 217 (A) and strain 219 (B). Labelling starts at the permissive temperature ; at the time indicated by the arrow, the temperature is shifted to the non-permissive temperature.

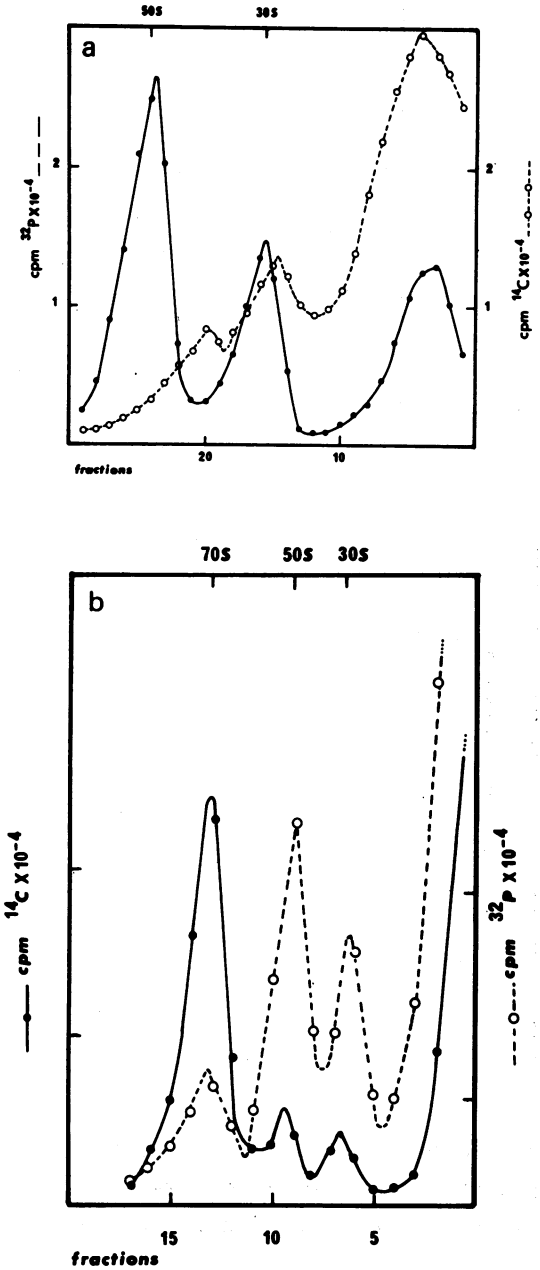

Figure 2 Analysis of extract of strain 217 on sucrose gradients
A in buffer TAM_{II}
B in buffer TAM_{I}

RNAs were labelled with ^{14}C-uracil for 2-3 generations at the permissive temperature and with ^{32}P for 100 min. after the temperature shift to 20°. When extracts were prepared and analysed in TAM_{II} buffer (fig. 2, A), part of the ^{32}P radioactivity was found associated with particles which sediment in peaks centered at 43S and 28S. The broadness of these peaks suggests some heterogeneity of the particles accumulated at the non-permissive temperature. When extraction and analysis were made in TAM_I buffer (Fig. 2, B), two ^{32}P-labelled peaks sedimenting at 30S and 50S were observed. The amount of material

