Note

RIBOSOMES OF STREPTOMYCIN-TREATED *ESCHERICHIA COLI* CONTAIN FAULTY RIBOSOMAL PROTEINS

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The misreading effect of streptomycin, an aminoglycoside antibiotic that interacts with the 30S ribosomal subunit, has clearly been demonstrated[1,2]. The first indication that streptomycin can introduce ambiguity into translation came from the finding that *Escherichia coli* auxotrophs can grow in the absence of the essential amino acids when the culture medium is supplemented with the drug[3]. This phenomenon, known as phenotypic suppression, was further observed in bacteria harboring missense or nonsense mutations[4]. Recently, erroneous biosynthesis induced by sublethal doses of streptomycin has been described for individual proteins: β-galactosidase[5], flagellin[6], elongation factors G and Tu[7]. In an estimate made with *E. coli*, it has been calculated that the frequency of errors in drug-treated cells is about 3×10^{-3} per codon[8], which is significantly higher than the average value of 4×10^{-4} found for normally growing cells[8,9].

Although it is clear that streptomycin induces misreading of the RNA message of bacteria, the mechanisms responsible for such induction of misreading are not yet fully understood[1,2]. It has been proposed that streptomycin, like other aminoglycoside antibiotics, affects the fidelity of translation by influencing the relative speed of steps in protein synthesis that follow the codon-anticodon interaction[10]. Also, the misreading effect has been attributed to ribosome conformational changes induced by the drug[11,12].

In this work, an attempt was made to investigate further the nature of parameters that may account for the increase in translational error frequency elicited by streptomycin. The question was raised whether drug-treated bacteria contain ribosomes whose molecular components, namely ribosomal proteins, are qualitatively normal, or else ribosomes whose components are structurally affected. The structural quality of ribosomal proteins was estimated from the amount of methionine misincorporated in the presence of low doses of antibiotic. Special attention was paid to the molecules which are naturally devoid of this amino acid[13]. Since stringent cells of *relA*$^+$ genotype and relaxed mutants carrying the *relA* allele are known to exhibit a different susceptibility to mistranslation[14], the two types of strain were analyzed in parallel.

The otherwise isogenic pair of *E. coli* strains CP78 (*relA*$^+$) and CP79 (*relA*) was used[15]. Both strains require histidine, threonine, arginine and leucine for growth. Cells were cultured at 37°C in a minimal Tris - glucose medium containing the four essential amino acids (50 μg/ml each), with or without streptomycin. Radioactive labeling was achieved by growing bacteria in mid-logarithmic phase for 30 minutes in the presence of both [^{3}H]lysine (4~6 μCi and 20 μg/ml) and [^{35}S]methionine (2 μCi/ml). In order to minimize the labeling of cysteine from radioactive methionine by amino acid interconversion, an excess of unlabeled cysteine was simultaneously added to the culture medium (400 μg/ml). Cells were collected by low-speed centrifugation after quick chilling, and ribosomes were isolated as previously described[16]. Ribosomal proteins were extracted by the acetic acid procedure[17], then analyzed by two-dimensional polyacrylamide gel electrophoresis[18]. Separation was carried out at pH 8.6 in 4% acrylamide in the first dimension, and at pH 4.5 in 18% acrylamide in the second dimension. Control experiments showed that, in this system, no significant difference in the migration of normal and abnormal ribosomal proteins could be detected. After electrophoresis, proteins were stained with Coomassie blue, then individual spots were cut out of the gel and dissolved overnight in 1 ml Soluene-350 (Packard Co.) at room temperature. Radioactivity

was counted in a Tri-Carb Packard spectrometer using the appropriate double-label setting, and the ratio $R = {}^{35}S/{}^{3}H$ was determined for each protein. For analyzing free radioactive ribosomal proteins present in the cytoplasmic fraction, a crude cellular extract was prepared then centrifuged at $225,000 \times g$ for 150 minutes. The supernatant fraction was mixed with unlabeled carrier ribosomal proteins, lyophilized and chromatographed on DE-52 cellulose (Whatman) in 5 mM phosphate buffer containing 6 M urea and 0.1% β-mercaptoethylamine (pH 8.0). Ribosomal proteins were eluted in the break-through volume, then lyophilized and finally analyzed by two-dimensional electrophoresis as above. [^{3}H]-Lysine (10~25 Ci/mmol) was from C.E.A. and [^{35}S]methionine (600~800 Ci/mmol) from Amersham Centre.

In a preliminary study, the effects of various doses of streptomycin on protein synthesis were analyzed (not shown). It was determined that, in agreement with other reports[4,6], 3 or 5 μg of drug per ml of culture medium induced the same inhibition of protein synthesis by only 10~15% in strains CP78 and CP79, respectively. These doses were used in all further experiments designed to evaluate the misreading effect of streptomycin.

The degree of streptomycin (SM)-induced misincorporation of methionine was first analyzed in ribosomal proteins extracted from ribosomes. For each protein, the ratio R of the amount of [^{35}S]methionine to the amount of [^{3}H]lysine incorporated was determined under drug treatment (R_{SM}) and compared to that in non-treated cells ($R_{control}$), in both stringent and relaxed strains. The same type of analysis was also performed in the case of free ribosomal proteins present in the cytoplasmic fraction of cells (supernatant). From the statistical analysis of four different measurements, we established 53% as the limit for variability of ratio R, in accordance with the value previously determined by others[10] in the same type of measurement. Results are therefore expressed in Table 1 only for those proteins whose $R_{SM}/R_{control}$ ratio significantly differed from 1.

