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Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria

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ABSTRACT

Structural and genetic studies on prokaryotic ribosomes have provided important insights into fundamental aspects of protein synthesis and translational control and its interaction with ribosomal drugs. Comparable mechanistic studies in eukaryotes are mainly hampered by the absence of both high-resolution crystal structures and efficient genetic models. To study the interaction of aminoglycoside antibiotics with selected eukaryotic ribosomes, we replaced the bacterial drug binding site in 16S rRNA with its eukaryotic counterpart, resulting in bacterial hybrid ribosomes with a fully functional eukaryotic rRNA decoding site. Cell-free translation assays demonstrated that hybrid ribosomes carrying the rRNA decoding site of higher eukaryotes show pronounced resistance to aminoglycoside antibiotics, equivalent to that of rabbit reticulocyte ribosomes, while the decoding sites of parasitic protozoa show distinctive drug susceptibility. Our findings suggest that phylogenetically variable components of the ribosome, other than the rRNA-binding site, do not affect aminoglycoside susceptibility of the protein-synthesis machinery. The activities of the hybrid ribosomes indicate that helix 44 of the rRNA decoding site behaves as an autonomous domain, which can be exchanged between ribosomes of different phylogenetic domains for study of function.

INTRODUCTION

Accurate decoding of genetic information is a crucial step in protein synthesis. Genetic, biochemical and structural data provide evidence for a functional role of ribosomal RNA in mRNA decoding and tRNA selection (1–3). The functional relevance of rRNA residues in codon–anticodon recognition and stabilization is reflected in their universal conservation throughout the three phylogenetic domains of life. While this holds true for most nucleotides in helices 18, 34 and 44 of the small subunit rRNA that form the aminoacyl-tRNA acceptor site (A site), critical variations have evolved between different phylogenetic domains, most prominent in helix 44, the penultimate stem of 16S rRNA (4). These variations most likely account for a decoding region that has been highly optimized in the context of evolutionary differentiation.

Aminoglycosides are a class of structurally related antibiotics which interfere with decoding by binding to the A site of small subunit rRNA (5). These antibiotics preferentially target prokaryotic over eukaryotic ribosomes and affect protein synthesis by inducing codon misreading and inhibiting tRNA translocation (6–8). The binding site is located within a conserved loop of helix 44, which in part shows phylogenetic sequence variability, e.g. at position 1408 and at base pair 1409–1491 (9) (rRNA residues are numbered according to *Escherichia coli* nomenclature; see also Figure 2). Crystal structures of various bacterial 30S ribosomal particles in complex with different ligands, e.g. tRNA and antibiotics, have revealed the molecular mechanisms of the decoding step at atomic resolution (10,11). In the absence of high-resolution X-ray crystal structures of eukaryotic ribosomes, model oligonucleotides mimicking the ribosomal decoding site have been used for structural analysis of the A site in human cytosolic ribosomes (12).

Site-directed mutagenesis has been used in prokaryotes to study the functional relevance of individual drug–nucleotide contacts in aminoglycoside–ribosome interaction (13). A detailed analysis of the aminoglycoside target site in ribosomes of higher and lower eukaryotes is complicated by the complexity of eukaryotic rRNA genetics. Genetic manipulation of the rRNA component of eukaryotic ribosomes has proven exceedingly difficult

