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A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin

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ABSTRACT

Oligonucleotide-directed mutagenesis was used to introduce an A to C transversion at position 523 in the 16S ribosomal RNA gene of *Escherichia coli rrn*B operon cloned in plasmid pKK3535. *E. coli* cells transformed with the mutated plasmid were resistant to streptomycin. The mutated ribosomes isolated from these cells were not stimulated by streptomycin to misread the message in a poly(U)-directed assay. They were also restrictive to the stimulation of misreading by other error-promoting related aminoglycoside antibiotics such as neomycin, kanamycin or gentamicin, which do not compete for the streptomycin binding site. The 530 loop where the mutation in the 16S rRNA is located has been mapped at the external surface of the 30S subunit, and is therefore distal from the streptomycin binding site at the subunit interface. Our results support the conclusion that the mutation at position 523 in the 16S rRNA does not interfere with the binding of streptomycin, but prevents the drug from inducing conformational changes in the 530 loop which account for its miscoding effect. Since this effect primarily results from a perturbation of the translational proofreading control, our results also provide evidence that the 530 loop of the 16S rRNA is involved in this accuracy control.

INTRODUCTION

Several lines of evidence indicate that the ribosomal RNA is the primary target for a number of drugs (1). The aminoglycoside antibiotic streptomycin has been shown to protect specific bases in *E. coli* 16S rRNA (909 to 915, 1413, 1487 and 1494) against chemical modifications (2). Furthermore, streptomycin could be cross-linked with nitrogen mustard to regions in 16S rRNA encompassing bases 892 to 917 and 1394 to 1415 (3). It was deduced from these results that the streptomycin binding site is proximal to the ribosomal decoding site, which has been mapped in the cleft of the 30S subunit at the subunit interface (4,5). The ribosomal mutations which cause resistance to streptomycin in *E. coli* occur in the 30S protein S12 (reviewed in 6,7). Streptomycin resistance mutations in rRNA operons could not be isolated by classical genetics since these operons are present in seven copies in the genome of *E. coli*. However, streptomycin-resistant mutants of *Euglena gracilis* chloroplasts have been isolated with a point mutation in the 16S rRNA gene (8). This point mutation was located at position 912 (*E. coli* numbering), which lies in the region of interaction between streptomycin and the 16S rRNA, as indicated above. When this mutation (a C to T transition) was introduced in the corresponding position of *E. coli* 16S rRNA gene in the cloned *rrn*B operon, the *E. coli* cells transformed with the mutagenized plasmid were found to be streptomycin-resistant (9). Gau-

thier *et al.* (10) have recently shown that resistance to streptomycin can also be induced in the chloroplasts of *Chlamydomonas eugametos* by altering the sequence of the 16S rRNA gene in the so-called "530 loop" region, at position 523 (*E. coli* numbering). In the present study, we have introduced this mutation in the 16S rRNA gene of the *rrn*B operon of *E. coli* and we show that *E. coli* cells transformed with the mutagenized plasmid also become streptomycin-resistant, in spite of the fact that the mutation lies outside the binding site of the drug.

MATERIALS AND METHODS

Cell strains, plasmids and phages.

Plasmid pKK3535 which contains the entire *rrn*B operon cloned into pBR322 (11) was kindly provided by Dr. Harry Noller, University of California, Santa Cruz. *E. coli* strains HB101 and DH1 (12,13) were used as plasmid hosts while *E. coli* JM101 (14) was used as the host of phage M13 derivatives. Recombinant phages were constructed by inserting the *Pst*I-*Xba*I fragment from pKK3535 which contains the entire 16S rRNA gene (Figure 1) into the polylinker site of M13mp19 (15). *E. coli* Sm^r-19 is a spontaneous streptomycin-resistant mutant derived from strain DH1. It was isolated by plating 10^{11} cells on agar plates containing 500 μg/ml of streptomycin.

Enzymes and chemicals.

Deoxy- and dideoxynucleoside triphosphates and all enzymes used in the digestion, ligation and sequencing of DNA, as well as the AMV reverse transcriptase were purchased from Pharmacia. Antibiotics were from Sigma. [^{3}H]Isoleucine (106 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and deoxyadenosine 5'-thiotriphosphate, [α-^{35}S]dATP (1000 Ci/mmol), were purchased from Amersham.

Oligonucleotide-directed mutagenesis.