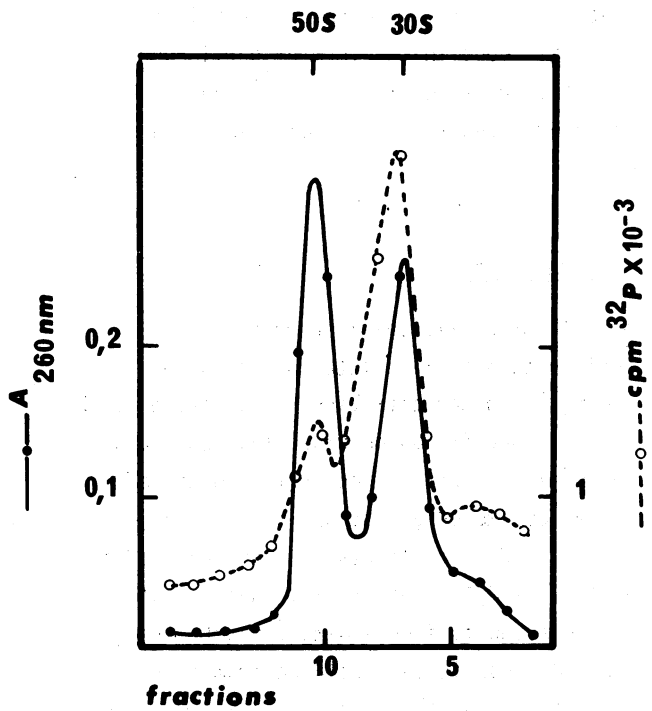

Figure 3 Dissociation on a TAM_{II} gradient of the 70S ribosomes recovered from the TAM_I gradient of figure 2, B. An aliquot of the fractions containing the 70S ribosomes was dialysed against TAM_{II} in the presence of 40 µg each of the normal subunits and layered on a 5,20 % sucrose gradient prepared in TAM_{II} buffer. Sedimentation was at 24.000 rpm for 15 hours in the Spinco SW27 rotor.

found under these peaks was always higher than in the corresponding 28S and 43S isolated in TMA_{II}, suggesting that some degradation occurs during extraction in low magnesium buffer. RNA analysis of the ^{32}P-labelled material which sedimented at 70S in the analysis of fig. 2, B demonstrated the presence of 16S RNA only. Upon dialysis of this 70S material against low Mg^{2+} TAM_{II} buffer the ^{32}P-labelled 70S peak also produced 30S-sedimenting material, almost exclusively (Fig. 3).

These observations suggest that the assembly of functional ribosomes in strain 217 is almost completely blocked at the non-permissive temperature. A low level of leakiness is observed since low amounts of 30S subunits bound to 50S subunits in the form of active ribosomes are found. Nevertheless most of the ribosomal material made at the non-permissive temperature is found in the form of particles which sediment at 30S and 50S in the presence of 10 mM Mg^{2+} ions but which are unable to form 70S ribosomes and which are very sensitive to low Mg^{2+} ion concentrations.

b) Properties of the accumulated particles

The in vitro assembly of normal ribosomal subunits (3) as well as the recovery of the ability to associate into 70S ribosomes after prolonged exposure to low Mg^{2+} ion concentrations (12), are only possible at elevated temperatures. Since it was observed that extracts of cold-incubated 217 cells in 10 mM Mg^{2+}-containing buffer contained unassociated 30S and 50S particles assembled at the non-permissive temperature, it was of interest to study the effects on these particles of incubation in the presence of 10 mM Mg^{2+} ions at 37°, under conditions which favor the recovery of active subunits from low-Mg^{2+} inactivated ones (12). ^{32}P-labelled 30 S and 50 S particles, isolated from extracts prepared at 10 mM Mg^{2+} were still unable to associate into 70S ribosomes, even when incubated in the presence of a large excess of unlabelled normal subunits (fig. 4, A and B). When ^{32}P-labelled 28S and 43S particles isolated from extracts prepared at 0.3 mM Mg^{2+}, were treated in the same way, their sedimentation coefficients increased to 30S and 48S, respectively (Fig. 5). The same results were obtained when total extracts were used instead of isolated particles.

