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Mutations in 16S ribosomal RNA disrupt antibiotic-RNA interactions

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Two of six mutations at a base-paired site in *Escherichia coli* 16S rRNA confer resistance to nine different aminoglycoside antibiotics *in vivo*. Chemical probing of mutant and wild-type ribosomes in the presence of paromomycin indicates that interactions between the antibiotic and 16S rRNA in mutant ribosomes are disrupted. The altered interactions measured *in vitro* correlate precisely with resistance seen *in vivo* and may be attributable to specific structural changes observed in the mutant rRNA.

Key words: aminoglycoside/mutagenesis/resistance/ribosome/rRNA

Introduction

The interaction of antibiotics and ribosomes is of interest for understanding both antibiotic action and ribosome function. Mutations giving rise to antibiotic resistance have been used to locate active sites or substrate binding domains of the ribosome (for review, see Gale *et al.*, 1981). In *Escherichia coli*, such ribosomal mutants have usually been mapped to ribosomal proteins. For example, mutations in 30S subunit protein S12 confer resistance to streptomycin (Birge and Kurland, 1969; Ozaki *et al.*, 1969) and alterations in S5 give spectinomycin resistance (Davies *et al.*, 1965). However, binding of antibiotics to these proteins has never been demonstrated.

Gorini was one of the first to propose that antibiotics might bind to rRNA (Garvin *et al.*, 1974). This is supported by the recent identification of mutants and modifications in rRNAs which confer resistance to antibiotics (for review, see De Stasio *et al.*, 1988). In 16S rRNA, alterations conferring resistance to aminoglycosides are clustered in the putative decoding region. Cloned methylases from aminoglycoside-producing organisms modify bases 1405 or 1408 in 16S rRNA, conferring resistance to kanamycin and gentamicin, or kanamycin and apramycin, respectively (Beauclerk and Cundliffe, 1987). Resistance to paromomycin in yeast mitochondrial 15S rRNA is due to a C to G transversion at the site equivalent to base 1409 in *E.coli* 16S rRNA (Li *et al.*, 1982). A G to U transversion at 1491 confers paromomycin resistance in *Tetrahymena thermophila* (Spangler and Blackburn, 1985). These two nucleotides (1409 and 1491) are paired at the base of the penultimate helix of the molecule in the secondary structure of *E.coli* 16S rRNA (Moazed *et al.*, 1986). A second mutation in *Tetrahymena*, nearby at 1495, confers resistance to hygromycin. These data are summarized in Figure 1.

The actual demonstration of interaction between aminoglycoside antibiotics and rRNA comes from chemical probing experiments reported recently (Moazed and Noller, 1987). The addition of aminoglycosides of the neomycin group to 70S ribosomes or 30S particles protects A1408 and G1494 in 16S rRNA from chemical modification, and enhances the reactivity of C525.

Here we combine the two approaches (mutagenesis and chemical probing) to demonstrate that mutations in the base of the penultimate stem of 16S rRNA which confer antibiotic resistance do so by altering the interaction between the ribosomes and antibiotics. Two mutations which affect the base paired secondary structure at 1409-1491 confer resistance to paromomycin as well as most other aminoglycosides *in vivo*, and show loss of drug-dependent protection of nucleotides 1408 and 1494 *in vitro*.

