# Basis for prokaryotic specificity of action of aminoglycoside antibiotics

**Michael I.Recht, Stephen Douthwaite[1] and Joseph D.Puglisi[2]**

Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305-5126, USA and [1]Department of Molecular Biology, Odense University, Campusvej 55, DK-5320, Odense M, Denmark

[2]Corresponding author
e-mail: puglisi@stanford.edu

**The aminoglycosides, a group of structurally related antibiotics, bind to rRNA in the small subunit of the prokaryotic ribosome. Most aminoglycosides are inactive or weakly active against eukaryotic ribosomes. A major difference in the binding site for these antibiotics between prokaryotic and eukaryotic ribosomes is the identity of the nucleotide at position 1408 (*Escherichia coli* numbering), which is an adenosine in prokaryotic ribosomes and a guanosine in eukaryotic ribosomes. Expression in *E.coli* of plasmid-encoded 16S rRNA containing an A1408 to G substitution confers resistance to a subclass of the aminoglycoside antibiotics that contain a 6′ amino group on ring I. Chemical footprinting experiments indicate that resistance arises from the lower affinity of the drug for the eukaryotic rRNA sequence. The 1408G ribosomes are resistant to the same subclass of aminoglycosides as previously observed both for eukaryotic ribosomes and bacterial ribosomes containing a methylation at the N1 position of A1408. The results indicate that the identity of the nucleotide at position 1408 is a major determinant of specificity of aminoglycoside action, and agree with prior structural studies of aminoglycoside–rRNA complexes.**

*Keywords*: A site/antibiotic resistance/decoding region/ribosome/rRNA

## Introduction

The aminoglycosides (Figure 1) are related bactericidal antibiotics that bind directly to prokaryotic 16S rRNA at the decoding region A site (Figure 2) (Moazed and Noller, 1987; Woodcock *et al.*, 1991), inducing codon misreading and inhibiting translocation both *in vitro* (Davies *et al.*, 1965) and *in vivo* (Edelmann and Gallant, 1977). The utility of aminoglycosides as antibacterial agents arises from their specificity of action. Although they target a conserved region of rRNA sequence, they discriminate between prokaryotic and eukaryotic ribosomes. Prokaryotic and mitochondrial ribosomes, which are sensitive to the action of many aminoglycosides, have an adenosine at position 1408 of the 16S rRNA (Gutell, 1994) (numbered according to the *Escherichia coli* sequence). In contrast, eukaryotic cytoplasmic ribosomes, which are insensitive to most aminoglycosides (Wilhelm *et al.*, 1978), have a guanosine at position 1408 (Gutell, 1994) (Figure 2).

The difference in potency of aminoglycoside antibiotics could be caused by a variety of factors. Although the sensitivity of prokaryotic organisms to the aminoglycosides could be due to differences in antibiotic uptake and membrane permeability between prokaryotic and eukaryotic cells, prokaryotic ribosomes are inhibited *in vitro* at a lower antibiotic concentration than eukaryotic ribosomes (Wilhelm *et al.*, 1978). Nonetheless, there are numerous differences between the ribosomes of prokaryotes and eukaryotes. In addition to the identity of nucleotide 1408, eukaryotic ribosomes differ from bacterial ribosomes in both the rRNA and protein components. It is not known whether eukaryotic resistance to certain aminoglycosides is due to lower affinity of the antibiotics for the ribosome or to non-productive binding of the antibiotics to the eukaryotic ribosome.

Biochemical and structural studies using a model oligo-nucleotide of the decoding region (Fourmy *et al.*, 1996; Recht *et al.*, 1996) demonstrated the importance of an A1408·A1493 base pair for the high-affinity binding of the antibiotic paromomycin to the decoding region. The geometry of the A·A pair creates the specific binding pocket for the critical ring I of aminoglycosides (Fourmy *et al.*, 1996; Yoshizawa *et al.*, 1998). Despite our structural studies, it is critical to demonstrate the link between binding affinity and antibiotic action *in vivo*. To understand whether the change from A1408 to G in eukaryotes is the origin of specificity of aminoglycosides for prokaryotes, we have introduced the G1408 mutation in *E.coli* ribosomes. Here we show that expression of 16S rRNA containing an A1408 to G mutation in *E.coli* strain DH1 confers resistance to many aminoglycoside antibiotics. The results explain the prokaryotic specificity of action for the aminoglycosides.