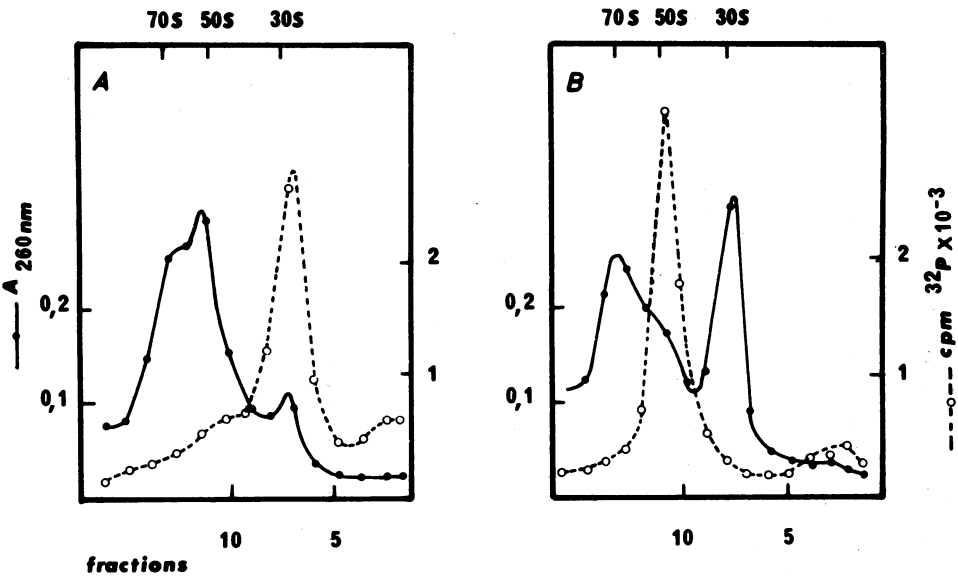

Figure 4 Association of defective particles from strain 217

1) 30S particles recovered from the TAM_I gradient of figure 2, B. The incubation mixture contained : 40 µg of unlabelled 30S subunits in TAM_I ; 80 µg of unlabelled 50S subunits in TAM_I ; 300 µl of ^{32}P-labelled 30S particles ; 300 µl of TAM_I.

2) 50S particles recovered from the TAM_I gradient of figure 2, B. The incubation mixture contained : 80 µg of unlabelled 30S subunits in TAM_I ; 40 µg of unlabelled 50S subunits in TAM_I ; 300 µl of ^{32}P-labelled 50S particles ; 300 µl of ^{32}P-labelled 50S particles ; 300 µl of TAM_I.

The incubation mixtures were heated at 37° for 60 min. and layered on a 5,20 % sucrose gradient prepared in TAM_I buffer. Sedimentation was at 21.500 rpm for 15 hours in the Spinco SW 27 rotor.

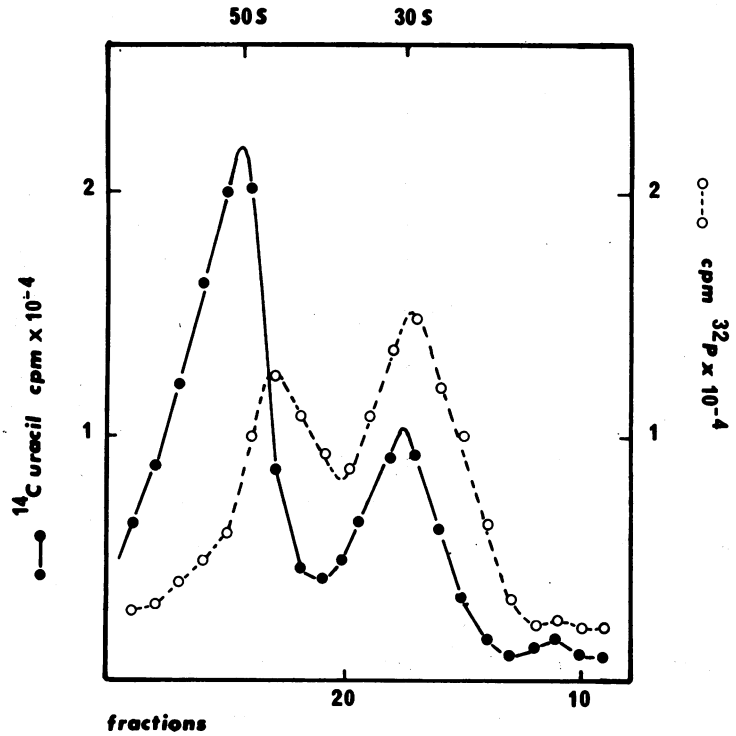

Figure 5 Sedimentation behaviour of 28S and 43S particles recovered from the TAM_{II} gradient of figure 2, A after heating at 37° for 1 hour in the presence of 10 mM Mg^{++}. The sedimentation conditions were the same as in fig.3.

TABLE II

Distribution of ^{32}P-radioactivity between 5S RNA and 23S RNA extracted from 43S and 50S particles accumulated by strain 217 at 20°

Particle	Radioactivity (CPM)		$\frac{\text{5S RNA}}{\text{23S RNA}}$ ratio+)
	5S RNA	23S RNA	
43S	1,100	25,100	0,044
50S	1,900	46,500	0,041

+) Theoretical value of $\frac{\text{5S RNA}}{\text{23S RNA}}$ ratio = 0,036

The reverse experiments was also attempted. 30S and 50S particles assembled at 20° and isolated from 10 mM Mg^{2+} gradients were dialyzed against 0.3 mM Mg^{2+} buffer. Their sedimentation coefficients decreased to 28S and 48S, respectively, under these conditions.

