Ribosomal Assembly Influenced by Growth in the Presence of Streptomycin

(strA allele/protein S12/ribosomes modified in vivo/misreading/T4 bacteriophage)

ROBERT T. GARVIN, ROLAND ROSSET*, AND LUIGI GORINI

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT Translational leakiness (i.e., nonspecific suppression) of nonsense mutants of bacteriophage T4 is increased in cells of certain streptomycin-resistant strains previously grown in the presence of streptomycin. Concomitantly, ribosomes extracted from these streptomycin-grown cells possess a high level of misreading. Increased suppression ability as well as ribosomes that highly misread accumulate with kinetics expected for a constant differential rate of synthesis of a new product induced by drug action. The misreading ribosomes do not contain appreciable amounts of streptomycin and the misreading property is lost by exposure to high salt concentrations. It is suggested that streptomycin (or dihydrostreptomycin, or paromomycin) induces a reversible modification in 30S subunit assembly without physically participating in the modified structure. The extent of this modification appears dependent upon the strA allele.

Under controlled conditions, streptomycin binds directly and specifically to naked 16S RNA, whatever its origin, and the drug’s failure to bind to 30S subunits derived from streptomycin-resistant cells is apparently due to a masking of the 16S specific site(s) by the mutated S12 protein (1). Moreover, 16S RNA bound to streptomycin fails to reconstitute in vitro biologically active 30S subunits. Obviously a similar total failure to assemble active ribosomes does not occur in vivo since streptomycin-resistant strains grow normally in the presence of streptomycin. It is possible, however, that the drug might be able to interfere in a limited way with the proper assembly, resulting in functionally altered ribosomes.

With these considerations in mind, we have now reexamined an unpublished observation of some years ago, which we were unable to explain at that time. In studying phenotypic suppression of T4 nonsense mutants, we observed that streptomycin programs the suppression ability of a strA40 host cell before suppression actually occurs. It may be noted that such a mechanism is not peculiar to phage infection, but that it becomes evident only with phage because it is possible to separate operationally host programming during growth before infection from actual suppression, which occurs during phage propagation.

Our investigation suggests that streptomycin does indeed interfere with normal ribosomal assembly when a responsive S12 mutation is present in the genome and, as a result of this interference, a functionally altered ribosomal particle is assembled.

STRAINS AND MEDIA

The bacterial strains tested for their ability for nonspecific, ribosomal suppression (measured as translational leakiness of nonsense mutations) are derived from the same Escherichia coli B strain L1 (argF40, argB11, no detectable tRNA-suppressors specific for nonsense codons). strA mutant alleles were introduced by P1 transduction: strA40 is a streptomycin-resistant, moderately restrictive allele permitting expression of phenotypic suppression by streptomycin of argF40 amber (our collection number: L1-431). strA1 is the most restrictive streptomycin-resistant allele we have isolated in E. coli B. It gives no detectable suppression by streptomycin of the argF40 amber (our collection number: L1-401). Strain L190 is a derivative of L1 carrying a strA allele of the drugD type (3) which becomes streptomycin-independent through a second site mutation. Its phenotype is streptomycin or paromomycin resistant when the two drugs are used separately, but it is sensitive to a mixture of them. The λ lysogens are obtained by infecting the L1 strains with X1857. They are induced at 42°C but not at 30°C. The indicator strain used for titrating T4 wild-type phage and UGA mutants is CAJ68 (su^DGA^). Cell density is calculated from the optical density (OD) at 480 nm with a Lumentor spectrophotometer. OD of 1.5 corresponds to 2 X 10^6 cells per ml.

Bacteriophage T4 strains are: wild-type D; UGA N65 and ωA1; UAA C410 and C427; and UAG H36 and H39.

Medium L contains 10 g of peptone (Difco) and 5 g of yeast extract (Difco) in 1000 ml of A-N buffer (pH 7). After the mixture is autoclaved, glucose is added (0.5% final concentration). A-N buffer contains (g/liter): K2HPO4, 7; KH2PO4, 3; Na citrate·2H2O, 0.5; MgSO4·7H2O, 0.1 (pH 7). N+ buffer contains (g/liter): NH4Cl, 4.3; MgCl2·6H2O, 2.2; Tris, 1.21; 2-mercaptoethanol, 0.41 ml (pH 7.8) with HCl.