## Results

### Growth phenotype of cells expressing mutant 16S rRNA

Expression of 1408G 16S rRNA in *E.coli* DH1 in the absence of aminoglycoside antibiotic has no apparent growth phenotype (Figure 3). Since the plasmid-encoded rRNA comprises only 60% of the total rRNA (Recht *et al.*, 1999), phenotypic effects of the 1408G mutation could be masked by the presence of 1408A wild-type ribosomes. The antibiotic spectinomycin inhibits translocation of genome-encoded ribosomes (Gale *et al.*, 1981), but expression of plasmid-encoded 16S rRNA containing a C1192 to U mutation supports growth in media containing spectinomycin (Sigmund *et al.*, 1988). Therefore, in the presence of spectinomycin, all actively translating ribosomes are of plasmid-encoded origin. Cells expressing
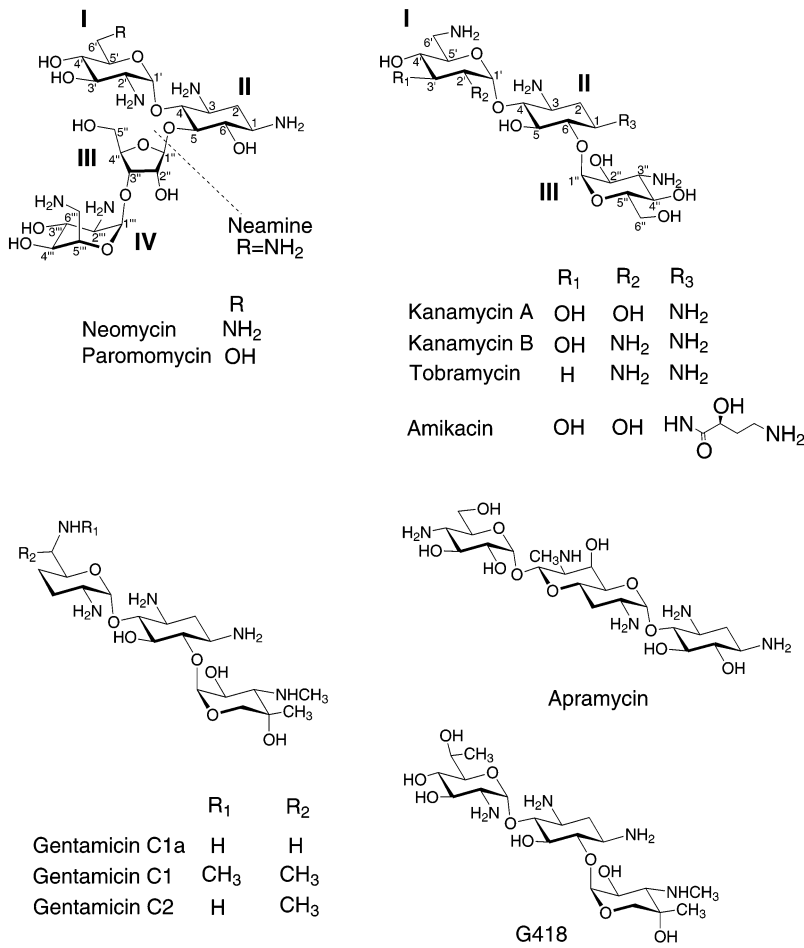
**Fig. 1.** Chemical structures of 2-deoxystreptamine (ring II)-containing aminoglycosides, all of which bind to the decoding region A site. Neamine consists of rings I and II of neomycin. The 4,5-disubstituted (neomycin, paromomycin) and the 4,6-disubstituted (gentamicin, kanamycin) are the two general classes of 2-deoxystreptamine-containing aminoglycosides.
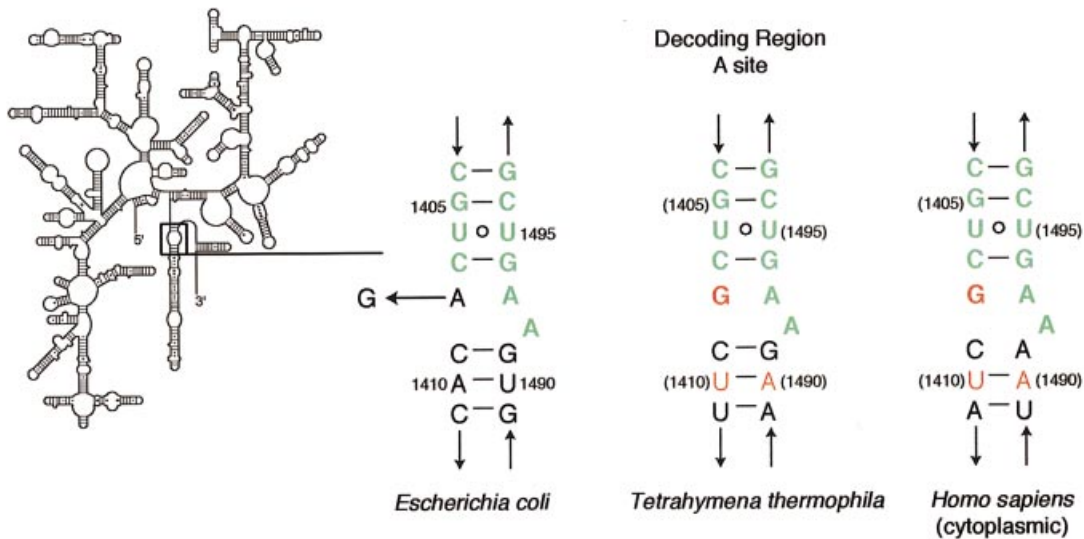