The mutagenic oligomer 5'TTACCGCGGCGGCTGGCACGG 3' is complementary to bases of the 16S rRNA gene corresponding to positions 513 to 533, with a single mismatch at position 523 (underlined). It was synthesized with an automated Pharmacia gene assembler. Mutagenesis of M13 recombinants, carrying the *Pst*I-*Xba*I fragment as an insert, was performed with the phosphorothioate method of Eckstein (16,17) using the Amersham oligonucleotide-directed *in vitro* mutagenesis system. The DNA sequence of the mutant M13 recombinant phages was confirmed by the dideoxynucleotide chain termination method (18), with [^{35}S]dATP as a label and using a synthetic primer (5'AGTAATTCCGATTAA 3') which hybridizes to the portion encompassing bases 551 to 565 in the 16S rRNA gene. The mutated *Pst*I-*Xba*I fragment was substituted for the non-mutated corresponding fragment in pKK3535 by a three-point ligation, yielding pPM523 with an A to C transversion. *E. coli* DHI cells transformed with pPM523 or pKK3535 were plated on agar using LB medium with 40 μg/ml ampicillin. Colonies were transferred by replica plating onto the same medium supplemented with streptomycin at concentrations of 0, 5, 10 and 25 μg/ml. This operation was repeated twice with colonies gro-

wing at a concentration of 5 μg/ml of streptomycin, following the procedure of Montandon *et al.* (9). Colonies originating from cells transformed with pPM523 were observed to grow in the presence of streptomycin up to 25 μg/ml after this treatment. They were conserved for further analysis. No colonies originating from cells transformed with pKK3535 could grow in the presence of 25 μg/ml of streptomycin.

Misreading capacity of the ribosomes from the transformed cells.

Cells transformed with pKK3535 (DHI-pKK3535) were grown in the presence of ampicillin (40 μg/ml). Streptomycin (25 μg/ml) was included for the culture of cells transformed with pPM523 (DHI-pPM523). Ribosomes were extracted from the bacterial pellets of these strains by high-speed centrifugation following standard procedures, and they were assayed for their misreading activity by monitoring the incorporation of isoleucine under the direction of poly(U) in the absence or the presence of streptomycin (detailed in 19). The cognate unlabeled aminoacid, phenylalanine, and the non-cognate labeled aminoacid, [^{3}H]isoleucine (5 μCi per sample of 100 μl), were at concentrations of 10 and 1.5 μM, respectively. Ribosomes isolated from Sm^r-19, a spontaneous streptomycin-resistant mutant of *E. coli* DHI, were included in the study for comparison.

Sequencing of the 16S rRNA region in the 530 loop.

Ribosomal RNA was isolated from the ribosomes of DHI-pKK3535 and DHI-pPM523, and was used as a template for reverse transcriptase sequencing. The primer was the synthetic primer complementary to bases 551 to 565 in the 16S rRNA (see above), which had been 5'-labeled with ^{32}P (20).

RESULTS AND DISCUSSION

Figure 1 outlines the strategy of mutagenesis. First, a *Pst*I-*Xba*I fragment containing the 16S rRNA gene was isolated from pKK3535 and cloned into the polylinker site of M13mp19. The fragment was mutagenized and mutant phages were sequenced. The mutated *Pst*I-*Xba*I fragment (A) was extracted from one mutant phage and three-point ligated with the *Pst*I-*Pst*I and *Xba*I-*Pst*I fragments (B and C) from pKK3535. This yielded plasmid pPM523 harbouring the mutated 16S rRNA gene. The position of the mutation within the 530 loop in the model of the secondary structure of the 16S rRNA (21) is presented in Figure 2. Competent *E. coli* DH1 cells were transformed with pPM523. Colonies grown in the presence of low levels of streptomycin were selected after successive replica plating on increasing concentrations of streptomycin up to 25 μg/ml, as described in Materials and Methods. This selection procedure probably causes a plasmid amplification, as is the case for cells grown in the presence of low levels of chloramphenicol or erythromycin (22,23). No streptomycin-resistant colonies were recovered when DHI cells were transformed with pKK3535 and submitted to the same selection procedure. The fact that cells cured for the mutated plasmid recover their sensitivity to streptomycin further confirms that resistance is caused by the plasmid (data not shown). It was