RESULTS

Table 1 shows that growth of a bacterial strain in the presence of streptomycin before infection may increase its ability to support a phage whose propagation depends on overcoming a nonsense translational block. Two actions of streptomycin are distinguishable. One is exerted during growth before infection and consists of programming the host efficiency to subsequently translate nonsense mutant phages in the absence of the drug. The second streptomycin action is exerted directly at the time of phage propagation. The table reports the behavior of only two UGA mutants but similar results, albeit on a smaller scale, are also obtained with the other two types of nonsense mutations, i.e., with UAG (H36, H39) and UAA.
(C410, C427) T4 mutants. In the samples presented in the table, the programming effect is about 10-times greater than the effect on propagation and this latter effect occurs relatively independently from the previous programming. Clearly the mechanisms underlying the two effects are different and the possibility is excluded that the programming effect may consist of a misinterpretation of a trivial artifact due to unbound streptomycin not completely washed away. Both streptomycin effects are exerted exclusively on translational leakiness of nonsense mutants because growth of wild-type phage is indifferent to host pregrowth in streptomycin and to the presence of the drug during phage propagation. The type of strA mutation carried in the ribosomes of the host strain plays an essential role in both these streptomycin effects. In fact, of the two strA mutants resistant to streptomycin tested, strA40 responds to streptomycin while strA1 appears indifferent to the drug. This correlates with the known difference in ability to restrict suppression displayed by these two strA alleles, strA40 being less restrictive than strA1 (2). Finally, results similar to those presented in Table 1 are obtained by testing dihydrostreptomycin instead of streptomycin. Moreover, by use of a host strain carrying a strA allele that confers resistance to both paromomycin and streptomycin, the effects of streptomycin and of paromomycin can be compared. It is found that the effects of the two drugs are identical in pattern and very similar in amount.

The experiments presented in Figs. 1 and 2 analyze the kinetics by which the host suppression efficiency increases or decreases when growing cells are shifted from media lacking to media containing the drugs and vice versa. It has been

### Table 1. Suppression of T4 UGA mutants by su- cells grown with or without streptomycin before infection

<table>
<thead>
<tr>
<th>T4 strain tested</th>
<th>Host used</th>
<th>L1 strA1</th>
<th>L1 strA0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sm during host pregrowth</td>
<td>(Sm during phase growth)</td>
<td>(Sm during phase growth)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>- +</td>
</tr>
<tr>
<td>N65</td>
<td>-</td>
<td>2.2</td>
<td>19.9</td>
</tr>
<tr>
<td>N65</td>
<td>+</td>
<td>149.1</td>
<td>307.6</td>
</tr>
<tr>
<td>αA</td>
<td>-</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>αA</td>
<td>+</td>
<td>10.8</td>
<td>19.3</td>
</tr>
<tr>
<td>Wild type</td>
<td>or +</td>
<td>10^4</td>
<td>10^4</td>
</tr>
</tbody>
</table>

T4 bacteriophage strains: N65 and αA are UGA mutants within and outside the rII region, respectively. E. coli B su strains: L1 strA0 and L1 strA1 are transductants of either strA allele into the same parent. Phage N65 was tested by use of λ-lysogen hosts. Both lysogen and nonlysogen were used with the same results for testing of αA. Growth conditions before infection: at 30°C in medium L containing 0 or 100 μg/ml of streptomycin. Overnight cultures were diluted in fresh medium, brought to logarithmic phase again, and stopped when a density of 2 × 10^6 cells per ml was reached. The cells were centrifuged and washed three times with A-N buffer, then suspended at 2 × 10^6 cells per ml in medium L containing 0 or 100 μg of streptomycin per ml. Phage infection is done at 30°C with a multiplicity of infection of 0.01 (i.e., 10^6 input phage). The figures are the number of phages found at 60 min less those found unadsorbed at 7 min after infection. The titer of these unadsorbed phages, determined in each sample, was consistently the same (i.e., no effect of streptomycin or pregrowth on phage adsorption) and thus averaged. Wild-type T4 gives similar burst sizes with both hosts and under all conditions tested and are therefore averaged. The same can be said for T4 mutants N65 and αA tested with L1 strA1. The table presents the results of a single set of experiments, but five were performed. In spite of significant fluctuations between the different sets, they show unequivocally an identical general pattern. Sm, streptomycin.