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

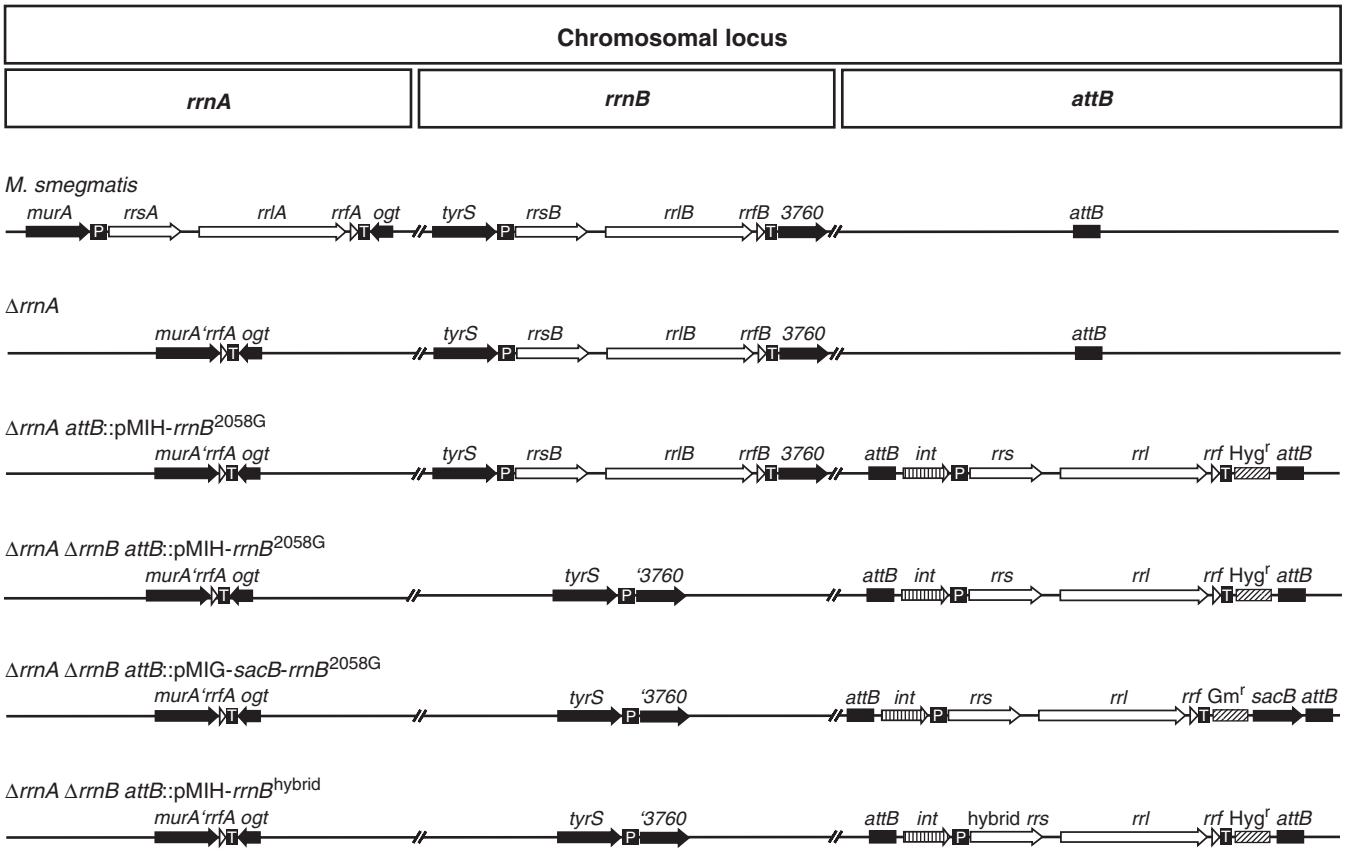

Figure 1. Sequential strategy for the generation of a plasmid-rRNA exchange system. From top: Following deletion of chromosomal *rrnA*, a complementation vector pMIH-*rrnB*^{2058G} carrying a functional *rrn* operon was introduced to the chromosomal *attB* site. Subsequent deletion of *rrnB* resulted in *M. smegmatis* Δ*rrnA* Δ*rrnB attB*::pMIH-*rrnB*^{2058G}, in which ribosomal RNA is exclusively transcribed from the plasmid. From there, a plasmid-rRNA exchange system was established by replacing pMIH-*rrnB*^{2058G} with pMIG-*rrnB-sacB*. Transformation with hybrid rRNA genes pMIH-*rrnB*^{hybrid} and selection on sucrose resulted in *M. smegmatis* Δ*rrnA* Δ*rrnB attB*::pMIH-*rrnB*^{hybrid} with homogenous populations of hybrid ribosomes.

due to the problem of the high copy number of corresponding operons. Higher eukaryotes have so far resisted any genetic manipulation of their ribosomal nucleic acids, virtually abolishing the possibility to test hypotheses by experimentation. Even in lower eukaryotic organisms, such as yeast, the presence of 100–200 tandemly repeated copies at the *RDN* locus, has made genetic studies of rRNA difficult (14–16).

The eukaryotic ribosome differs significantly from the prokaryotic one (17). It is impossible to predict the contribution of additional, phylogenetically diverse ribosomal elements and characteristics, as in the eukaryotic ribosome, to aminoglycoside susceptibility. It is, for instance, commonly assumed that translation in mammalian cells functions with higher fidelity than that of bacteria (18). To address this question experimentally, we have performed domain shuffling experiments in rRNA. Using genetic techniques, we successfully engineered a functionally critical domain in the ribosome by replacing the bacterial A-site rRNA of helix 44 with its counterpart from higher and lower eukaryotes. For functional characterization of these hybrid ribosomes, we studied their susceptibility to antibiotics targeting the ribosomal A site and which are known to affect bacterial versus eukaryotic ribosomes to different extents.

MATERIALS AND METHODS

Construction of the *rrn* plasmid exchange system

Starting with *Mycobacterium smegmatis* mc^2-155 SMR5 (19), a single-rRNA allelic strain bearing a deletion of ~5 kb in the chromosomal *rrnA* operon was constructed by gene replacement. The deletion strategy for chromosomal rRNA operons is shown in Supplementary Figure 1. Subsequently and following integration of the *rrnB*-replacement vector into the chromosomal *rrnB* locus, the strain was transformed with integration-proficient vector pMIH-*rrnB*^{2058G} for rRNA complementation. pMIH-*rrnB*^{2058G} is a derivative of plasmid pMV361-*rrnB*^{2058G} (20), in which the kanamycin resistance cassette has been replaced with a hygromycin resistance cassette. Counterselection and genetic screening for deletion of the chromosomal *rrnB* locus resulted in strain *M. smegmatis* Δ*rrn* pMIH-*rrnB*^{2058G} bearing unmarked full deletions of the chromosomal *rrnA* and *rrnB* loci. In this strain, ribosomal RNA is exclusively transcribed from the complementation vector (Figure. 1).

To establish a genetic system for efficient plasmid exchange, an integration-proficient complementation vector pMIG-*rrnB-sacB* was designed that carries the *rrnB* wild-type operon, a gentamicin resistance cassette,

and the sucrose sensitivity marker *sacB*. This vector was used to replace pMIH-*rrnB*^{2058G} in strain *M. smegmatis* Δ*rrn* pMIH-*rrnB*^{2058G}, resulting in strain *M. smegmatis* Δ*rrn* pMIG-*rrnB-sacB*. The latter was used as plasmid exchange system in which the *rrnB* wild-type operon can be efficiently replaced with an *rrnB* mutant operon by transformation and selection on sucrose-containing media (Figure. 1).

Construction of mutant strains with hybrid ribosomes

Site-directed mutagenesis of rRNA genes was performed by PCR mutagenesis as described previously (21). In brief, hybrid rDNA oligonucleotides comprising the eukaryotic helix 44 decoding site sequence were used for gene amplification. Using restriction endonuclease recognition sites present in the operon, rRNA gene fragments in plasmid pMIH-*rrnB* were replaced to construct pMIH-*rrnB*^{hybrid}, the plasmids coding for hybrid 16S rRNA genes. *Mycobacterium smegmatis* Δ*rrn* pMIG-*rrnB-sacB* was transformed with the various pMIH-*rrnB*^{hybrid} constructs and selected on hygromycin plates containing sucrose (Figure 1). Successful plasmid exchange was controlled by sequence analysis.

Minimal inhibitory concentrations

Broth microdilution tests were performed in a microtiter plate format as described previously (22). In brief, bacterial strains were cultured on Luria–Bertani (LB) agar plates at 37°C. Freshly grown cultures were resuspended in LB broth supplemented with 0.05% of Tween 80, diluted to an absorbance at 600 nm of 0.025, and incubated in the presence of 2-fold serial dilutions of the following 2-deoxystreptamine aminoglycosides: paromomycin, neomycin, geneticin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (Sigma). After incubation at 37°C for 72 h, the minimal inhibitory concentration (MIC) was recorded as the lowest concentration of drug inhibiting visible growth.