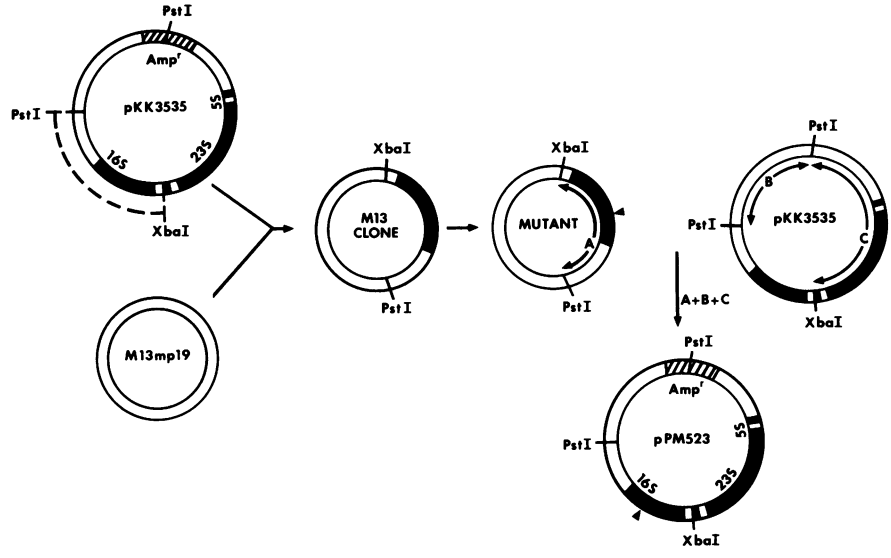

Figure 1: Construction of mutant plasmid pPM523. Restriction sites used for fragmentation are indicated. The broken line delineates the *PstI-XbaI* fragment that was removed from pKK3535, cloned into phage M13 and mutated. The arrowhead points to the site of mutation. Ligation of the mutated *PstI-XbaI* fragment (A) to the *PstI-PstI* and *XbaI-PstI* fragments (B and C) from pKK3535 yielded pPM523.

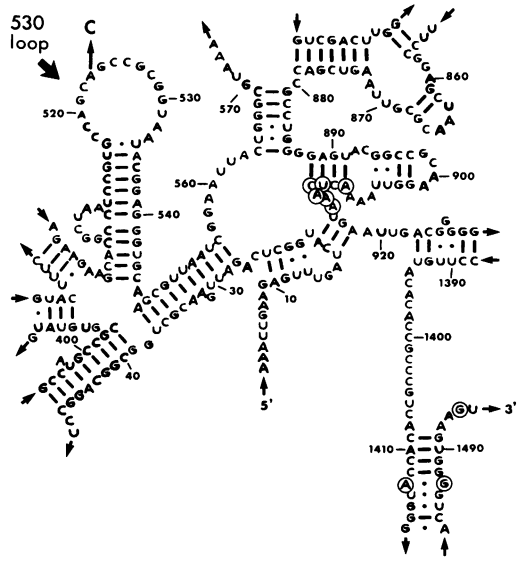

Figure 2: Secondary structural model of a part of *E. coli* 16S rRNA (21) showing the 530 loop and the site where the mutation has been introduced. Circled bases in the 910, 1410 and 1490 regions are protected against chemical probes by streptomycin (2).

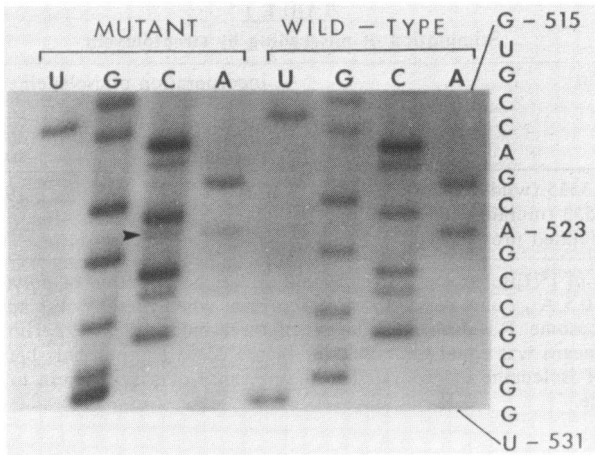

Figure 3: Autoradiogram showing the sequence through the mutated position in the 16S rRNA. Ribosomes were isolated from *E. coli* DHI cells transformed with pPM523 (mutant) or pKK3535 (wild-type). The 16S rRNA was used as a template in reverse transcription sequencing experiments. Lanes U, G, C and A refer to the rRNA sequence and result from reactions containing the complementary ddNTP. The mutated position is indicated by an arrowhead.

also observed that cells transformed with the mutated plasmid could grow on solid media containing as high as 500 μg/ml of streptomycin.