* Phage burst size × 10^-2.

![Fig. 1. Kinetics by which suppression of efficiency of L1 strA40 increases during growth in streptomycin or dihydrostreptomycin. Suppression by L1 strA40 was tested on T4 mutants C410 ochre (left) and N65 UGA (right). Host pregrowth and phage propagation were at 30°C in medium L. Streptomycin or dihydrostreptomycin were present for different times during host pregrowth, but were absent during phage propagation. Different volumes of inoculum, withdrawn from an exponentially growing culture reaching OD of 1.5 of L1 strA40 (or of its λ-lysogen when N65 is tested), were quickly diluted in different volumes of warmed medium L, each containing the amount of drug needed for a final concentration of 100 μg/ml. All subcultures (7 ml total volume each) were harvested when the same OD of 1.5 was reached again, so that each culture went through a different, preestablished number of cell divisions, but all cells were exactly in the same phase of growth at the moment of phage infection. As a control, the incubation time needed by each subculture to reach OD 1.5 was checked and found to be, as expected, the division time (30 min) multiplied by the preestablished number of divisions. Samples of the original undiluted culture were immediately chilled to 0°C and, after addition of the drug, kept in ice throughout the incubation. Zero-time samples with and without the drug were also examined. At the end of the incubation the cells were centrifuged at 0°C, washed three times with A-N buffer, suspended in L medium at 2 × 10^6 cells per ml (a 10-times concentration of the centrifuged culture), and infected at a multiplicity of infection of 0.01. The burst size was determined (see details in footnotes of Table 1). *Abbreviations* are given in two scales mutually corresponding: (a) Fraction of the total cellular content in ribosomes supposedly produced after drug addition [streptomycin (Sm)-ribosomes]. The percent that theoretically should accumulate after each division is calculated on the assumption that ribosomes are formed at a constant differential rate during exponential growth. (b) Number of cell divisions determined experimentally. *Ordinates* are burst sizes in percent of the maximum attainable. This maximum is the average of two determinations in subcultures grown for six and eight divisions in the presence of the drug. On the basis of the assumptions discussed in the text, the percent of the burst size and that of newly formed ribosomes should be related by a straight line (dashed line). Data presented are the average of four independent experiments with streptomycin (●) or dihydrostreptomycin (×).
shown already (2), and Table 1 reiterates, that strA mutations control translational leakiness and suppression. Thus the programming cited above should consist of some alteration occurring in the 30S ribosomal subunits and caused by the presence of the drug. The controls of the experiment presented in Fig. 1 show that cells to which streptomycin or dihydrostreptomycin had been added after they were cooled to 0°, then centrifuged and washed immediately, or after maintenance at 0° (i.e., in nongrowing conditions) for a time corresponding to 5 generations if they were incubated at 30°, display the same suppression efficiency as cells grown in the absence of the drug (for example the average burst size of N65 is 0.05 phage per adsorbed input phage). By contrast, after 5 generations in the presence of streptomycin (or dihydrostreptomycin) the suppression efficiency rises about 80 times (burst size of N65 is four phage per adsorbed input phage). This finding indicates that growth is required for production of the supposed 30S modification. On the assumption that the 30S subunits assembled after addition of streptomycin are the only ones responsible for the high suppression efficiency, the rate of increase in suppression efficiency should follow the usual (6) kinetics of accumulation of a product formed at a constant rate per cell described by the equation:

\[ \text{Bs max} = 100 \left( 1 - e^{-a\tau} \right), \]

where Bs max is the maximum final burst size supported by cells grown for a long time in streptomycin and \( a \) is the rate constant of the equation for exponential growth, \( dN/dt =aN \) (\( N \) = number of bacteria per unit volume). This means that when the burst sizes expressed in percent of the maximum obtainable are plotted against a scale where 25, 50, 75, and 93.75% corresponds to \( 1/5, 1, \) and 2.4 divisions, a straight line should be obtained. Figs. 1 and 2 show that the kinetics obtained with ochre C410 satisfy the hypothesis. The kinetics obtained with UGA N65 (and with oA1, not shown) could be accounted for by suggesting that phage growth is less than proportional to the

\[\text{Table 2. Misreading intrinsic to, and radioactivity associated with, ribosomes from cells grown in [H]dihydrostreptomycin}\]