Ribosome purification

Approximately 8 g of wet bacterial cell mass were resuspended in 25 ml of homogenization buffer (HB; 20 mM Tris–HCl pH 7.4, 100 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA and 3 mM 2-mercaptoethanol) and lyzed with a French Pressure Cell (American Instrument Company, Maryland) at 16 000 psi, as described previously (23). The lysate was supplemented with DNase (RQ1 RNase-free, Promega, 2 U per gram cell mass), incubated on ice for 10 min, and adjusted with HB to a volume of 70 ml. Pre-cooled alumina (1.5 g per gram cell mass) was added to the cell lysate, stirred on ice for 20 min, and removed by low-speed centrifugation (10 min at 2000*g*; all centrifugations were performed at 4°C). Cell debris was sedimented by centrifugation for 30 min at 10 000*g* and the lysate was passed through a tea filter. Centrifugation of the lysate for 60 min at 32 500*g* resulted in the S-30 supernatant. Sixteen milliliter portions of the supernatant were layered on 9 ml sucrose cushions (SC; 20 mM Tris–HCl pH 7.4, 350 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, 1.1 M sucrose and 3 mM 2-mercaptoethanol) and centrifuged for 15 h at 110 000*g* (Beckman Coulter Optima™ L-80 XP Ultracentrifuge) to separate ribosomal particles from the S-100 supernatant. The S-100 fraction was dialyzed for 24 h (6000–8000 MWCO, Spectra/Por, Serva) with three intermittent changes of buffer A (50 mM Tris–HCl pH 7.5, 70 mM NH_4Cl, 30 mM KCl and 7 mM $MgCl_2$), concentrated (Viva spin, 10 000 MWCO, Viva Science), and stored at −80°C. Ribosome pellets from the S-100 preparation were resuspended in washing buffer (WB; 20 mM Tris–HCl pH 7.4, 350 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA and 7 mM 2-mercaptoethanol) and passed twice through sucrose cushions (6 h at 180 000*g* and 16 h at 83 000*g*). The final ribosome pellets were resuspended in buffer A, incubated for 30 min at 4°C, dispensed into aliquots, and stored at −80 C after shock freezing in liquid nitrogen. 70S ribosome concentrations were determined by absorption measurements on the basis of 23 pmol per A_{260} unit.

Qualitative evaluation of ribosome preparations

Integrity of 70S ribosomes was determined by analytical ultracentrifugation (14 h at 70 000*g*) through a 10–40% sucrose gradient in both association buffer (buffer A) and dissociation buffer (50 mM Tris–HCl pH 7.5, 70 mM NH_4Cl and 30 mM KCl). 70S, 50S and 30S ribosome fractions in different gradient layers were detected and quantified by absorption at 254 nm. Functional activity of purified ribosomes was determined by assessing their capacity to form initiation complexes. 70S ribosomes (0.5 μM) were incubated in buffer A with m022 SD-MFTI-mRNA at 1.5 μM (5′-GGCAAGGAGGUAAAUAAUG UUCACGAUC-3′; obtained from Dharmacon), initiation factors (IF) 1 and 3 from *M. smegmatis*, IF-2 from *E. coli* (1 μM each), GTP (1 mM), and [^{3}H]-fMet-tRNA^{fMet} (1 μM) for 60 min at 37°C; purified tRNA and IF2 from *E. coli* were a kind gift from Marina Rodnina. The initiation complexes formed were bound to nitrocellulose filters (Sartorius, pore size 0.45 μm), washed with 15 ml cold buffer A, dissolved in 10 ml scintillation cocktail (Filtersafe, Zinsser Analytic) and quantified in a Liquid Scintillation Analyzer (Tri-Carb 2900 TR, PerkinElmer).

Cell-free $(UUU)_{12}$ translation assays

Cell-free translation reactions in buffer A (pH 7.5) were prepared on ice and contained *M. smegmatis* tRNA^{bulk} (0.5 mg/ml), amino acids mixture (30 μM each) lacking phenylalanine and/or leucine, 10% (v/v) S100 extract, energy mix [DTT (1 mM), GTP (1 mM), ATP (4 mM), PEP (5 mM)], pyruvate kinase (0.1 mg/ml), and polyamines [spermidine (2 mM) and putrescine (8 mM)]. The reaction mixture was preincubated with radiolabeled phenylalanine and/or leucine (30 μM; obtained from Amersham) at 37°C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM and $(UUU)_{12}$-mRNA (5′-GCGGCAA GGAGGUAAAUA AUG $(UUU)_{12}$ UAA GCAGG-3′, obtained from Dharmacon) to 1 μM; serially diluted aminoglycoside antibiotics were added simultaneously. Following incubation at 37°C for 60 min, the reaction was

stopped by addition of KOH to 0.5 M and subsequent hydrolysis at 37°C for 30 min. Synthesized polypeptides were precipitated with 200 µl of 5% tri-chloro-acetic acid (TCA) for 10 min on ice and bound to nitrocellulose filters (Sartorius, pore size 0.45 µm). Filter-bound polypeptides were washed with cold 30% 2-propanol and quantified in 10 ml of scintillation cocktail. Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of polypeptide synthesis and to calculate the aminoglycoside IC_{50} values of dose-dependent inhibition (Figure 3 and Table 2).

Cell-free luciferase translation assays

Purified 70S hybrid ribosomes were used in a coupled transcription–translation reaction of firefly luciferase (plasmid pBESTluc, Promega). A typical reaction (15 µl volume) contained 0.25 µM 70S ribosomes, 300 ng DNA, 40% (v/v) of *M. smegmatis* S100 extract, 100 µM amino acid mixture, and RNasin (40 U, Promega). rNTPs, tRNAs and energy were supplied by addition of commercial S30 Premix Without Amino Acids (Promega). Serially diluted aminoglycosides were added and the reaction mixture was incubated at 37°C for 60 min. The reaction was stopped on ice, 100 µl of luciferase assay substrate (Promega) was added, and the bioluminescence was measured in a luminometer (Bio-Tek instruments, FLx800). Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of luciferase synthesis and to calculate the aminoglycoside IC50 values of dose-dependent inhibition (Figure 4 and Table 3).