Therefore, although particles sedimenting at 30S and 50S in 10 mM Mg^{2+} can be assembled at 20° in cultures of strain 217, the final maturation of these particles in self-associating subunits cannot be accomplished by in vitro incubation at high temperature. Moreover, their sedimentation behaviour is very much affected not only by the actual Mg^{2+} ion concentration of the buffer in which they are suspended but also by the Mg^{2+} concentration of the buffer in which the original extracts were prepared.

c) RNA analysis

Nashimoto and Nomura (6) demonstrated the existence of a precursor of 16S RNA (p16S RNA) in the 21S particle accumulated by a cold sensitive ribosomal assembly defective mutant. This precursor analyzed by Lowry and Dhlberg (20) is similar to the p16S RNA accumulated in chloramphenicol-treated cells. It differs from mature 16S RNA only by the presence of some supplementary oligonucleotides at both 3' and 5'-ends.

The RNAs from 70S, 50S and 30S particles isolated on 10 mM Mg^{2+} gradients and the RNAs from 43S and 28S particles isolated on 0.3 mM Mg^{2+} gradients were extracted by phenol deproteinization and analysed by electrophoresis on agarose-acrylamide gel.

50S and 43S particles were shown to contain intact 23S RNA together with a stoechiometric amount of 5S RNA (table II). This observation ruled out the possibility that 43S particles could differ from 50S particles by the absence of 5S RNA. Although essentially no active 50S subunits are assembled, a fraction of 5S RNA extracted from whole cells labelled at the non-permissive temperature was found in the mature form (21). In order to check for precursor forms, the RNAs from 50S and 30S particles were analysed on an agarose acrylamide gel together with mature ^{14}C-labelled 16S and 23S RNAs (Fig. 6). It is clear that while a good coincidence exists between ^{14}C and ^{32}P profiles

for the larger RNA, the ^{32}P-labelled smaller RNA moves more slowly than ^{14}C-16S RNA. This electrophoretic mobility suggests that this RNA exists in the particle as a precursor similar to that described by Lowry and Dahlberg (20).

These observations therefore establish that the assembly of normal 30S subunits at 20° in strain 217 is blocked in such a way that the maturation of p16S RNA does not occur.