Several proteins from ribosomes misincorporate methionine during treatment with sublethal doses of streptomycin. Thus, ribosomes of strain CP78 harbor six different proteins whose methionine content is significantly higher than normal, including proteins S18, L24 and L27 which are naturally devoid of this amino acid[13]. Similarly, ribosomes of strain CP79 contain faulty proteins. Six of them (S18, L1, L6, L11,

Table 1. Streptomycin-induced misincorporation of methionine into ribosomal proteins.

Fraction	Protein	Molar ratio Met: Lys	$R = {}^{35}S/{}^{3}H \times 10^{2}$				$R_{SM}/R_{control}$	
			Control		SM		CP78	CP79
			CP78	CP79	CP78	CP79		
Ribosomes	S4	3:20	—	17.9	—	33.3	—	1.86
	S5	6:12	—	34.2	—	65.3	—	1.91
	S9	3:10	—	19.7	—	34.3	—	1.74
	S18	0: 6	2.2	2.6	8.7	10.9	3.95	4.19
	L1	6:23	12.1	11.6	21.3	39.8	1.76	3.43
	L6	1:16	9.9	10.2	29.1	36.5	2.94	3.58
	L11	5:13	27.2	25.7	51.4	55.7	1.89	2.17
	L24	0:16	0.8	1.0	1.9	3.6	2.37	3.60
	L27	0:11	1.1	1.5	4.3	7.9	3.90	5.27
Supernatant	S4	3:20	14.6	—	52.6	—	3.60	—
	S12	0:13	1.2	—	2.9	—	2.41	—
	S19	2:13	9.1	8.7	62.1	61.8	6.82	7.10
	S21	0: 7	1.8	—	5.5	—	3.05	—
	L3	4:19	20.1	—	45.6	—	2.27	—
	L19	1:11	6.3	6.7	24.1	26.9	3.82	4.01
	L24	0:16	1.0	0.8	3.1	2.7	3.10	3.37
	L28	1: 7	10.2	—	30.5	—	2.99	—
	L32	1: 6	11.1	—	20.1	—	1.81	—

L24 and L27) are the same as in CP78 ribosomes, which may suggest that the misreading effect is somewhat specific, even though generally more pronounced in the relaxed than in the stringent strain. In the supernatant fraction, several free ribosomal proteins are also found to contain abnormally high quantities of methionine, their number being larger in the stringent than in the relaxed extract (9 vs 3 proteins). Some of these abnormal free proteins such as S4 and L24 are also detected in ribosomes, but most of them are not.

Taken together, the present observations thus indicate, for the first time, that several ribosomal proteins are erroneously synthesized in *E. coli* cells under streptomycin treatment. This finding could be expected when referring to the well-documented misreading effect of the drug on the synthesis of non-ribosomal proteins[5,7]. But, more surprising is the fact that a number of these abnormal ribosomal proteins are present within the ribosomes. It is then conceivable that such qualitative modification of ribosomes may play a role in their translational misfunctioning, especially if structural alterations concern some specific ribosomal proteins crucially required for accurate protein synthesis. This could be the case, for example, for protein S18 which is part of the decoding site on ribosomes and seems implicated in the binding of messenger RNA, and that of aminoacyl- and peptidyl-tRNA[10]. It may also concern proteins L11, L24 and L27 which are involved in the peptidyltransferase activity[20] or else proteins S4 and S5 which are thought to participate in the process of codon recognition[10]. Further experiments are however required to check the plausibility of this hypothesis, including *in vitro* measurement of misreading by ribosomes prepared from cells previously treated with the drug as compared to ribosomes isolated from untreated cells. In any case, it must be emphasized that the qualitative modification of ribosomes cannot explain *per se* the entire mechanism of erroneous translation elicited by streptomycin. It is indeed likely that misreading starts occurring before those modified ribosomes are synthesized by bacteria, that is as soon as the antibiotic interacts with the structurally normal ribosomes already present within the cells at the beginning of treatment. Abnormal ribosomes are probably formed later, at a time that remains to be determined, and then possibly increase even more the frequency of translational errors. In addition, it cannot be excluded that the misreading effect of streptomycin could be due as well to non-ribosomal components such as initiation and elongation factors, or aminoacyl-tRNA synthetases, that would be erroneously synthesized and consequently would abnormally function in protein synthesis. Here again, further experiments are required to check the structural quality of these components.

In terms of ribosome biogenesis, it seems that the structural alterations of some ribosomal proteins induced by streptomycin do not impair the process of their incorporation into ribosomes. This is the case for proteins S4, S5, S9, S18, L1, L11 and L24, which have previously been shown to participate in the early steps of ribosome assembly, and proteins L6 and L27 which are required in later steps.[21] By contrast, a number of erroneous proteins are found exclusively in a free state in the soluble fraction of bacteria (proteins S12, S19, S21, L3, L19, L28 and L32), which suggests that the utilization by cells of abnormal ribosomal proteins during ribosome biogenesis proceeds through a discriminatory mechanism. This mechanism seems more selective in the stringent than in the relaxed strain, as revealed by both the larger number of erroneous proteins found in the cytoplasmic extract and their lower number in complete ribosomes.

It must be noted that only undegraded faulty proteins were analyzed under our experimental conditions. Since abnormal proteins synthesized by streptomycin-treated cells are known to be rapidly degraded *in vivo*[22], it is likely that some of them escaped our analysis, which should then be taken as a minimal estimate of the drug-induced misreading of ribosomal proteins in *E. coli*. From this point of view, it would be of interest to compare our present data with those that would arise from the analysis of certain bacterial mutants, such as *htpR* or *lon* mutants, which have a lower capacity to degrade abnormal or incomplete proteins than that of wild-type cells[23,24].

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