Rabbit reticulocyte translation assays

Cytosolic ribosomes present in commercially available rabbit reticulocyte lysate (Promega) were used for *in vitro* translation of a firefly luciferase control mRNA (Promega). A standard 30 µl reaction contained 20 µl reticulocyte lysate, 500 ng of luciferase mRNA, an amino acid mixture (100 µM each), and RNasin. Serially diluted aminoglycosides were added and the reaction mixture was incubated at 30°C for 90 min. After incubation, 100 µl of luciferase assay substrate (Promega) was added and luciferase activity was determined.

RESULTS AND DISCUSSION

The two chromosomal *rrn* operons in *M. smegmatis* were inactivated to produce a strain in which ribosomal RNA is exclusively transcribed from plasmid-encoded rRNA genes. Operon inactivation was achieved by means of unmarked deletion mutagenesis spanning the complete rRNA gene sequences and part of the promoter region (Supplementary Figure 1). For complementation, a fully functional wild-type *rrnB* operon encompassing promoter and termination sequences was cloned into the integration-proficient plasmid pMIG-*sacB*. In *M. smegmatis* Δ*rrn* pMIG-*sacB*-*rrnB*, the wild-type rRNA operon carried on pMIG-*sacB* is efficiently replaced with a mutant rRNA operon by plasmid exchange following transformation with integration-proficient plasmid pMIH-$rrnB^{mut}$ (for details, see the Materials and Methods section and Figure 1). The rRNA plasmid exchange system was used to replace the bacterial 16S rRNA helix 44 within the decoding region with its homologous structures from parasitic kinetoplastid protozoa. The kinetoplastid genera *Leishmania* and *Trypanosoma* comprise species that are responsible for leishmaniasis and trypanosomiasis, diseases that are widespread in many tropical and subtropical countries. The insect trypanosome *Blastocrithidia* is characterized by an rRNA decoding site with an adenine at the position homologous to 16S rRNA residue 1408, and therefore represents a very rare trait within the eukaryotic domain. We also constructed human–bacterial hybrid ribosomes by introducing the human helix 44 rRNA homolog into functional ribosomes of strain *M. smegmatis* Δ*rrnB* by a method described previously (13). Both approaches resulted in bacterial mutant strains with homogenous populations of hybrid ribosomes in which a 34 nucleotide portion of helix 44 was substituted with its counterpart of eukaryotic small-subunit rRNA (Figure 2). Together with A-site residues in helices 18, 34 and 44 that are universally conserved throughout the phylogenetic domains, the engineered decoding site resembles that of eukaryotic cytosolic ribosomes. Engineering the eukaryotic decoding sites into bacterial ribosomes had relatively little effect on cell growth, with only slightly increased generation times as compared to wild-type cells (generation times: wild-type 3.7 ± 0.3 h; *Leishmania* hybrids 4.8 ± 0.5 h; *Blastocrithidia* hybrids 4.2 ± 0.1 h; *H. sapiens* hybrids 5.1 ± 0.6 h).

A bacterial oligoribonucleotide analog of 16S rRNA helix 44 has been shown to interact with both antibiotic and RNA ligands of the 30S subunit in a manner that correlates with normal subunit function (24). Since the decoding-site rRNA in the hybrid ribosomes is identical to that of eukaryotic ribosomes, the hybrid ribosomes may serve as a model to investigate specific features associated with the eukaryotic A-site rRNA structure. Towards this end, we investigated the susceptibility of the hybrid ribosomes to the 2-deoxystreptamine antibiotics paromomycin, neomycin, geneticin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (see Supplementary Figure 2 for chemical structures). In MIC assays, which determine growth inhibition at the whole cell level, *M. smegmatis* mutants carrying human–bacterial hybrid ribosomes were highly resistant to all aminoglycoside antibiotics tested except for geneticin (Table 1). Relative resistance of the human hybrids to geneticin, calculated as the ratio of mutant to wild-type MIC, was 8 to 16 as compared to ≥ 1024 for paromomycin, neomycin, gentamicin, netilmicin, tobramycin, and kanamycin. The *Leishmania* hybrids showed high-level resistance to neomycin, gentamicin, netilmicin, tobramycin and kanamycin, with relative resistance levels of ≥ 1024. However, the *Leishmania* hybrids showed distinct susceptibility to paromomycin and geneticin, i.e. aminoglycosides with a hydroxyl group at position 6′ of ring I. *Mycobacterium smegmatis* hybrids with a *Blastocrithidia* decoding region were susceptible to all aminoglycoside antibiotics; the minimal inhibitory concentrations of the 4,6- and