**Fig. 2.** Sequence of the decoding region A site from prokaryotic (*E.coli*) and eukaryotic (*Tetrahymena thermophila* and *Homo sapiens*) rRNAs. Sequences are numbered according to the *E.coli* sequence. Nucleotides that are universally conserved in all non-mitochondrial 16S-like rRNAs are shown in green (Gutell, 1994). Nucleotides that are conserved in all eukaryotic cytoplasmic ribosomes are indicated in red. The 1408G mutation was introduced into the 16S rRNA sequence and expressed from plasmid pKK3535 (Recht *et al.*, 1999).

1408G 16S rRNA containing the additional 1192U mutation were able to grow, with a slightly slower growth rate than otherwise wild-type ribosomes (83 versus 59 min doubling time), in media containing 50 μg/ml spectinomycin, indicating that these mutant ribosomes are functional (Figure 3).
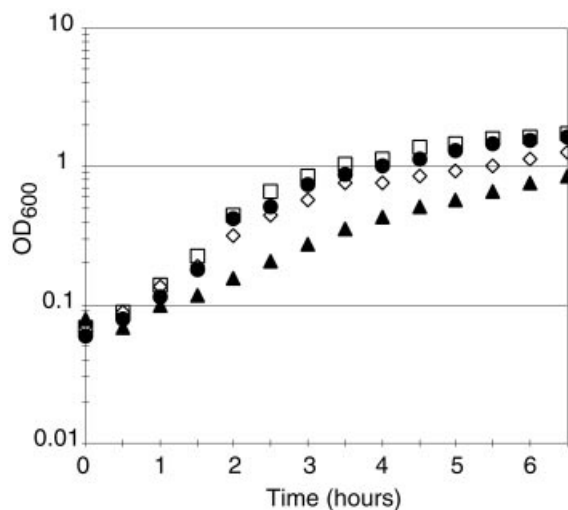
**Fig. 3.** Plots of growth curves for cells expressing plasmid-encoded 16S rRNA containing either the wild-type sequence plus the 1192U mutation grown in ampicillin (□) or spectinomycin (◇), or the 1408G mutation plus the 1192U mutation grown in ampicillin (●) or spectinomycin (▲). Growth was monitored as absorbance at 600 nm. The figure presents data from a single experiment. Similar results were obtained in two separate experiments.

### Aminoglycoside resistance

Expression of the 1408G 16S rRNA, with cells grown in media containing 50 μg/ml spectinomycin, conferred >200-fold resistance to the aminoglycosides kanamycin A, amikacin and apramycin (Table I). In addition, the mutation conferred >64-fold resistance to neomycin, gentamicin C, tobramycin and neamine. Only 4-fold resistance was observed for paromomycin, and there was no resistance conferred to G418. No resistance to streptomycin, a structurally distinct aminoglycoside, was observed.