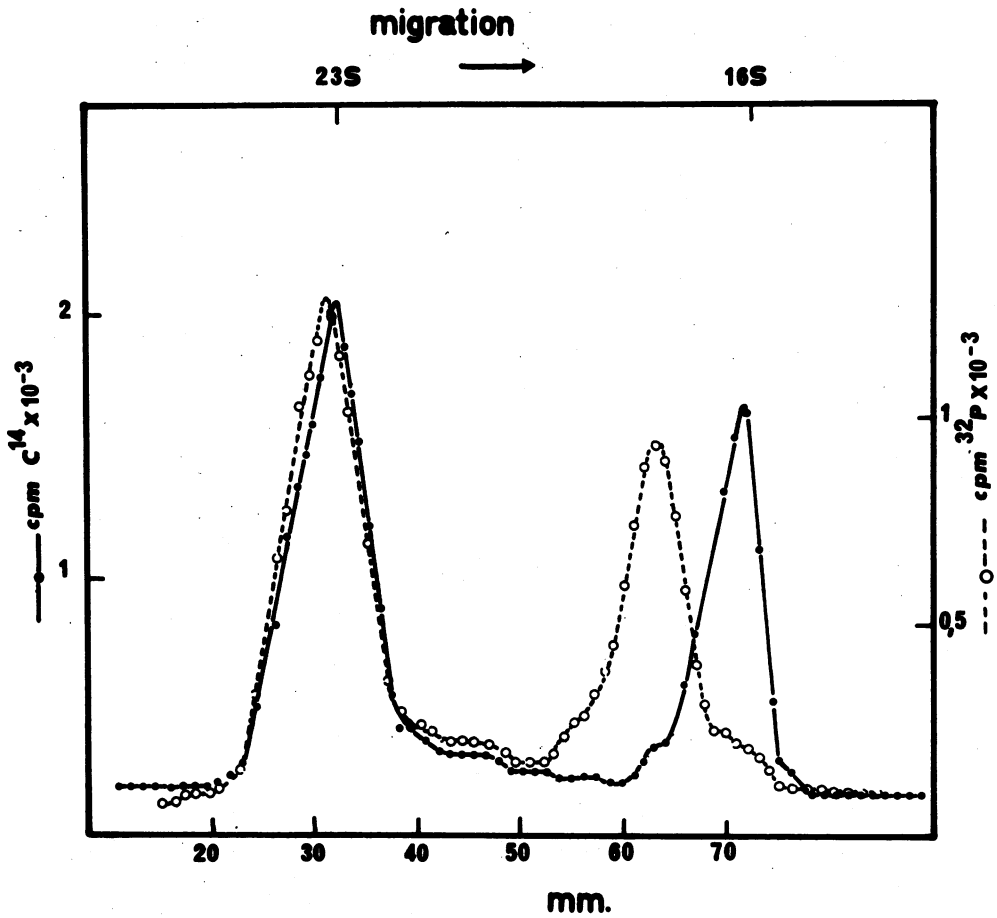

Figure 6 Electrophoretic mobility of RNA extracted from 30S and 50S particles recovered from the TAM_I gradient of figure 2, A. An RNA sample labelled with ^{14}C-uracil at 42° was added before electrophoresis.

II - STUDY OF STRAIN 219

This strain was also isolated as a revertant from a streptomycin-dependent strain and selected for its thermosensitivity. It grows at 30° but not at 42°. We have characterized the particles synthetized at the non-permissive temperature by their sedimentation coefficient in 0.3 mM Mg^{2+}-containing buffer. The ^{32}P radioactivity associated with particles assembled at 42° is distributed between two peaks sedimenting at 26S and 50S (Fig. 7). The 26S and 50S particles look homogeneous and contain 16S RNA and 23S RNA + 5S RNA respectively.

As previously described for strain 217, we checked the associability of these two particles. While the 26S particles fail to associate into 70S ribosomes, the 50S particles behaves like

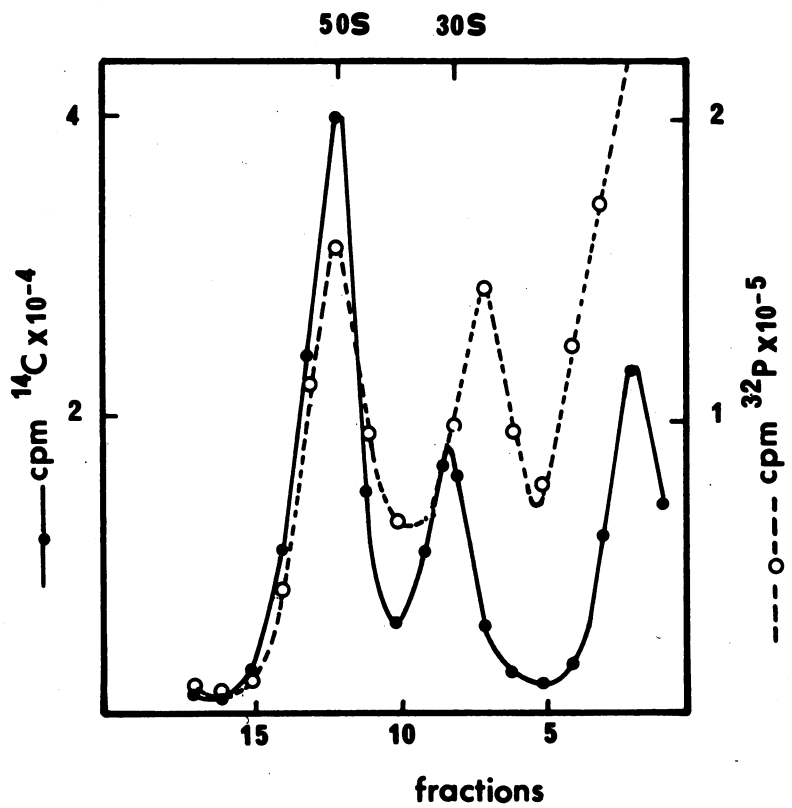

Figure 7 Analysis of extracts of strain 219 on a sucrose gradient prepared in TAM_{II} buffer. The conditions were the same as in figure 2.