Ribosomes were isolated from cells transformed with the mutated or the control plasmid and the sequence of the 16S rRNA was determined around the 530 region. The sequence of the RNA from cells transformed with the mutated plasmid clearly revealed the presence of a band corresponding to a C at position 523, next to a band corresponding to an A (Figure 3). Only band A could be seen in the RNA from cells transformed with the wild-type plasmid at this position. It should be emphasized here that the content of ribosomes with mutated RNA in cells transformed with the mutated plasmid cannot be assessed from a comparison of the relative intensities of the bands corresponding to C and A at position 523. This would lead to an underestimation of the content of mutated ribosomes since band C is the lower band of a doublet and it is frequent that the intensity of the lower band of C doublets is drastically reduced.

Since stimulation of misreading is an essential step in the bactericidal action of streptomycin (24), this effect was assessed *in vitro* in a poly(U)-directed system with ribosomes from *E. coli* transformed with pPM523 or pKK3535 (Table 1). In contrast to wild-type ribosomes, ribosomes from the cells transformed with the mutated plasmid hardly responded to the stimulation by streptomycin. The same was true of the ribosomes from the spontaneous streptomycin-resistant mutant Sm^r-19, which harbours a mutation in protein S12 (6,7). Our results, therefore, demonstrate that the mutation at position 523 in the 16S rRNA confers

TABLE 1
Stimulation of misreading by streptomycin

Strain	Incorporation of isoleucine (in cpm)	
	Without streptomycin	With streptomycin
DHI-pKK3535 (wild-type)	8 144	193 012 (24)
DHI-pPM523 (mutated 16S rRNA)	4 512	5 738 (1.3)
Sm^r-19 (mutated protein S12)	4 236	4 415 (1)

The incorporation of [^{3}H]isoleucine was measured under the direction of poly(U). The amount of ribosomes was 0.5 A_{260} unit per assay. Streptomycin, when present, was added at a molar ratio of 10 per ribosome. Results are the means of three independent experiments. Standard deviation on the means was equal to or inferior than ± 15%. The numbers between parentheses are the ratio of isoleucine incorporated in the presence of streptomycin to that incorporated in its absence.

streptomycin resistance to the ribosomes. Furthermore, the near absence of stimulation indicates that the proportion of ribosomes with the mutated 16S rRNA is high. A similar phenomenon was observed by Montandon *et al.* (9), when transforming *E. coli* cells with a plasmid harbouring a 16S rRNA gene mutated at position 912, which also confers streptomycin resistance. The high proportion of ribosomes with mutated RNA which originates from the plasmid compared with ribosomes containing wild-type RNA originating from the host chromosome is most probably a consequence of the high-copy number of the plasmid.

The binding site of streptomycin is located on the 30S ribosomal subunit, at the subunit interface near the decoding site (see Introduction). However, as illustrated in Figure 4, the 530 loop has been localized on the exterior face of the 30S subunit, at a distance from the streptomycin binding domain (25). Moazed and Noller (26) have shown that codon-anticodon interaction at the decoding site triggers a conformational change in the 16S rRNA, allosterically affecting the 530 loop. It could be envisaged that, conversely, a mutation affecting the 530 loop could induce a conformational change in the decoding site, which would prevent the binding of streptomycin. An alternative interpretation of our results could be that the mutation in the 530 loop does not interfere with the binding of streptomycin but prevents the bound drug from inducing in the ribosome conformational changes which correlate with its miscoding effect (27,28). To determine whether the mutation at position 523 either prevents the binding of streptomycin or interferes with the induction of conformational changes by the bound drug, we have examined how the mutated ribosomes respond to other related error-promoting aminoglycoside antibiotics such as neomycin, kanamycin or gentamicin, which do not compete for the streptomycin binding site (29,30). Table 2 shows that the ribosomes with the mutated RNA are also restrictive to the stimulation of misreading by these agents. This is in contrast with the situation encountered with the ribosomes harbouring a mutated protein S12. These ribosomes