<table>
<thead>
<tr>
<th>No. of cell divisions</th>
<th>Expected accumulation of drug-ribosomes</th>
<th>Misreading index (Ile/Ph)</th>
<th>[H]dihydroSm bound (molecules/ribosome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in the presence of dihydroSm</td>
<td>(% of maximum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.0</td>
<td>1.9</td>
<td>0.008</td>
</tr>
<tr>
<td>2</td>
<td>75.0</td>
<td>3.8</td>
<td>0.014</td>
</tr>
<tr>
<td>3</td>
<td>87.50</td>
<td>4.9</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>93.75</td>
<td>4.3</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>96.87</td>
<td>4.2</td>
<td>0.007</td>
</tr>
</tbody>
</table>

An overnight culture of LI strA/0 in L medium was inoculated (1% by volume) into the same medium. When growth at 30° reached OD 1.5, the culture was divided into fresh L medium containing dihydrostreptomycin or [H]dihydrostreptomycin (specific activity 3 Ci/mmol, Amersham/Searle) for a final concentration of 100 \( \mu \)g of drug per ml of culture. Dilutions of the inoculum were such that at OD = 1.5 the different subcultures went through 1, 2, 3, 4, and 5 generations. Dihydrostreptomycin cultures, used for misreading determinations, were 50 ml; [H]-dihydrostreptomycin cultures, used for binding determinations, were 7 ml. When OD 1.5 was reached, the cultures were immediately chilled to 0° and the cells were centrifuged. The pellets of cells were washed three times with A-N buffer. For the time-zero samples, 50- and 7-ml aliquots of the original culture were centrifuged and suspended in 50 and 7 ml of chilled A-N buffer containing 100 \( \mu \)g of dihydrostreptomycin or [H]dihydrostreptomycin per ml, respectively. Then the cells were centrifuged and washed three times, as the other samples. All washed pellets were suspended in 2 or 0.5 ml of \( N^+ \) buffer and sonicated for 60 sec at 0° (VWR Biosonik sonicator). The sonicated samples were treated with 50 or 10 \( \mu \)g of DNase I (Worthington), incubated at 37° for 15 min, and centrifuged at 15,000 rpm (15,000 \( \times \) g) for 45 min. The clear supernatants were centrifuged at 50,000 rpm (170,000 \( \times \) g) for 120 min. The supernatant was removed and the pellets dissolved in small amounts (0.3 ml) of \( N^+ \) buffer. These solutions were centrifuged at 15,000 rpm (15,000 \( \times \) g) for 15 min. The clear supernatants contain the ribosomes, which are titrated by absorption at 260 nm. Radioactivity ([H] counts) was determined, with a LS290 Beckman scintillation counter, in aliquots of labeled ribosomal solutions. The cpm in the aliquots actually counted were about 10^4. Repartition of counts between cells and supernatants was also measured. The ratio of counts found in the supernatant and in the pellet was 19.3 before washing and 22.7, 1.1, and 1.2 at the first, second, and third washing, respectively. Unlabeled ribosomes were used for determining misreading designated as "intrinsic" because it occurs in the absence of any misreading agent (as streptomycin) added to the incorporating system. Poly(U)-directed incorporation of [14C]phenylalanine and [3H]lysine was measured by the standard procedure (7). [3H] cpm were determined in a low-background gas-flow counter (Nuclear Chicago). Misreading index = 1e cpm per 1000 Phe cpm (cpm of Phe actually measured about 6 \( \times \) 10^3). Drug-ribosomes = ribosomes presumed to be formed after addition of the drug. The table presents the results of a single set of experiments, but three experiments were performed. In spite of significant fluctuations between the different sets, they show unequivocally an identical general pattern. Sm, streptomycin.