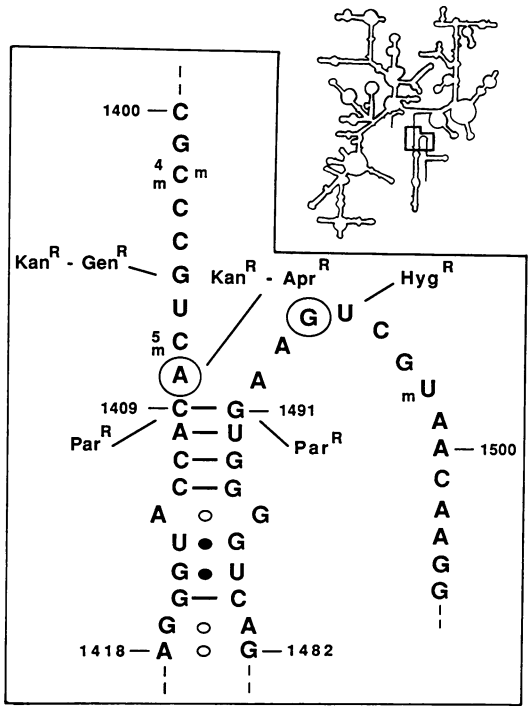

Fig. 1. Location of mutations and modifications in 16S rRNA which confer resistance to aminoglycosides. A portion of the 1400 region of 16S rRNA from *E.coli* is enlarged from the secondary structure based on Moazed *et al.* (1986). Methylation of 1405 confers resistance to kanamycin (Kan) and gentamicin (Gen) while methylation of 1408 confers resistance to kanamycin and apramycin (Apr) (Beauclerk and Cundliffe, 1987). Mutations of U to C in *Tetrahymena* 15S rRNA yield resistance to hygromycin (Hyg) (Spangler and Blackburn, 1985). Paromomycin (Par) resistance is conferred by a C to G transversion at 1409 in yeast mitochondria (Li *et al.*, 1982) or a G to U mutation at 1491 in *Tetrahymena* (Spangler and Blackburn, 1985). Nucleotides reactive with DMS but protected by paromomycin in wild-type ribosomes are circled.

Results

Construction and expression of mutant ribosomes

Mutations in 16S rRNA of *E.coli* were constructed by site-directed mutagenesis (Zoller and Smith, 1984), using the modifications described by Kunkel (1985). Positions C1409 and G1491, at the base of the penultimate helix of 16S rRNA, were each mutated separately to all possible nucleotides. The six mutations were cloned into pKK3535 (Brosius *et al.*, 1981), which contains the entire *rrn*B operon. Mutations were designated by the site and nature of the mutation (i.e. plasmid with C to U at 1409 is pKK1409U). Reverse transcription of cellular ribosomes indicated that ~45% of the ribosomes contained rRNA coded by the plasmid (data not shown). Three of the six single base mutations were found to be lethal when expressed in *E.coli* strain HB101 (E.A.De Stasio and A.E.Dahlberg, unpublished data). These mutations were expressed in the conditional expression vector pNO2680 (Gourse *et al.*, 1985). Double mutations were constructed by standard cloning techniques using an *Nco*I restriction site between the two mutations.

Aminoglycoside resistance in vivo

E.coli HB101 was transformed with the three non-lethal mutations (pKK1409U, pKK1491C and pKK1491U) and tested for resistance to aminoglycosides *in vivo*. Resistance was measured by the minimal inhibitory concentration (MIC) assay, an assay for the lowest antibiotic concentration which inhibits cell growth. Resistance to paromomycin and related neomycins is shown in Figure 2a. The presence of mutant ribosomes from plasmids pKK1491C and pKK1491U increased the resistance to paromomycin, neomycin and ribostamycin such that 16–32 times more antibiotic was required for inhibition compared to cells containing wild-type plasmid, pKK3535, or pKK1409U. The former two mutations conferred only a 4-fold greater resistance to neamine. In addition, a revertant of the lethal mutant G1409, which appears to be plasmid-borne, gave a low-level of resistance to paromomycin.

Resistance to other aminoglycosides was also tested. Data in Figure 2b show that the same two mutations, C1491 and U1491, also confer resistance to kanamycin, tobramycin, gentamicin, apramycin and hygromycin. The level of resistance was similar to that for the neomycins, 16-fold, except for hygromycin, 4-fold. The mutations did not confer resistance to spectinomycin, kasugamycin or tetracycline. In addition, the mutant plasmids were expressed in the streptomycin-sensitive *E.coli* strain DH1 to determine that there was no measurable resistance to streptomycin. Resistance in DH1 to the other aminoglycosides was similar to that seen using HB101 (data not shown).

Aminoglycoside resistance was not the result of a reduction in cell growth rate, although the rate is affected by the three viable mutations (E.A.De Stasio and A.E.Dahlberg, unpublished data). Equally slow growth was observed for bacteria containing pKK1409U and pKK1491U, but only the latter plasmid conferred antibiotic resistance.