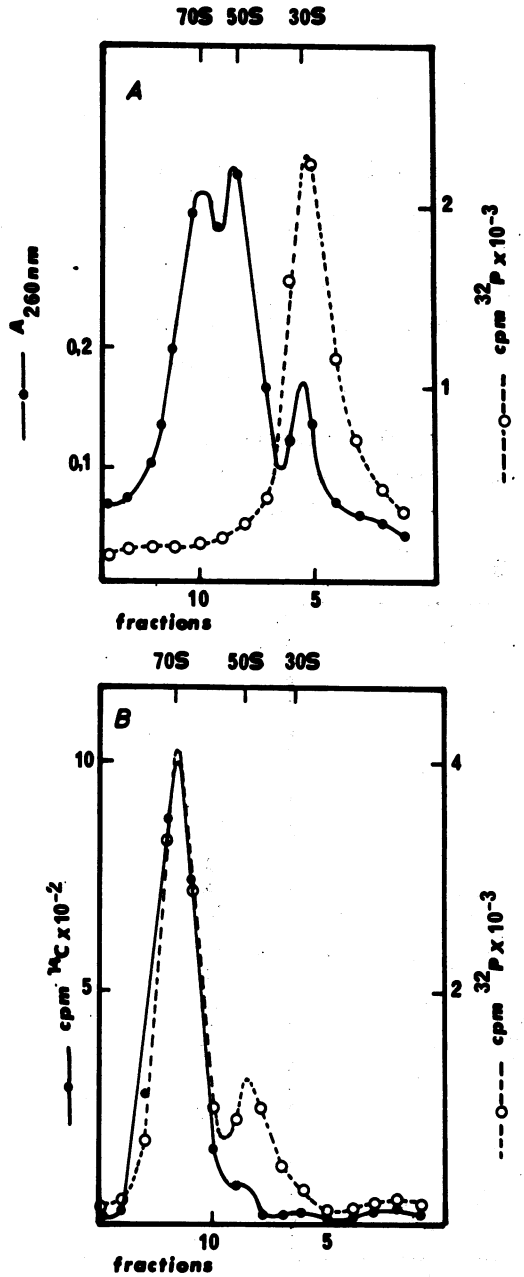

Figure 8

Association of particles from strain 219
A) 30S B) 50S
The experimental conditions were the same as in figure 4.

mature 50S subunit in this respect (Fig. 8). In agreement with this observation, it was shown that, upon dialysis against 0.3 mM Mg^{2+}, the ^{32}P-labelled 70S peak present in 10 mM Mg^{2+} extracts of cells incubated at the non-permissive temperature liberates exclusively ^{32}P-50S subunits upon dissociation (Fig. 9).

Although these 50S particles contain some precursors of 5S RNA (21), they cannot be distinguished from normal active subunits.

RNA extracted from the defective 26S particles was analysed on agarose-acrylamide gel (Fig. 10). The ^{32}P-radioactivity moves more slowly than the ^{14}C-radioactivity which corresponds to mature 16S RNA. Its migration rate is similar to that of the p16S RNA accumulated by strain 217. This similarity was confirmed by fingerprinting (22).