![Fig. 2. Kinetics of variations in suppression efficiency of L190 when paromomycin is supplied or diluted out during growth. Suppression by L190 was tested on T4 C410 ochre. (Left) Results of two experiments (and x) in which paromomycin is supplied to the host during growth. Experimental conditions were the same as that described for Fig. 1. (Right) Cells in exponential phase of growth were centrifuged from a culture growing for 10 generations in L medium containing 100 \( \mu \)g/ml of paromomycin and harvested when the OD reached 1.5. The cells were washed three times in A-N buffer and suspended in L medium without paromomycin at different cell densities calculated for reaching OD 1.5 after a preestablished number of divisions. At the end of growth, cells were centrifuged and suspended in L medium at a density 10-times higher (2 \( \times \) 10^9 cells per ml). Upon infection, the burst size is determined. The meaning of the parameters are as described for Fig. 1. Dashed line at the right is the theoretical dilution curve of a metabolite that is no longer produced. Pm, paromomycin.](image-url)
function corrected, while with ochre C427 (also not shown) the growth should be more than proportional. Whatever the explanation is for the observed departures from theoretical expectations, the important point is that all cases exclude an immediate uptake of the drug.

The results reported so far are only an indication that one of the targets of streptomycin action in vivo might be the assembly of 30S subunits, but more direct evidence of this is given by the study of extracted ribosomes described below. According to previous knowledge (2), if it is correct that growth in the presence of the drug brings about a structural modification of the ribosomes responsible for the observed enhancement of nonsense leakiness, then the same modified ribosomes should also permit a higher level of misreading in vitro. The amount of this misreading is expected to be very modest because it should not interfere in vivo with cellular growth since the growth of L1 strA40 is not slowed in the presence of the drug. Moreover, the misreading should be intrinsic to the system, in the sense that it should occur in the absence of added streptomycin since enhancement of suppression is seen after growth in streptomycin but the drug need not necessarily be present when suppression actually occurs. Finally, misreading should be seen only with ribosomes extracted from L1 strA40 and L190 grown in the presence of streptomycin (or dihydrostreptomycin or paromomycin), but it should be undetectable or very low with ribosomes from the same strains grown in the absence of the drug or from L1 strA1 grown under either condition.

Initially, several experiments were performed with L1 strA40 ribosomes extracted and purified with a technique involving exposure to high salt concentration. The results failed to show any difference in misreading between cells grown in the presence and in the absence of streptomycin. Subsequently, the possibility was considered that the sought ribosomal alteration might be reversible in high salt concentration, as observed already in a study involving L190 ribosomes (8). The step of ribosomal pelleting through a sucrose gradient in a buffer containing 1 M ammonium chloride was therefore eliminated (see description of the technique actually used in the footnote to Table 2). The ribosomes extracted with this modified technique from L1 strA40 grown in the presence of streptomycin now permitted misreading (Table 2). By contrast the ribosomes from L1 strA40 grown without streptomycin or from L1 strA1 grown under either condition failed to show any appreciable misreading. Moreover, upon subsequent treatment with the sucrose-gradient step previously omitted, the misreading ribosomes lost this property. Thus, growth in the presence of streptomycin brings about a reversible ribosomal modification, responsible for enhancement of unspecific suppression (i.e., nonsense leakiness) in vivo and of misreading in vitro. Table 2 shows the kinetics by which the amount of misreading per unit of ribosome increases as a function of growth in the presence of the drug (in this particular case, dihydrostreptomycin). Similar to the suppression-efficiency increase (see C410 in Figs. 1 and 2), the kinetics of increase of misreading are compatible with the assumption that the modified ribosomes (= drug–ribosomes) are formed and accumulate at a constant rate during exponential growth in the presence of the drug.