Chemical probing of mutant ribosomes

Paromomycin, neomycin, kanamycin and gentamicin strongly protect the usually reactive N1 position of A1408 and N7 of G1494 from attack by dimethyl sulphate (DMS) (Moazed and Noller, 1987). These same antibiotics also cause significantly enhanced reactivity at N3 of C525. We used RNA-directed chemical probing (Moazed *et al.*, 1986) to examine, first, the effect of the mutations on the higher order structure of 16S rRNA, and, second, their effect on the previously observed interactions between these antibiotics and 16S rRNA.

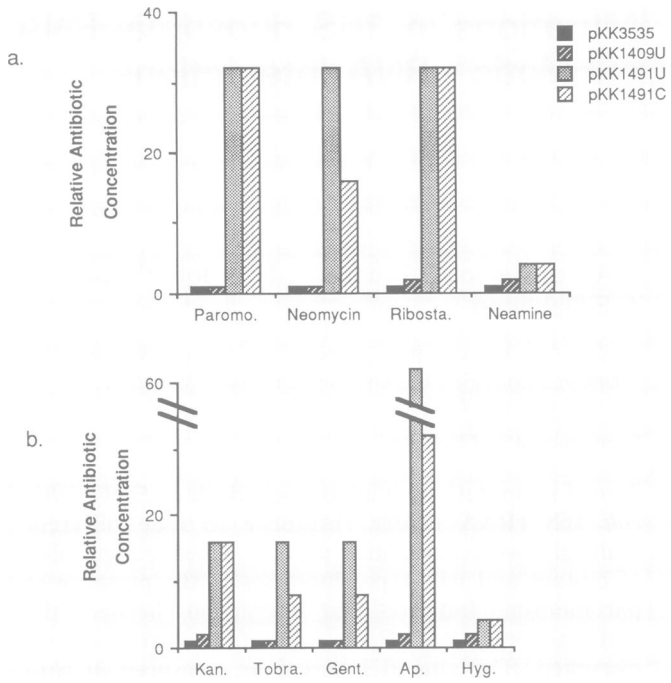

Fig. 2. Resistance to aminoglycosides conferred by mutations in 16S rRNA. Minimal inhibitory concentrations (MICs) of each drug for cells containing wild-type plasmid, pKK3535, were set equal to one, and the increase in MIC for bacteria containing mutant plasmids is plotted. MICs (in μg/ml) for cells containing pKK3535 are as follows: neomycin = 5, paromomycin = 10, ribostamycin = 10, neamine = 40, kanamycin = 10, tobramycin = 2.5, gentamicin = 2.5, apramycin = 10, hygromycin = 80.

Ribosomes from cells containing wild-type or mutant plasmid were modified with DMS in the presence of 10^{-6} M paromomycin. Modified RNA was then used as a template for reverse transcription. A representative gel is shown in Figure 3 and the results are in Table I. The results of probing vacant, mutant ribosomes (Figure 3; Table I) show that the antibiotic-sensitive mutant U1409 has a reactivity pattern indistinguishable from that of wild-type ribosomes. The antibiotic-resistant mutants U1491 and C1491, however, show significant differences in reactivity. In both mutants, the reactivity of A1408 is diminished, and the normally unreactive C1409 becomes reactive at N3. Furthermore, in the C1491 mutant, C1491 itself is reactive at N3, a Watson–Crick pairing position, whereas the N1 of the wild-type G1491 is normally unreactive. Finally, the normally reactive N7 position of G1494, which is protected by aminoglycoside antibiotics from DMS attack, has decreased reactivity in ribosomes from strains bearing the C1491 mutation.

Interestingly, A1418 and A1483, normally protected in 70S ribosomes (D.Moazed, J.McWhirter and H.F.Noller, unpublished data), are significantly more reactive in the U1491 and C1491 mutant ribosomes. This adds further support to the suggestion that the interactions of 30S subunits with antibiotics, tRNA and 50S subunits are somehow interrelated (Moazed and Noller, 1987).