Resistance was not due to altered expression of the mutant 16S rRNA, as cells grown in the presence of an aminoglycoside did not show an increased proportion of 1408G rRNA. The cells had a slower growth rate in the presence of high concentrations of any aminoglycoside, and growth was not stimulated in the presence of sub-lethal concentrations of the antibiotic. Addition of neomycin to 20 μg/ml to a culture expressing 1408G 16S rRNA caused an increase in doubling time, from 83 to 245 min. Cells expressing 16S rRNA with the 1408G mutation could be propagated in liquid culture in the presence of 40 μg/ml neomycin or 200 μg/ml apramycin, with the same slower growth rate as observed in the presence of 20 μg/ml neomycin.

Growth of cells in the presence of ampicillin instead of spectinomycin, with or without the additional mutation at C1192, caused a similar, but less dramatic, resistance phenotype (4- to 32-fold resistance). In the presence of spectinomycin, ribosomes containing the additional 1192U mutation comprise 75–80% of the actively translating ribosomes (polysome fraction) (Johanson and Hughes, 1995; data not shown) compared with 60–65% in the presence of ampicillin (Recht *et al.*, 1999). Therefore, the addition of spectinomycin enhances the resistance phenotype of the 1408G mutation by removing the aminoglycoside-sensitive genome-encoded ribosomes from the pool of actively translating ribosomes.

**Table I.** Minimum inhibitory concentration of various aminoglycosides against *E.coli* DH1 expressing wild-type or 1408G 16S rRNA

| | Wild-type | | 1408G | | Relative resistance |
|---|---|---|---|---|---|
| | μg/ml | μM | μg/ml | μM | |
| Kanamycin A | 2.5 | 4.3 | 1280 | 2200 | 512 |
| Amikacin | 1.25 | 1.6 | 320 | 410 | 256 |
| Apramycin | 5 | 7.8 | >1280 | >2000 | >256 |
| Neomycin | 5 | 5.3 | 640 | 680 | 128 |
| Gentamicin C | 1.25 | 1.8 | 160 | 230 | 128 |
| Tobramycin | 1.25 | 1.9 | 160 | 240 | 128 |
| Neamine | 20 | 43 | >1280 | >2700 | >64 |
| Paromomycin | 5 | 5.9 | 20 | 24 | 4 |
| G418 | 2.5 | 3.6 | 2.5 | 3.6 | 1 |

The MIC is the minimum concentration of antibiotic that fully inhibited growth after 24 h incubation at 37°C. Relative resistance is the value of the MIC against cells expressing 1408G (priming site V, 1192U) 16S rRNA divided by the MIC against cells expressing wild-type (priming site V, 1192U) 16S rRNA.

### Binding of aminoglycosides to 1408G ribosomes

Aminoglycoside resistance could result from either decreased affinity of the antibiotic for the ribosome or non-productive binding of the drug to the ribosome. To determine if there was a correlation between resistance and low-affinity binding of the aminoglycoside to the 30S subunit, chemical footprinting experiments were performed on 1408G 30S subunits in the presence of increasing concentrations of neomycin. The mutant ribosomes in the mixture could be monitored exclusively by primer extension due to the presence of priming site V (Powers and Noller, 1993), a silent mutation present only in the plasmid-encoded 16S rRNA. Even with 1 mM neomycin present, only a slight footprint is observed at G1494(N7) (Figure 5A). In contrast, when the wild-type neomycin-sensitive 30S subunits in the same reaction are monitored (Figure 5B), neomycin causes a strong footprint at G1494(N7) and A1408(N1) at 10 μM antibiotic.

The cells expressing 1408G 16S rRNA are only slightly resistant to paromomycin. There is a moderate decrease (~10-fold) in paromomycin binding affinity for 1408G mutant 30S subunits. It was demonstrated previously that 100 μM paromomycin causes a weak footprint at G1494(N7) in 30S subunits containing the 1408G mutation (Recht *et al.*, 1999). These results agree with those obtained with a model oligonucleotide corresponding to the *E.coli* decoding region A site (Recht *et al.*, 1996). With an oligonucleotide containing an A1408 to G mutation, paromomycin caused a weak footprint when present at 100 μM (Recht *et al.*, 1996), whereas neomycin, even when present at 1 mM, caused no changes in reactivity at any bases (data not shown). The resistance phenotype is consistent with the footprinting data. A strong footprint is indicative of high-affinity binding of the aminoglycoside, which is presumably a prerequisite for antibiotic action. Resistance therefore arises from decreased aminoglycoside affinity for its ribosomal target.