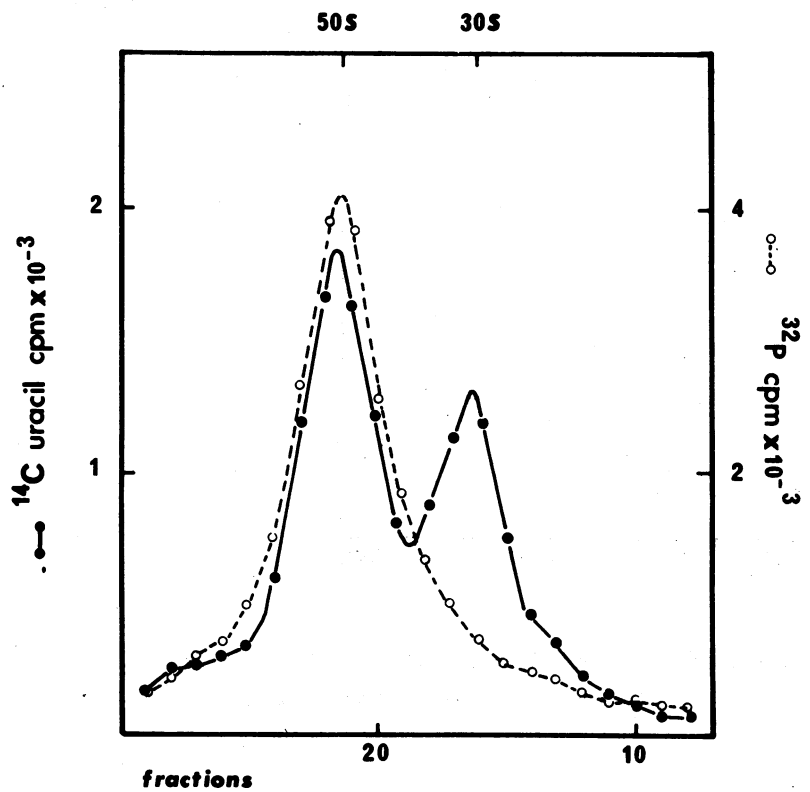

Figure 9 Association on a TAM_{II} gradient of the 70S ribosomes recovered from an extract of strain 219 prepared and analysed in TAM_I buffer. The experimental conditions are the same as in figure 3.

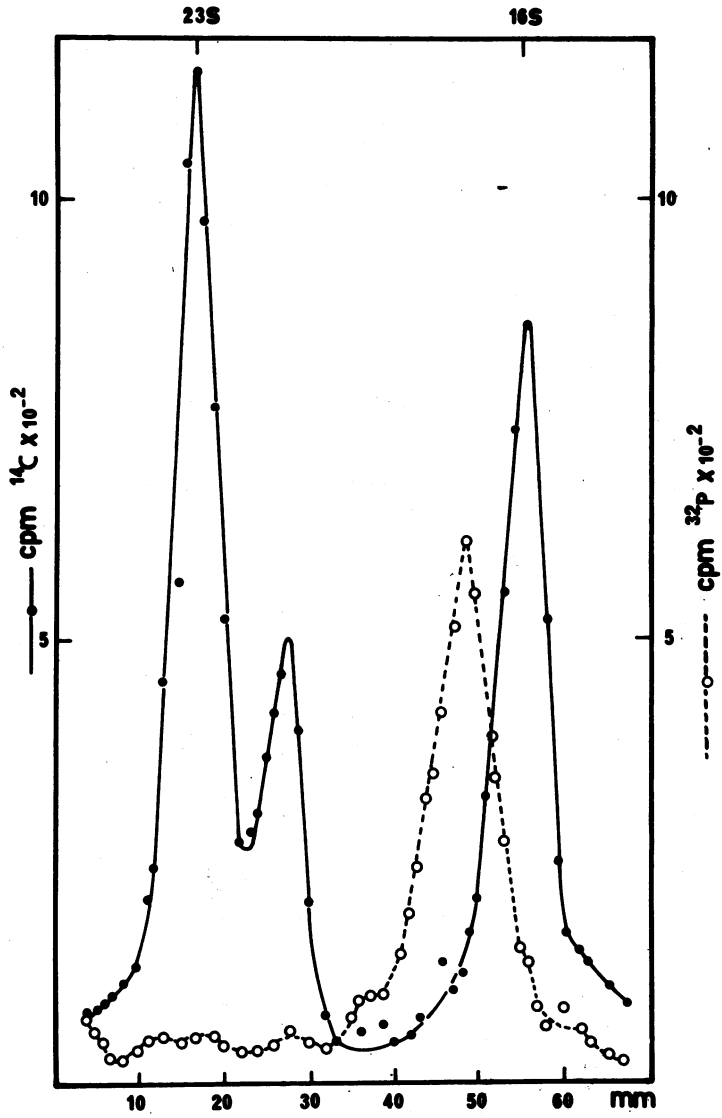

Figure 10 Electrophoretic mobility of RNA extracted from 26S particles isolated on a TAM_{II} gradient. The conditions were the same as in figure 6.