Table 2 also answers the question as to whether the drug is a part of the modified ribosome or causes the modification without physically participating in the new structure. It is seen that, although growth occurs in the presence of [H]-dihydrostreptomycin, the extracted ribosomes do not carry any significant amount of H label and, even more convincingly, the few H counts observed do not increase as misreading increases. Irrespective of whether in vivo dihydrostreptomycin ever becomes associated with L1 strA40 ribosomes, or actually dissociates from the ribosome due to an extraction artifact, the relevant fact is that modified ribosomes not carrying the drug are obtained. The absence of H counts, however, does not in itself indicate absence of streptomycin, unless it is proved that the cells are unable to oxidize dihydrostreptomycin to streptomycin. The following test was therefore performed: 50 mg of either streptomycin or dihydrostreptomycin (sulfate salts) was added to a growing culture of L1 strA40 (50 ml), or incubated with the corresponding extract of cells therefrom. The supernatants obtained after 2 hr of incubation at 37° were liberated from the sulfate ions with BaCl2 and lyophilized. The drug hydrochloride was extracted with methanol; the methanol-free solution was chromatographed by a thin-layer technique (4). The development solvent was 0.25% Na sulfate in water. The Rf of the spots revealed by sulfuric-acid spray correspond to those of the original streptomycin or dihydrostreptomycin with no indication of even partial conversion of one drug to the other. We therefore conclude that absence of H counts in the ribosomes actually means absence of drug, and that the misreading ribosome does not include drug molecules in its structure.

**DISCUSSION**

From the above results some general conclusions can be drawn. One is that a new target for streptomycin action, i.e., assembly of the ribosome, is indicated in vivo and confirmed in vitro. It is found that, as long as the drug is present during growth, the ribosomes produced are different from those found in the absence of the drug. Since no mutation is involved and since this alteration is reversible after ribosomal extraction and purification, the difference should consist of either a modified assembly of the normal components or of addition of new component(s). Our results cannot distinguish between these two possibilities, but we can exclude that the new component, if there is one, is the drug itself since, upon extraction, the modified ribosomes are free of any significant amount of drug. No attempts have been made to locate the modification in either one of the two ribosomal subunits, but it seems reasonable to assume that the 30S subunit should be involved since the properties that appear modified are those known to pertain to the smaller subunit.

A second point concerns the role of protein S12 in the architecture and functioning of 30S subunits. Originally S12, which is determined by the gene strA where the streptomycin-resistant mutations are located, was considered to be the site of action of streptomycin and therefore directly related to the bactericidal action of streptomycin. This concept, however, became untenable: first, when mutants resistant to streptomycin and/or paromomycin were isolated and shown to be located at the same strA site (5); and second, when mutation ram (the gene of protein S4) was isolated and studied (7). It was then discovered that S12 and S4 interact inside the 30S subunit structure in such a way that the first restricts and the second relaxes the ribosomal function of securing fidelity in translation. Accordingly, strA was considered to be the site of ribosomal restriction and streptomycin, whose action formally
mimicked that of ram, was supposed to antagonize restriction by modifying, in analogy to ram, the structural relationship of S12 with the rest of the ribosomal architecture. This rather vague model is now supported by the present discovery indicating that streptomycin interferes with ribosomal assembly, even conceding that ribosomal assembly is not the only target of streptomycin action, since Table 1 shows an additional effect on suppression efficiency, independent from assembly.

In terms of streptomycin binding, the strA1, strA40, and drugD strA alleles are indistinguishable since, in contrast with strA+, none of the three corresponding 30S subunits binds the drug in vitro (unpublished). In terms, however, of ribosomal functioning their response to streptomycin is quite different. This observation strengthens again the indication that the drug acts before assembly of 30S particles is completed. The programming effect of the drug is very conspicuous with the class of mutants carrying the drug-dependent strA allele, designated as phenotypically masked (3) and of which L190 is a revertant to independence. It involves not only suppression efficiency but also sensitivity to killing. Strain L190 is resistant to streptomycin and paromomycin used separately and when tested on cells grown in the absence of either drug. However, the cells are killed by the mixture streptomycin + paromomycin or when exposed to either drug after growth in the presence of the other. The pattern of misreading induced by streptomycin, paromomycin, or streptomycin + paromomycin is in remarkable agreement with that expected from the supposed programming effect. The L190 ribosomes with altered misreading are more stable than those of L1 strA40 but they are still revertible to barely perceptible misreading levels after two precipitations with 1 M ammonium sulfate (8). These widely different phenotypes obtained with the three strA alleles tested suggest that protein S12 plays a pivotal role in ribosomal assembly.

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