### Common elements among aminoglycosides

With the exception of apramycin, the aminoglycosides to which expression of 16S rRNA containing the 1408G mutation conferred resistance all contain a 6′ amino group
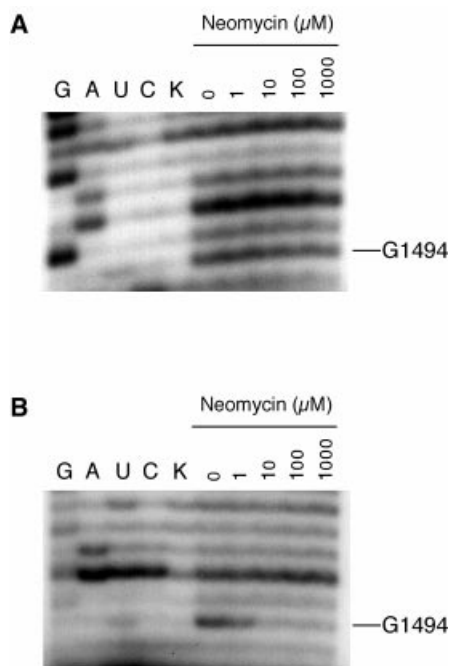
**Fig. 4.** Autoradiograph showing dimethyl sulfate probing experiments in the presence of increasing concentrations of neomycin with (**A**) 1408G and (**B**) wild-type 30S subunits. In all experiments, 30S subunits were present at 100 nM. Neomycin was present at the concentration indicated. In (A), modifications were monitored by primer extension from a DNA oligonucleotide primer complementary to priming site V. For (B), the same reactions as those in (A) were monitored with a primer complementary to the wild-type 1535–1514 sequence. The lanes labeled G, A, U and C are dideoxy sequencing reactions. K is primer extension from 16S rRNA to which no modifying agent was added.



**Fig. 5.** Common elements of aminoglycosides to which expression of the 1408G mutation confers resistance. The 6' amino is required, whereas the substituent at the 2' position can be either an amino or hydroxyl group. Ring II (2-deoxystreptamine) may either be 4,5- or 4,6-disubstituted. X = NH or O.

on ring I of the aminoglycoside (Figure 5). The cells are sensitive to aminoglycosides containing a 6' hydroxyl group (paromomycin and G418). The greatest resistance was observed for aminoglycosides containing the combination of a 6' amino and a 2' hydroxyl group on ring I (kanamycin A and amikacin). All of these aminoglycosides contain 2-deoxystreptamine (ring II), which is either 4,5- or 4,6-disubstituted. This pattern of substitution is neither important for binding to the prokaryotic sequence (Yoshizawa *et al*., 1998) nor for the observed resistance, since 1408G confers resistance to both neomycin and kanamycin (4,5- and 4,6-disubstituted-2-deoxystreptamine, respectively).

## Discussion

The aminoglycoside-binding site on the 30S subunit is the location of the codon–anticodon interaction on the ribosome. The highly conserved sequences of 16S rRNA that make up the decoding region are essential for ribosome function (Zimmermann *et al*., 1990). The results presented here demonstrate that functional ribosomes are produced with an A1408G to G mutation in the decoding region A site of *E.coli* ribosomes. Assuming a conserved mechanism of decoding between prokaryotic and eukaryotic ribosomes, the conservation of nucleotide 1408 within a phylogenetic domain is not critical to ribosome function, as the 1408G mutation can be accommodated by a prokaryo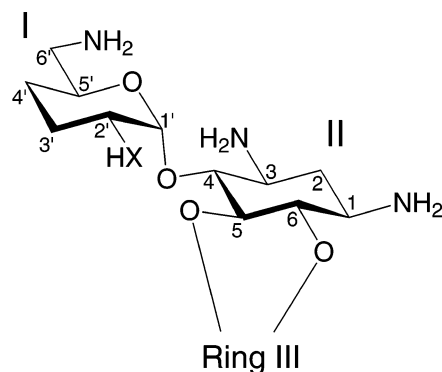tic ribosome. Ribosomes containing a 1408G mutation in 16S rRNA do slow the growth rate of *E.coli* when they are the only active ribosomes in the cell, whereas no growth phenotype is observed when they are present as the majority of a mixture with wild-type ribosomes. This may be due to reduced activity of the 1408G ribosomes in the presence of the antibiotic spectinomycin. Functional interactions between the 1192 region of 16S rRNA, where spectinomycin binds to the ribosome (Moazed and Noller, 1987), and the decoding region have been observed previously (Hui *et al*., 1988). Further studies of the kinetics and accuracy of the 1408G ribosomes may help to explain why this sequence is not normally observed in prokaryotic 16S rRNA.