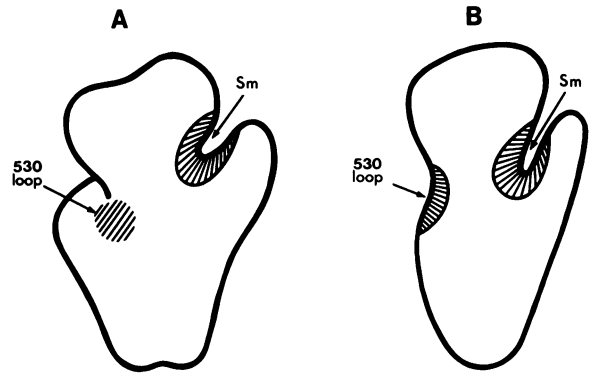

Figure 4: 30S subunit models of Stöffler and Stöffler-Meilicke (44) (A) and Lake (45) (B), showing the location of the 530 loop and of the streptomycin (Sm) binding domain.

which do not bind streptomycin (29) are stimulated as well as wild-type ribosomes to misread the message in the presence of neomycin, kanamycin or gentamicin. Thus, ribosomes with a mutation at position 523 in the 16S rRNA differ from ribosomes with mutated protein S12 in their response to agents binding at sites different from that of streptomycin. As it seems highly unlikely that the 16S rRNA mutation could affect simultaneously these different binding sites, we suggest that the mutation at position 523 does not prevent the binding of streptomycin but interferes with the conformational perturbations which account for its action.

Our results also stress the involvement of the 530 loop in the control of translational accuracy. This involvement is supported by the proximity between the 530 loop and the binding site of the elongation factor EF-Tu on the 30S subunit (25,31,32). Indeed, the binding site of EF-Tu maps the so-called recognition site, whose existence has been postulated by Lake

TABLE 2
Stimulation of misreading by aminoglycoside antibiotics

Strain	Stimulation of misreading by		
	neomycin	kanamycin	gentamicin
DHI-pKK3535	28	3	13
DHI-pPM523	16	1	6
Sm^r-19	30	3	14

Misreading was assessed by measuring the poly(U)-directed incorporation of [^{3}H]isoleucine. Stimulation of misreading is expressed as the ratio of isoleucine incorporated in the presence of the antibiotic to that incorporated in its absence. Neomycin, gentamicin or kanamycin, when present, were added at a molar ratio of 5, 10 and 20 per ribosome, respectively. Results are the means of four independent experiments. Standard deviation on the means was equal to or inferior than ± 20%.

and by Johnson *et al.* (33-35). The incoming aminoacyl-tRNA complexed to EF-Tu and GTP initially interacts at this site with the ribosome, before switching to the aminoacyl site at the subunit interface. The interaction between the ribosome and EF-Tu complexed to the aminoacyl-tRNA plays a key-role in the control of translational accuracy (36). Furthermore, the control of translational accuracy is exerted at two steps: a reversible selection of the aminoacyl-tRNA followed by an irreversible proofreading step (reviewed in 36-38). While neomycin, kanamycin and gentamicin affect both control steps (39,40), streptomycin selectively perturbs the proofreading step (41-43). Our results lead us to conclude that the 530 loop where a mutation can suppress the effect of streptomycin plays a role in the proofreading control of accuracy. This is in line with a previous suggestion of Moazed and Noller that the proofreading control may depend upon the conformation of the 530 loop (26). It will be interesting to identify which other positions in the 530 loop are involved in the control of translational accuracy.

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REFERENCES

1. Cundliffe, E. (1987) Biochimie 69, 863-869.
2. Moazed, D. and Noller, H.F. (1987) Nature 327, 389-394.
3. Gravel, M., Melançon, P. and Brakier-Gingras, L. (1987) Biochemistry 26, 6227-6232.
4. Gornicki, P., Nurse, K., Hellmann, W., Boublik, M. and Ofengand, J. (1984) J. Biol. Chem. 259, 10493-10498.
5. Oakes, M.I., Clark, M.W., Henderson, E. and Lake, J.A. (1986) Proc. Natl. Acad. Sci. USA 83, 275-279.
6. Vazquez, D. (1979) Mol. Biol. Biochem. Biophys. 30, 89-95.
7. Wallace, B.J., Tai, P.C. and Davis, B.D. (1979) in Antibiotics V - Mechanism of Action of Antibacterial Agents (Hahn, F.E., ed.), pp. 272-303, Springer-Verlag, Berlin.
8. Montandon, P.E., Nicolas, P., Schürmann, P. and Stutz, E. (1985) Nucleic Acids Res. 13, 4299-4310.
9. Montandon, P.E., Wagner, R. and Stutz, E. (1986) EMBO J. 5, 3705-3708.
10. Gauthier, A., Turmel, M. and Lemieux, C. (1988) Mol. Gen. Genet., in press.
11. Brosius, J., Ullrich, A., Raker, M.A., Gray, A., Dull, T.J., Gutell, R.R. and Noller, H.F. (1981) Plasmid 6, 112-118.
12. Bolivar, F., and Backman, K. (1979) Meth. Enzymol. 68, 245-267.
13. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New-York.
14. Messing, J., Crea, R. and Seeburg, P.H. (1981) Nucleic Acids Res. 9, 309-321.
15. Messing, J. (1983) Meth. Enzymol. 101, 20-78.
16. Taylor, J.W., Schmidt, W., Cosstick, R., Okruzek, A. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8749-8764.

17. Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765-8785.
18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
19. Grisé-Miron, L., Noreau, J., Melançon, P. and Brakier-Gingras, L. (1981) Biochim. Biophys. Acta 656, 103-110.
20. Gellebter, J. (1987) Focus 9, 5-8.
21. Noller, H.F., Asire, M., Barta, A., Douthwaite, S., Goldstein, T., Gutell, R.R., Moazed, D., Normanly, J., Prince, J.B., Stern, S., Triman, K., Turner, S., Van Stolk, B., Wheaton, V., Weiser, B. and Woese, C.R. (1986) in Structure, Function and Genetics of Ribosomes (Hardesty, B. and Kramer, G., eds.), pp. 143-163, Springer-Verlag, New-York.
22. Clewell, B.D. (1972) J. Bacteriol. 110, 667-676.
23. Sigmund, C.D. and Morgan, E.A. (1982) Proc. Natl. Acad. Sci. USA 79, 5602-5606.
24. Davis, B.D., Chen, L. and Tai, P.C. (1986) Proc. Natl. Acad. Sci. USA 83, 6164-6168.
25. Trempe, M.R., Ohgi, K. and Glitz, D.G. (1982) J. Biol. Chem. 257, 9822-9829.
26. Moazed, D. and Noller, H.F. (1986) Cell 47, 985-994.
27. Noreau, J., Grisé-Miron, L. and Brakier-Gingras, L. (1980) Biochim. Biophys. Acta 608, 72-81.
28. Hanas, J.S. and Simpson, M.V. (1986) J. Biol. Chem. 261, 6670-6676.
29. Chang, F.N. and Flaks, J. (1972) Antimicrob. Ag. Chemother. 2, 294-307.
30. Lando, D., Cousin, M.A., Ojasoo, T. and Raynaud, J.P. (1976) Eur. J. Biochem. 66, 597-606.
31. Langer, J.A. and Lake, J.A. (1986) J. Mol. Biol. 187, 617-621.
32. Girshovish, A.S., Bochkareva, E.S. and Vasiliev, V.D. (1986) FEBS Lett. 197, 192-208.
33. Lake, J.A. (1977) Proc. Natl. Acad. Sci. USA 74, 1903-1907.
34. Johnson, A.E., Fairclough, R.H. and Cantor, C.R. (1977) in Nucleic Acid-Protein Recognition (Vogel, H.J., ed.), pp. 469-490, Academic Press, New-York.
35. Oakes, M., Henderson, E., Scheinman, A., Clark, M. and Lake, J.A. (1986) in Structure, Function and Genetics of Ribosomes (Hardesty, B. and Kramer, G., eds.), pp. 47-67, Springer-Verlag, New-York.
36. Thompson, R.C. (1988) Trends Biochem. Sci. 13, 91-93.
37. Brakier-Gingras, L. and Phoenix, P. (1984) Can. J. Biochem. Cell Biol. 62, 231-244.
38. Kurland, C.G. and Ehrenberg, M. (1984) Prog. Nucleic Acid Res. Mol. Biol. 31, 192-219.
39. Campuzano, S., Cabanas, M.J. and Modolell, J. (1979) Eur. J. Biochem. 100, 133-139.
40. Jelenc, P.C. and Kurland, C.G. (1984) Mol. Gen. Genet. 194, 195-199.
41. Thompson, R.C., Dix, D.B., Gerson, R.B. and Karim, A.M. (1981) J. Biol. Chem. 256, 6676-6681.
42. Ruusala, T. and Kurland, C.G. (1984) Mol. Gen. Genet. 198, 100-104.
43. Smailov, S.K. and Gavrilova, L.P. (1985) FEBS Lett. 192, 165-169.
44. Stöffler, G. and Stöffler-Meilicke, M. (1984) Annu. Rev. Biophys. Bioeng. 13, 303-330.
45. Lake, J.A. (1985) Annu. Rev. Biochem. 54, 507-530.