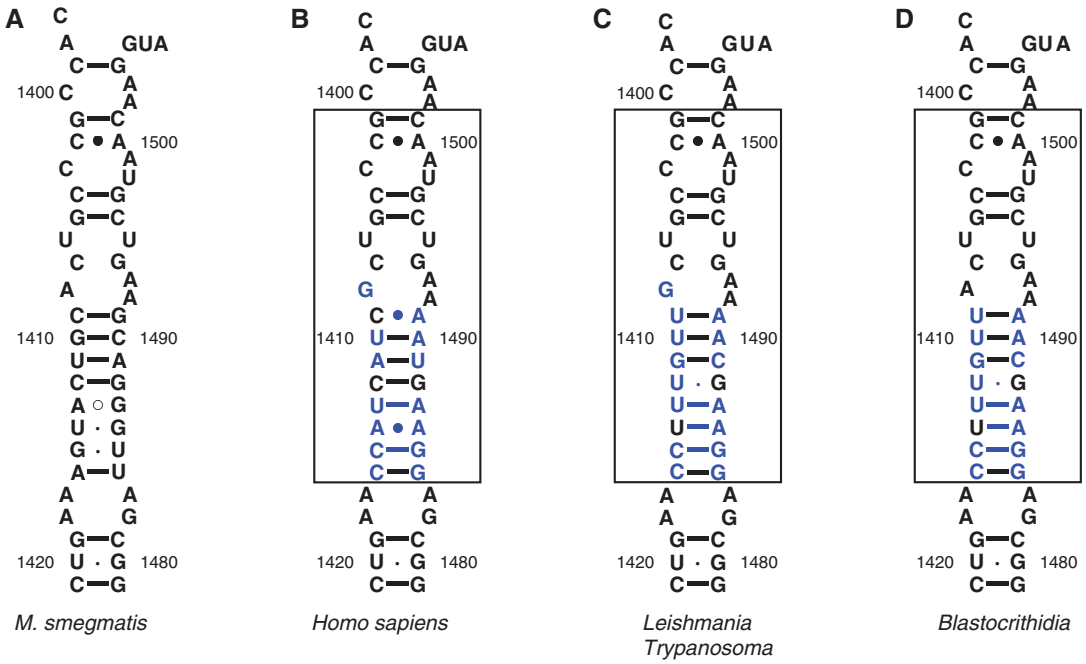

Figure 2. 16S rRNA sequence within helix 44 of *M. smegmatis* wild-type and hybrid ribosomes after transplanting the A-site rRNA of eukaryotic ribosomes. (**A**) *Mycobacterium smegmatis*. (**B**) Human–bacterial hybrid ribosomes. (**C**) Hybrid ribosomal RNA containing the decoding-site rRNA of the protozoan *Leishmania*, which is also identical to *Trypanosoma*; and (**D**) *Blastocrithidia*. Base substitutions rendering the bacterial 16S rRNA eukaryotic are depicted in blue; the transplanted region is boxed. rRNA residues are numbered according to the nucleotide numbering used in *E. coli* 16S rRNA.

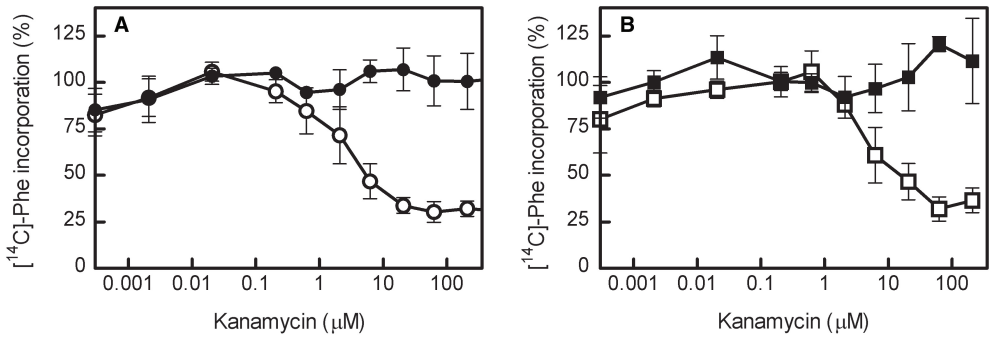

Figure 3. Kanamycin-induced inhibition of polypeptide synthesis using $(UUU)_{12}$-directed phenylalanine incorporation. (**A**) *Homo sapiens* cytosolic hybrid ribosomes (closed circles) versus *M. smegmatis* wild-type ribosomes (open circles). (**B**) *Leishmania* (closed squares) versus *Blastocrithidia* (open squares) hybrid ribosomes. The relative amount of [^{14}C]-phenylalanine incorporated by 5 pmol of purified 70S ribosomes after 60 min. incubation in the presence of varying concentrations of kanamycin A is shown. SEs are indicated. The corresponding IC_{50} values for kanamycin A and selected aminoglycoside antibiotics are given in Table 2.

4,5-disubstituted deoxystreptamines against *Blastocrithidia* hybrids were similar to those observed for the wild-type *M. smegmatis* strain (Table 1).

To analyze the antibiotic effects on translation in more detail, we determined the IC_{50} values for selected aminoglycosides in $(UUU)_{12}$-mRNA-directed phenylalanine incorporation in cell-free translation assays. *In vitro* translation assays were established with purified 70S ribosomes of wild-type and mutant *M. smegmatis* strains. A polyphenylalanine mRNA with a ribosomal-binding site, twelve consecutive (UUU) triplets coding for phenylalanine, and a stop codon was used to assess aminoglycoside-induced inhibition of [^{14}C]-phenylalanine incorporation in translating wild-type and hybrid ribosomes. Bacterial wild-type ribosomes were highly susceptible to all aminoglycosides tested, with IC_{50} values for poly-Phe synthesis below 3 μM (Figure 3 and Table 2). Hybrid ribosomes with a *Blastocrithidia* decoding region were likewise susceptible to low aminoglycoside concentrations. In contrast, hybrid ribosomes with the human decoding site were resistant, exhibiting IC_{50} values that are more than 500-fold higher than those observed in bacterial wild-type ribosomes. *Leishmania* hybrid ribosomes were resistant to all aminoglycosides tested except paromomycin. In order to assess aminoglycoside-stimulated misreading (25) in hybrid ribosomes, paromomycin was used

to measure drug-induced incorporation of leucine in a cell-free $(UUU)_{12}$ translation assay. In line with the other assays used to determine ribosomal drug susceptibility, the *Leishmania* hybrids were significantly more susceptible to paromomycin-induced mistranslation than the human hybrids (data not shown).

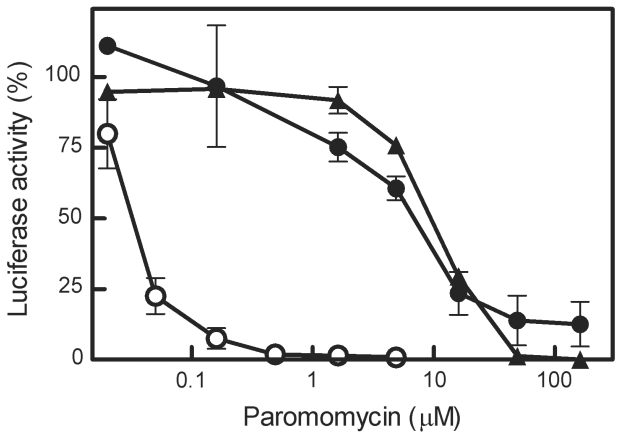

Figure 4. Paromomycin-induced inhibition of protein synthesis measured as luciferase activity in cell-free translation assays of firefly luciferase mRNA. Rabbit reticulocyte (closed triangles) versus human-bacterial hybrid (closed circles) and wild-type *M. smegmatis* (open circles) ribosomes; error bars represent the SEM ($n = 3$). The corresponding IC_{50} values for paromomycin and the aminoglycosides tested are given in Table 3.