A low level of aminoglycoside resistance was observed even when ~40% of the actively translating ribosomes were sensitive to the antibiotic. This partial dominance of resistance was unexpected, as aminoglycoside sensitivity was dominant to resistance in *E.coli* heterozygous for resistance to the structurally distinct aminoglycoside streptomycin (Sparling *et al*., 1968). No resistance to streptomycin was observed for 16S rRNA mutations that confer streptomycin resistance unless the wild-type ribosomes were removed from the actively translating pool by addition of spectinomycin (Powers and Noller, 1991). Aminoglycoside resistance with a mixed ribosome population may be specific to the 2-deoxystreptamine antibiotics, as the binding site of, and rRNA resistance mutations to, these antibiotics are localized to the decoding region A site. The precise location of the binding site for streptomycin is still unclear, and rRNA mutations that confer resistance to it may do so in an indirect manner.

Resistance arises from decreased affinity of a subclass of aminoglycosides for ribosomes with the 1408G mutation. All aminoglycosides to which there is significant resistance have changes in the covalent geometry of ring I. The 1408G mutation perturbs the binding pocket for ring I of the aminoglycosides by changing the conformation of the 1408–A1493 base pair. The 6' hydrogen bond donor on the aminoglycoside contacts the phosphodiester backbone between A1492 and A1493 in both the gentamicin C1a (6' NH2) and paromomycin (6' OH) complex with wild-type rRNA (Fourmy *et al*., 1996; Yoshizawa *et al*., 1998) (Figure 6). The 1408G mutation leads to a strong preference for a 6' hydroxyl in the binding pocket. It is
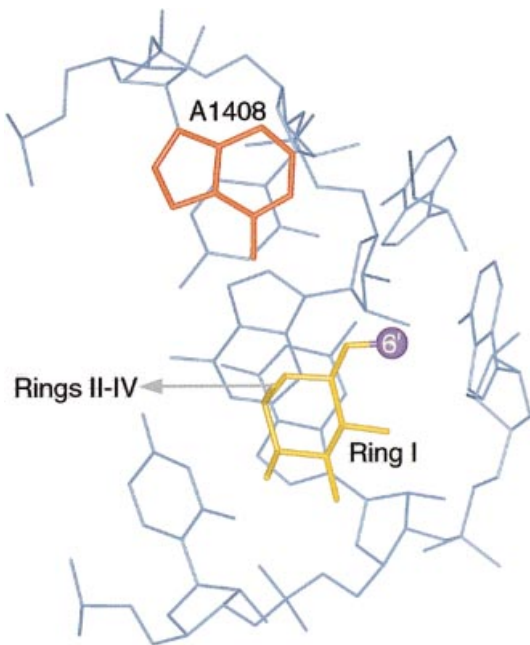
**Fig. 6.** Structure of paromomycin bound to the prokaryotic A site sequence (Fourmy *et al*., 1996). Only ring I of paromomycin (yellow) is shown. The 6′ hydroxyl group (purple) of paromomycin is in the same plane as, but does not directly contact, A1408 (red).

possible that a 6′ amino group, with its positive charge, altered hydrogen bonding capability and larger van der Waals radius, cannot be accommodated in the altered binding pocket. NMR structural studies on the 1408G mutant RNA indicate a subtle conformational change in the aminoglycoside-binding site (S.R.Lynch and J.D.Puglisi, unpublished), which then leads to large effects on aminoglycoside activity.

Our results are consistent with previous reports of aminoglycoside resistance and *in vitro* antibiotic activity. Resistance to kanamycin and apramycin in clinical isolates of *Mycobacterium* species is caused by mutation of A1408 to G (Alangaden *et al*., 1998; Prammananan *et al*., 1998). In addition, *Streptomyces tenjimariensis*, which produces the aminoglycoside istamycin, methylates the N1 position of A1408 of its 16S rRNA. This methylation precludes formation of the A1408·A1493 base pair and confers resistance to kanamycin and apramycin, but not to paromomycin (Skeggs *et al*., 1985). The 1408G substitution has a phenotypic effect on aminoglycoside action similar to the methylation of A1408. *In vitro* translation assays indicate that aminoglycosides containing a 6′ hydroxyl group on ring I are the most effective in stimulating misreading with eukaryotic ribosomes, whereas aminoglycosides with a 6′ amino group are less active (Wilhelm *et al*., 1978).