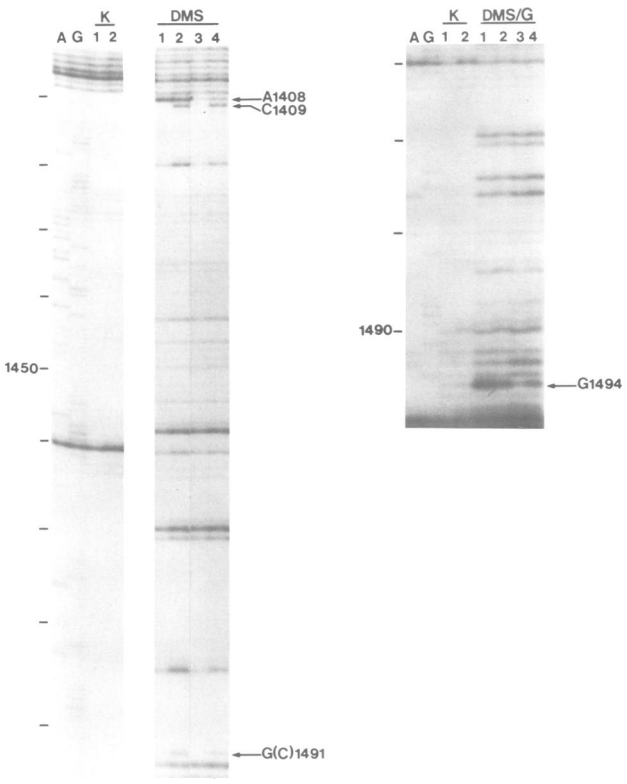

Fig. 3. Chemical footprinting of aminoglycosides on wild-type and mutant ribosomes in the presence or absence of paromomycin. A and G are dideoxy sequencing lanes and refer to the nucleotide sequence of 16S rRNA. K, unmodified control; DMS, dimethyl sulphate; DMS/G, DMS-modified samples in which strand scission (Peattie, 1979) has been induced at N7-modified guanines. **Lane 1**, wild-type 70S ribosomes (pKK3535); **lane 2**, mutant 70S ribosomes (pKK1491C); **lane 3**, wild-type 70S ribosomes (pKK3535) + paromomycin; **lane 4**, mutant 70S ribosomes (pKK1491C) + paromomycin.

Table I. Chemical probing of 16S rRNA in mutant ribosomes[a]

Plasmid	Modification + Par				Modification − Par			
	1408	1409	1491	1494	1408	1409	1491	1494
pKK3535	−	−	−	−	++	−	−	++
pKK1409U	−	−	−	nd	++	−	−	nd
pKK1491U	±	+	(−)[b]	nd	+	+	(−)[b]	nd
pKK1491C	±	+	+	+	+	+	+	+

Symbols: −, unreactive; ±, weakly reactive; +, reactive; ++, strongly reactive; nd, not done.

[a]Plasmid-coded rRNA (mutant except for pKK3535) comprised 45% of the 70S ribosomes; 55% was from the host *rrn* operons. The 70S ribosomes were modified in the presence or absence of 10^{-6} M paromomycin.
[b]No U-specific probe was used in these experiments.

Footprinting results with paromomycin correlate precisely with the observed phenotypes of the mutants. The aminoglycoside-sensitive U1409 mutant ribosomes show protection at A1408, similar to that of wild-type ribosomes (Moazed and Noller, 1987). Both of the drug-resistant mutants, C1491 and U1491, in contrast, show no detectable paromomycin-dependent effects.

Discussion

We have produced a series of six single base mutations at positions 1409 and 1491 in 16S rRNA and demonstrated resistance to aminoglycoside antibiotics in two of the three viable mutants. Chemical probing results show that, in addition to the expected enhanced reactivity of bases 1409 and 1491 due to creation of a base−base mismatch at these positions, A1408 and G1494 are clearly decreased in their reactivity toward DMS. This result shows that the U1491 and C1491 mutations cause a rearrangement in the higher order structure of this region of 16S rRNA that extends beyond the mutated bases themselves. This rearrangement undoubtedly underlies the loss of antibiotic sensitivity in the mutant ribosomes. Loss of paromomycin-dependent protection of A1408 and G1494 shows that the U1491 and C1491 mutations abolish previously observed drug−16S rRNA interactions. We cannot conclude from our results, however, that the mutant ribosomes have lost all capacity to bind these antibiotics.