Table 1. Activity of aminoglycoside antibiotics against cells carrying hybrid ribosomes

Compound	MIC (μg/ml)			
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*
Paromomycin	1	≥1024	128	4
Neomycin	0.5	>1024	>1024	1
Geneticin	8	128	4	4
Gentamicin	1	>1024	>1024	1
Netilmicin	2	>1024	>1024	8
Tobramycin	1	1024	1024	2
Kanamycin A	1	>1024	>1024	1

We next wished to validate the hybrid ribosome approach by comparing the human–bacterial hybrid ribosomes to rabbit reticulocyte ribosomes (the decoding-site rRNA of human cytosolic ribosomes is identical to that of most vertebrates, including rabbit). In addition, with a view to define the selectivity of aminoglycoside compounds for protozoan versus human ribosomes more accurately, we wished to use an assay which is not limited by its sensitivity. Note that both the whole-cell determination of minimal inhibitory concentrations (MIC; Table 1) and the $(UUU)_{12}$ assay using purified ribosomes (Table 2) are limited by the amount of drug which can be applied. Towards this end we analyzed synthesis of luciferase based upon a coupled transcription–translation assay. Figure 4 shows the effect of paromomycin on protein synthesis in bacterial versus human–bacterial hybrid ribosomes and compared to rabbit reticulocyte ribosomes translating a luciferase mRNA. The paromomycin concentration required to inhibit synthesis of active luciferase in human-hybrid ribosomes to 50% (IC_{50}) closely resembled the IC_{50}

Table 2. Aminoglycoside-induced inhibition of $(UUU)_{12}$-directed phenylalanine incorporation

Compound	IC_{50} (μM)[a]			
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*
Paromomycin	0.9	>500	96	2.7
Neomycin	0.4	>500	>500	0.7
Gentamicin	0.7	>500	>500	0.7
Netilmicin	0.7	>500	>500	1.1
Tobramycin	1.0	>500	>500	2.3
Kanamycin A	2.5	>500	>500	7.5

[a]Aminoglycoside concentrations required to inhibit [^{14}C]-phenylalanine incorporation to 50 percent (IC_{50}). Inhibition kinetics are exemplified by the graphs for kanamycin A presented in Figure 3. Best-fit nonlinear regression was used to define the 100% value and to calculate the IC_{50}. An antibiotic concentration of 1 μM corresponds to approximately four aminoglycoside molecules per ribosome.

Table 3. Aminoglycoside-induced inhibition of luciferase synthesis

Compound	IC_{50} (μM)[a]				
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*	Rabbit reticulocyte lysate[b]
Paromomycin	0.03	4.7	0.18	0.05	9.2
Neomycin	0.04	13	6.7	0.05	18
Geneticin	0.03	0.8	0.02	0.02	0.2
Netilmicin	0.05	64	127	0.11	58
Tobramycin	0.02	21	60	0.05	38
Kanamycin A	0.05	116	204	0.03	67

[a]Aminoglycoside concentrations required to inhibit synthesis of active luciferase to 50 percent (IC_{50}). Inhibition kinetics are exemplified by the graphs for paromomycin presented in Figure 4. Best-fit nonlinear regression was used to define the 100% value and to calculate the IC_{50}.
[b]The rRNA decoding region of rabbit reticulocyte ribosomes is identical to that of human cytosolic ribosomes shown in Figure 2B.

in rabbit reticulocyte lysate. In fact, the IC_{50} values of the human hybrid ribosomes are virtually identical to the IC_{50} values determined for rabbit cytosolic ribosomes for all aminoglycosides tested in this study (Table 3). In contrast, the IC_{50} values in bacterial ribosomes were two to three orders of magnitude lower. Thus, transplanting helix 44 of the eukaryotic decoding region into bacterial ribosomes testified to be a valid approach for studying the specificity of aminoglycoside antibiotics, compounds that selectively affect prokaryotic versus eukaryotic ribosomes.

Analysis of aminoglycoside-induced inhibition of luciferase synthesis in *Leishmania* hybrid ribosomes and comparison to human hybrid ribosomes also allowed quantifying the differences in drug susceptibility. *Leishmania* hybrid ribosomes are ~20- to 30-fold more susceptible to the 4,5-disubstituted aminoglycoside paromomycin than human hybrid ribosomes. In line with the results from MIC determinations (Table 1) and aminoglycoside-induced inhibition of $(UUU)_{12}$-driven phenylalanine incorporation (Table 2), there is a significant difference in drug susceptibility between *Leishmania* hybrid and human hybrid ribosomes for aminoglycosides with a 6′-hydroxyl group. This is in contrast to aminoglycosides with a 6′-amino group where no such difference is present.

The basic mechanisms of translation of mRNA to protein are conserved throughout all organisms (17,26). Experimental data suggest that the selectivity of aminoglycoside antibiotics for bacterial ribosomes is due to defined phylogenetic variations of ribosomal RNA in an otherwise highly conserved binding site (13). The results obtained with the hybrid ribosomes are in excellent agreement with the drug susceptibilities determined in rabbit reticulocyte lysate (Table 3) and with data reported in the literature on complete eukaryotic ribosomes (25,27), demonstrating that the model system adequately reflects the situation in eukaryotic cytosolic ribosomes. Drugs targeting protein synthesis are infrequently used for treatment of infections with lower eukaryotes such as protozoan parasites, helminths or fungi, although the potential for selective activity appears to exist (28–33). Using various assays of drug activity, we demonstrated that the 4,5-disubstituted aminoglycoside paromomycin exhibits a distinct activity towards hybrid ribosomes carrying the cytosolic decoding site of *Leishmania* and *Trypanosoma*. These results provide an explanation for the activity of paromomycin in treatment of leishmaniasis (34,35), by pointing to the cytosolic ribosome as a drug target. Little treatment options involving quite toxic drugs, such as the trivalent arsenic compound melarsoprol, exist for late stage *T. brucei rhodiense* sleeping sickness (36). The availability of compounds with antitrypanosomal activity would be most significant, if only as second-line option for patients who experience relapse.