The eukaryotic organisms *Tetrahymena thermophila* and *Giardia lamblia* are sensitive to aminoglycosides containing a 6′ hydroxyl group (Palmer and Wilhelm, 1978; Edlind, 1989). The 18S rRNA from these organisms contains a 1409–1491 base pair (*E.coli* numbering), which is present in prokaryotes but normally absent in higher eukaryotes (Figure 2) (Gutell, 1994). Disruption of this base pair in *E.coli* has been shown to confer resistance to most 2-deoxystreptamine aminoglycosides, including those containing a 6′ hydroxyl (DeStasio *et al*., 1989;

DeStasio and Dahlberg, 1990). The presence of both 1408G and a mismatch at positions 1409–1491 most likely decreases the affinity of aminoglycosides for the eukaryotic ribosome more than either base substitution alone.

The results presented here indicate that the specificity of aminoglycosides for prokaryotic ribosomes is largely determined by the presence of an adenosine at nucleotide 1408. Since we observe resistance to the aminoglycosides normally inactive against eukaryotic ribosomes by mutation of 1408 to a guanosine in the context of the *E.coli* ribosome, we conclude that this sequence variation between prokaryotes and eukaryotes is what makes the aminoglycosides useful as antibiotics. These results could aid in the design of new aminoglycosides that possess greater specificity for prokaryotic ribosomes.

## Materials and methods

### Mutagenesis and expression of plasmid-encoded 16S rRNA
The 1408G mutation was introduced into the plasmid pKK3535, and expressed from the natural *rrnB* promoters as described previously (Recht *et al*., 1999). This mutation was introduced both with and without the additional mutation 1192U, which confers resistance to spectinomycin (Sigmund *et al*., 1988), as well as the allele-specific priming site V mutation (Powers and Noller, 1993). All mutant 16S rRNAs were expressed from the *rrnB* promoters of pKK3535. Quantitation of plasmid-encoded rRNA was performed as described (Recht *et al*., 1999).

In chemical modification experiments, the priming site V mutation allowed exclusive monitoring of plasmid-encoded 16S rRNA in the mixture of plasmid- and genome-encoded 16S rRNA. The priming site V mutation does not affect the interaction of aminoglycosides with the ribosome (Recht *et al*., 1999). As a control in all *in vivo* experiments, the wild-type 16S sequence containing priming site V was expressed from the plasmid pKK3535.

### Determination of minimum inhibitory concentration (MIC)
Cultures were started from single colonies and grown in LB media containing 50 µg/ml spectinomycin for 1192U plasmids or 100 µg/ml ampicillin for 1192C plasmids. MIC tests were performed by inoculation of 5 ml cultures with a 1:1000 dilution of overnight culture in LB media containing spectinomycin (or ampicillin) plus a 2-fold series of dilutions of one of the following aminoglycosides: paromomycin, neomycin (a mixture of neomycin B and C), gentamicin C (a mixture of C1, C1a and C2), kanamycin A, amikacin, apramycin, tobramycin, neamine or G418.

The reported MIC is the concentration of aminoglycoside at which the growth of the cultures was completely inhibited after 24 h incubation at 37°C.

To monitor bacterial growth, cultures were inoculated with a 1:200 dilution of an overnight culture in LB media containing spectinomycin or ampicillin. For experiments in which neomycin was added, cells were allowed to grow until the culture reached an $OD_{600}$ of ~0.2. Neomycin was added to a final concentration of 20 µg/ml and growth was monitored for an additional 6–8 h.

### Chemical modification of 30S subunits
Preparation of 30S subunits and chemical modification experiments were performed as described (Recht *et al*., 1999).

Primer extension from priming site V was performed as described (Recht *et al*., 1999). Primer extension from the 1513 primer was performed as described (Stern *et al*., 1988) using a 21 nucleotide DNA primer complementary to nucleotides 1535–1514 of the wild-type *E.coli* 16S rRNA sequence.

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