A derivative of strain 219, with a *str* A^+ *spc* R genotype was constructed by transduction. This strain (417) which carries the sud_2 mutation is thermosensitive, accumulates the same 26S particles and assembles normal 50S subunits. Moreover the 16S RNA extracted from this strain has the same electrophoretic behaviour and the same fingerprint as the p16S RNA from 219. Therefore the expression of the thermosensitive sud_2 mutation is independent of the alleles at spc and str A loci. The sud_2 mutation is responsible by itself for the defect of the 30S subunit assembly.

DISCUSSION

It is clear from the results of the study of strains 217 and 219, that the two mutations sud_1 and sud_2 are responsible for the suppression of streptomycin dependance and for the conditional subunit assembly defectiveness. Both of them map close to the *spc* R locus, in the same region as mutation *ram* which affects protein S_4. This protein is one of the first proteins which bind to 16S RNA in the protein assembly map of Mizushima and Nomura (23). Its importance in the orderly building up of the 30S subunit is confirmed by the present observations.

Proteins S_4 appears particularly important for the maturation process of the 16S RNA precursor. At their non-permissive temperatures, strain 217 and 219 were found to accumulate the same p16S RNA as that previously identified by Lowry and Dahlberg (20) in strain *spc* 49-1 (6). Since the maturation process occurs late in the assembly, it is not possible to decide whether the effect of protein S_4 on maturation is direct or indirect. However the fact that alterations in proteins S_4 or S_5 affect the maturation process at the same extent, suggests that this effect is mainly a result of some alteration in the conformation of the particles.

Although strain 217 has not exactly the same phenotype as any of the previous *sad* mutants isolated by Nomura and his collaborators (5, 6), in agreement with the hypothesis of Nashimoto and Nomura (6) it is strickingly similar to them as regards the *in vivo*

dependance of the 50S subunit assembly on that of the 30S subunit since a mutation which exclusively modifies the structure of a 30S subunit component blocks the formation of both 30S and 50S particles. Strain 219, in contrast, is the first known strain in which the assembly of one ribosomal subunit is inhibited at elevated temperatures. In this strain, the biosynthesis of normal 50S subunits is no longer dependent upon the 30S subunit assembly. Nevertheless this observation cannot be considered as contradicting the proposal of Nashimoto and Nomura (6), which applied to cold sensitive mutants. In view of the influence of high temperatures on subunit assembly, it can be suggested that, in a thermosensitive strain, incubation at the non-permissive temperature of 42° provides enough energy to overcome a block in the assembly of the 50S subunit, which can only be overcome at 20° in cold-sensitive strains through some interactions with the 30S subunit assembly process.

The assembly phenotype of mutants is usually defined with reference to the sedimentation properties on sucrose gradients of defective particles examined in extracts which are prepared in low Mg^{2+} buffers. Some of the observations reported in the present communication demonstrate that proper attention to the Mg^{2+} ion concentration must be paid not only at the time of the sucrose gradient analysis but already during the preparation of extracts. As a matter of fact, the two mutant strains 217 and 219 produce at the non-permissive temperature particles which sediment at 30S and 50S, when extracted and analyzed at 10 mM Mg^{2+}. The same observation has been made on strain sad 68 (5), provided by Dr Nomura. When the Mg^{2+} concentration is decreased, the sedimentation rates of the particles also decrease but they do not reach the values found for particles directly extracted at 0.3 mM Mg^{2+}. Extraction in the presence of high concentrations of Mg^{2+}, followed by analysis at low concentrations, could therefore lead to erroneous conclusions.

Lindahl (24) has recently observed in E.Coli extracts prepared and analyzed in the presence of 10 mM Mg^{2+} ions new ribosomal precursor particles which sediment at 30S and 50S. Their precursor character was indicated by their inability to associate into 70S ribosomes and by the fact that they contain p16S and p23S rRNAs, respectively. These particles are nevertheless clearly dis-

tinguishable from the 30S and 50S immature particles which we ourselves observed in E.coli mutants extracted at 10 mM Mg^{2+}, since, contrary to our mutant particles, they still sediment at 30S and 50S in extracts prepared and analysed in the presence of 0.3 mM Mg^{2+} ions.

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