Defined point mutations have been introduced previously into the bacterial rRNA A site to study individual drug—nucleotide interactions of aminoglycosides (13). These studies demonstrated the role of nucleotides 1408, 1409 and 1491 in drug binding. A base substitution of adenine at position 1408 with a guanine interrupts the pseudo-base-pair formation of the adenine with ring I of the disubstituted 2-deoxystreptamines and alterations of the 1409–1491 bp interfere with the stacking interaction between 1491 and ring I. Our study complements this reductionistic approach by transferring the complete eukaryotic binding site into bacterial ribosomes. Given the complex structure of the ribosome and its various components that contribute to translational fidelity, e.g. ribosomal proteins S4, S5 and S12 (3), it is remarkable that a hybrid bacterial ribosome carrying the eukaryotic H44 shows a drug-susceptibility pattern identical to that of complete rabbit reticulocyte ribosomes. From this we conclude that additional phylogenetically variable ribosomal structures do not affect aminoglycoside susceptibility of the complete eukaryotic ribosome. In line with previous findings (13,37–39), a more general picture emerges, where ribosomes with a 1408 guanine in small subunit rRNA are resistant to aminoglycosides carrying an amino group at position 6′ of ring I (neomycin, gentamicin, netilmicin, tobramycin, kanamycin), while A sites with a non-canonical base-pair interaction between residues 1409 and 1491 are predominantly resistant to geneticin and paromomycin, drugs which carry a hydroxyl group at the 6′ position of ring I. Together, these results suggest that paromomycin and geneticin have a specific activity against ribosomes that are characterized by a canonical 1409–1491 bp; that neomycin, gentamicin, netilmicin, tobramycin and kanamycin are active against ribosomes with a 1408 adenine; and that metazoan cytosolic ribosomes are generally resistant to 2-deoxystreptamines.