Mutations conferring resistance have disrupted Watson−Crick base pairing between 1409 and 1491 (C−C and C−U) in 16S rRNA. The only viable mutant not resistant (U1409) retains base pairing (U−G). Morgan *et al.* (1988) also found this mutant to be sensitive to paromomycin. Resistant mutants reported for other organisms, *Tetrahymena* U1491 (C−U) and yeast mitochondria 1409 (G−G), also have disrupted the 1409−1491 base pair. We found G1409 to be lethal in pKK3535 but a second site mutant in pKK1409G, yet uncharacterized, reverts the lethal phenotype and the plasmid then confers a 4-fold level of resistance to paromomycin. Our failure to recover viable transformants containing mutant A1491 is not consistent with the finding of Morgan *et al.* (1988) who reported A1491 is viable. While we do not know the reason for this discrepancy it may be attributed to differences in strains and plasmids. However, they do find that this mutant confers resistance to apramycin and (weakly) to the neomycin and kanamycin classes of antibiotics (E.Morgan and S.Gregory, personal communication). This is consistent with our data and with the model that disruption of Watson−Crick base pairing between 1409 and 1491 confers resistance to most aminoglycoside antibiotics. The model is further supported by results from double mutant plasmids where disruption of base pairing also produces aminoglycoside resistance. For example, the plasmid containing 1409U−1491C confers resistance to most aminoglycosides tested while plasmids containing 1409U−1491A or 1409G−1491C do not (data not shown). Interestingly, this disruption in base pairing in *E.coli* confers resistance to not just one or two but to a large number (at least nine) of aminoglycoside antibiotics. It is specific, however, to this group of antibiotics.

Resistance to one of the aminoglycosides, neamine, is weak and atypical. As shown in Figure 2a, resistance is only 4-fold for cells containing pKK1491C or 1491U. We also find that pKK1409G−1491C confers a 4-fold level of resistance to neamine although it does not confer resistance to any other antibiotic and does form a Watson−Crick base pair. Other mutant pairs at 1409−1491 (U−A or U−G) do not confer neamine resistance. Neamine consists of only rings one and two of neomycin. Thus, we conclude that the interaction of neamine with 16S rRNA can be disturbed by subtle changes in RNA structure regardless of base pairing

between positions 1409 and 1491.

Resistance to aminoglycosides in cells containing pKK1491C and pKK1491U is dominant (as measured by continued cell growth) only at low concentrations (10 μg/ml) of antibiotic (as discussed by Morgan *et al.*, 1988) (data not shown). Resistance at higher antibiotic concentrations (as measured by the MIC assay; see Figure 2) is not dominant and cell growth is eventually halted presumably due to the effects of the antibiotic on host-coded, wild-type ribosomes present in the cell.

Materials and methods

Mutant construction and expression

Mutations were constructed by site-directed mutagenesis in an M13 system (Zoller and Smith, 1984) with one mutagenic primer for each site. Single stranded M13 was prepared from *E.coli* strain RZ1032 (gift of T.Kunkel), to allow incorporation of uridine. Mutagenesis was then performed with the modifications of Kunkel (1985). Mutations were expressed in pKK3535 (Brosius *et al.*, 1981) containing the entire *rrn*B operon or pNO2680 in which the *rrn*B promoters are replaced by pL from phage lambda (Gourse *et al.*, 1985).

Aminoglycoside resistance and footprinting

Minimal inhibitory concentrations were determined by the tube dilution method, using 2-fold serial dilutions. Ampicillin was present at 200 μg/ml to select for plasmid retention. Chemical footprinting was done by incubating 20 pmol of 70S ribosomes in 100 μl 80 mM potassium cacodylate (pH 7.2), 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, with or without 5 μM paromomycin, for 20 min at 37°C and 15 min at 20°C. Chemical modification was performed by addition of DMS (2 μl, 1:6 dilution in ethanol) followed by incubation at 37°C for 5 min. Reactions were stopped, and the RNA extracted and resuspended as described (Moazed *et al.*, 1986). Primer extension and gel electrophoresis were performed as in Moazed *et al.* (1986).

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