Successful crystallizations of the ribosome and ribosome–drug complexes have initiated a rebirth of interest in the ribosome as drug target (40–43). Apparently, helix 44 of the rRNA decoding A site behaves as an autonomous domain, which can be dissected from the remaining part of the ribosome and shuffled between ribosomes of different phylogenetic domains *in vivo* while retaining its functional characteristics. It is likely that this possibility will help to study mechanisms of the various eukaryotic rRNA decoding sites in the future and to test hypotheses built upon X-ray structures. The construction of hybrid ribosomes, as demonstrated in this study, should also help characterizing the specificity of ribosomal inhibitors already available and of future compounds to come.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Dahlberg,A.E. (1989) The functional role of ribosomal RNA in protein synthesis. *Cell*, **57**, 525–529.
2. Noller,H.F. (1991) Ribosomal RNA and translation. *Annu. Rev. Biochem.*, **60**, 191–227.
3. Ogle,J.M. and Ramakrishnan,V. (2005) Structural insights into translational fidelity. *Annu. Rev. Biochem.*, **74**, 129–177.
4. Gutell,R.R. (1994) Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res.*, **22**, 3502–3507.
5. Moazed,D. and Noller,H.F. (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, **327**, 389–394.
6. Davies,J., Gorini,L. and Davis,B.D. (1965) Misreading of RNA codewords induced by aminoglycoside antibiotics. *Mol. Pharmacol.*, **1**, 93–106.
7. Campuzano,S., Vazquez,D. and Modolell,J. (1979) Functional interaction of neomycin B and related antibiotics with 30S and 50S ribosomal subunits. *Biochem. Biophys. Res. Commun.*, **87**, 960–966.
8. Peske,F., Savelsbergh,A., Katunin,V.I., Rodnina,M.V. and Wintermeyer,W. (2004) Conformational changes of the small ribosomal subunit during elongation factor G-dependent tRNA-mRNA translocation. *J. Mol. Biol.*, **343**, 1183–1194.
9. Pfister,P., Hobbie,S., Vicens,Q., Böttger,E.C. and Westhof,E. (2003) The molecular basis for A-Site mutations conferring aminoglycoside resistance: relationship between ribosomal susceptibility and X-ray crystal structures. *ChemBioChem*, **4**, 1078–1088.
10. Carter,A.P., Clemons,W.M., Brodersen,D.E., Morgan-Warren,R.J., Wimberly,B.T. and Ramakrishnan,V. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature*, **407**, 340–348.
11. Ogle,J.M., Brodersen,D.E., Clemons,W.M. Jr, Tarry,M.J., Carter,A.P. and Ramakrishnan,V. (2001) Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science*, **292**, 897–902.
12. Kondo,J., Urzhumtsev,A. and Westhof,E. (2006) Two conformational states in the crystal structure of the Homo sapiens cytoplasmic ribosomal decoding A site. *Nucleic Acids Res.*, **34**, 676–685.
13. Hobbie,S.N., Bruell,C., Kalapala,S.K., Akshay,S., Schmidt,S., Pfister,P. and Böttger,E.C. (2006) A genetic model to investigate structural drug-target interactions at the ribosomal decoding site. *Biochimie*, **88**, 1033–1043.
14. Musters,W., Venema,J., van der Linden,G., van Heerikhuizen,H., Klootwijk,J. and Planta,R.J. (1989) A system for the analysis of yeast ribosomal DNA mutations. *Mol. Cell. Biol.*, **9**, 551–559.
15. Chernoff,Y.O., Vincent,A. and Liebman,S.W. (1994) Mutations in eukaryotic 18S ribosomal RNA affect translational fidelity and resistance to aminoglycoside antibiotics. *EMBO J.*, **13**, 906–913.
16. Wai,H.H., Vu,L., Oakes,M. and Nomura,M. (2000) Complete deletion of yeast chromosomal rDNA repeats and integration of a new rDNA repeat: use of rDNA deletion strains for functional analysis of rDNA promoter elements in vivo. *Nucleic Acids Res.*, **28**, 3524–3534.
17. Kapp,L.D. and Lorsch,J.R. (2004) The molecular mechanics of eukaryotic translation. *Annu. Rev. Biochem.*, **73**, 657–704.
18. Weinstein,I.B., Ochoa,M.Jr and Friedman,S.M. (1966) Fidelity in the translation of messenger ribonucleic acids in mammalian subcellular systems. *Biochemistry*, **5**, 3332–3339.
19. Sander,P., Meier,A. and Bottger,E.C. (1995) *rpsL*$^{+}$: a dominant selectable marker for gene replacement in mycobacteria. *Mol. Microbiol.*, **16**, 991–1000.
20. Sander,P., Prammananan,T., Meier,A., Frischkorn,K. and Bottger,E.C. (1997) The role of ribosomal RNAs in macrolide resistance. *Mol. Microbiol.*, **26**, 469–480.
21. Pfister,P., Hobbie,S., Brull,C., Corti,N., Vasella,A., Westhof,E. and Böttger,E.C. (2005) Mutagenesis of 16S rRNA C1409-G1491 base-pair differentiates between 6'OH and 6'NH_3^+ aminoglycosides. *J. Mol. Biol.*, **346**, 467–475.
22. Pfister,P., Corti,N., Hobbie,S., Bruell,C., Zarivach,R., Yonath,A. and Böttger,E.C. (2005) 23S rRNA base pair 2057-2611 determines ketolide susceptibility and fitness cost of the macrolide resistance mutation 2058A- > G. *Proc. Natl Acad. Sci. USA*, **102**, 5180–5185.
23. Beringer,M., Bruell,C., Xiong,L., Pfister,P., Bieling,P., Katunin,V.I., Mankin,A.S., Böttger,E.C. and Rodnina,M.V. (2005) Essential mechanisms in the catalysis of peptide bond formation on the ribosome. *J. Biol. Chem.*, **280**, 36065–36072.
24. Purohit,P. and Stern,S. (1994) Interactions of a small RNA with antibiotic and RNA ligands of the 30S subunit. *Nature*, **370**, 659–662.
25. Wilhelm,J.M., Jessop,J.J. and Pettitt,S.E. (1978) Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. *Biochemistry*, **17**, 1149–1153.
26. Liebman,S.W., Chernoff,Y.O. and Liu,R. (1995) The accuracy center of a eukaryotic ribosome. *Biochem. Cell Biol.*, **73**, 1141–1149.
27. Kurtz,D.I. (1974) Fidelity of protein synthesis with chicken embryo mitochondrial and cytoplasmic ribosomes. *Biochemistry*, **13**, 572–577.
28. Botero,D. (1970) Paromomycin as effective treatment of *Taenia* infections. *Am. J. Trop. Med. Hyg.*, **19**, 234–237.
29. Araujo,F.G., Guptill,D.R. and Remington,J.S. (1988) Azithromycin, a macrolide antibiotic with potent activity against Toxoplasma gondii. *Antimicrob. Agents Chemother.*, **32**, 755–757.
30. Edlind,T.D. (1989) Susceptibility of *Giardia lamblia* to aminoglycoside protein synthesis inhibitors: correlation with rRNA structure. *Antimicrob. Agents Chemother.*, **33**, 484–488.
31. Beckers,C.J., Roos,D.S., Donald,R.G., Luft,B.J., Schwab,J.C., Cao,Y. and Joiner,K.A. (1995) Inhibition of cytoplasmic and organellar protein synthesis in Toxoplasma gondii. Implications for the target of macrolide antibiotics. *J. Clin. Invest.*, **95**, 367–376.
32. Mathis,A., Wild,P., Deplazes,P. and Boettger,E.C. (2004) The mitochondrial ribosome of the protozoan *Acanthamoeba castellanii* is the target for macrolide antibiotics. *Mol. Biochem. Parasitol.*, **135**, 225–229.
33. Mathis,A., Wild,P., Boettger,E.C., Kapel,C.M. and Deplazes,P. (2005) Mitochondrial ribosome as the target for the macrolide antibiotic clarithromycin in the helminth *Echinococcus multilocularis*. *Antimicrob. Agents Chemother.*, **49**, 3251–3255.
34. Moskalenko,N. and Pershin,G.N. (1966) Comparative study of the chemotherapeutic effect of paromomycin and monomycin in experimental cutaneous leischmaniasis in albino mice. *Farmakol. Toksikol.*, **29**, 90–94.
35. Murray,H.W., Berman,J.D., Davies,C.R. and Saravia,N.G. (2005) Advances in leishmaniasis. *Lancet*, **366**, 1561–1577.
36. Stanghellini,A. and Josenando,T. (2001) The situation of sleeping sickness in Angola: a calamity. *Trop Med Int Health*, **6**, 330–334.
37. Sander,P., Prammananan,T. and Böttger,E.C. (1996) Introducing mutations into a chromosomal rRNA gene using a genetically modified eubacterial host with a single rRNA operon. *Mol. Microbiol.*, **22**, 841–848.
38. Recht,M.I., Douthwaite,S. and Puglisi,J.D. (1999) Basis for prokaryotic specificity of action of aminoglycoside antibiotics. *EMBO J.*, **18**, 3133–3138.
39. De Stasio,E.A., Moazed,D., Noller,H.F. and Dahlberg,A.E. (1989) Mutations in 16S ribosomal RNA disrupt antibiotic—RNA interactions. *EMBO J.*, **8**, 1213–1216.
40. Yonath,A. (2005) Antibiotics targeting ribosomes: resistance, selectivity, synergism and cellular regulation. *Annu. Rev. Biochem.*, **74**, 649–679.
41. Poehlsgaard,J. and Douthwaite,S. (2005) The bacterial ribosome as a target for antibiotics. *Nat. Rev. Microbiol.*, **3**, 870–881.
42. Hermann,T. (2005) Drugs targeting the ribosome. *Curr. Opin. Struct. Biol.*, **15**, 355–366.
43. Sutcliffe,J.A. (2005) Improving on nature: antibiotics that target the ribosome. *Curr. Opin. Microbiol.*, **8**, 534–542.