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# The antibiotics micrococcin and thiostrepton interact directly with 23S rRNA nucleotides 1067A and 1095A

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## ABSTRACT

**The antibiotics thiostrepton and micrococcin bind to the GTPase region in domain II of 23S rRNA, and inhibit ribosomal A-site associated reactions. When bound to the ribosome, these antibiotics alter the accessibility of nucleotides 1067A and 1095A towards chemical reagents. Plasmid-coded *Escherichia coli* 23S rRNAs with single mutations at positions 1067 or 1095 were expressed *in vivo*. Mutant ribosomes are functional in protein synthesis, although those with transversion mutations function less effectively. Antibiotics were bound under conditions where wild-type and mutant ribosomes compete in the same reaction for drug molecules; binding was analysed by allele-specific footprinting. Transversion mutations at 1067 reduce thiostrepton binding more than 1000-fold. The 1067G substitution gives a more modest decrease in thiostrepton binding. The changes at 1095 slightly, but significantly, lower the affinity of ribosomes for thiostrepton, again with the G mutation having the smallest effect. Micrococcin binding to ribosomes is reduced to a far greater extent than thiostrepton by all the 1067 and 1095 mutations. Extrapolating these results to growing cells, mutation of nucleotide 1067A confers resistance towards micrococcin and thiostrepton, while substitutions at 1095A confer micrococcin resistance, and increase tolerance towards thiostrepton. These data support an rRNA tertiary structure model in which 1067A and 1095A lie in close proximity, and are key components in the drug binding site. None of the mutations alters either the higher order rRNA structure or the binding of r-proteins. We therefore conclude that thiostrepton and micrococcin interact directly with 1067A and 1095A.**

## INTRODUCTION

During protein synthesis, ribosomal RNA (rRNA) participates directly in tRNA and elongation factor interaction, and possibly in catalytic events (1,2). Most of these essential processes have now been linked with specific regions in the rRNA (1,3). Characteristic for these functionally important rRNA regions is that they are target sites for antibiotics and cytotoxins that inhibit protein synthesis (4,5). One such region is the GTPase centre in domain II of 23S rRNA, which interacts with elongation factor EF-G and with tRNA, and is associated with GTP hydrolysis and ppGpp production in the stringent response (6, and references therein). This rRNA region is also the target site for the thiopeptide antibiotics thiostrepton and micrococcin (7,8). These drugs produce similar, but not identical, footprinting patterns on 23S rRNA, affecting the accessibility of nucleotides between positions 1067 and 1098 (8,9). Although this footprint is rather extensive, probably only a few of the protections represent direct drug–nucleotide contacts (9). The attachment site of the ribosomal protein (r-protein) L11 encompasses this rRNA region, and binding of L11 is essential for effective drug interaction (5).

Nucleotide 1067A in 23S rRNA has been shown to be important for thiostrepton interaction. The ribose of this nucleotide is methylated in the thiostrepton-producing organism *Streptomyces azureus* rendering ribosomes resistant to the drug (10), and transversion mutations here confer resistance in *Escherichia coli* ribosomes (11). In footprinting experiments, antibiotics effect the accessibility of the N1 position of 1067A, where base reactivity is reduced by thiostrepton and enhanced by micrococcin; the drugs also strongly protect nucleotide 1095A (8). In the tertiary structure model proposed for this rRNA region, the hairpin loop at 1067A is folded to lie in close proximity to the loop containing 1095A (12). The most straightforward inference is that nucleotides 1067A and 1095A interact directly with the drugs and are key components in the drug target site. This is not, however, the only interpretation of the data. The footprint effects could alternatively be allosteric alterations in the rRNA structure induced by drug binding at another site. In the same vein, drug resistance could be an indirect consequence of rRNA mutations altering the interaction of r-protein L11.

We have investigated putative sites of direct drug–rRNA interaction by use of a combination of mutagenesis and allele-specific footprinting techniques. It was first established whether mutagenesis of 1095A affects drug binding. Given the subtle differences in the footprints of thiostrepton and micrococcin, we also compared how the binding of the two drugs is affected by rRNA mutations. Finally, we determined whether the drugs

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interact directly with 1067A and 1095A, or whether mutations here reduce drug binding indirectly such as by perturbing the attachment of L11.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*Escherichia coli* strains DH1 and TG1 (13) were used as hosts for plasmids and bacteriophages, respectively, and were grown in LB (DH1) or YT (TG1) media (13). Growth rates of cells harbouring plasmids were determined at incubation temperatures of 30°C, 37°C and 42°C on agar plates with ampicillin (Sigma) at 100 mg/l, or ampicillin at 25 mg/l together with erythromycin (Sigma) at 80 mg/l. This is approximately twice the amount of erythromycin required to inhibit the growth of cells containing only wild-type ribosomes (14).

### Site-directed mutagenesis and plasmid construction

Mutations at 23S rRNA position 1067 (11) in plasmid pKK3535 derivatives (15) were provided by Al Dahlberg. The 1 kb *SacI*/*SalI* 23S rRNA gene fragments were inserted into M13mp18, and additional mutations were introduced by site-directed mutagenesis in the 1170 region (16) to facilitate allele-specific priming (Figure 1). These mutagenised sequences were excised on 0.9 kb *SacI*/*SphI* fragments and were inserted in plasmid pSTL102 to reconstitute intact 23S rRNA genes. pSTL102 contains the entire *rrnB* operon with the 23S rRNA mutation 2058G conferring erythromycin resistance (17,18). The pSTL102 derivatives were named pGK1067A, pGK1067C, pGK1067G and pGK1067T. Mutations at 23S rRNA position 1095 were generated in a similar manner using the deoxynucleotide 5′-GTGAGCTATVACGCTTTC (where V is A, C or G) complementary to nucleotides 1087–1104. Expression vectors pGK1095C, pGK1095G and pGK1095T were constructed as described above. The structure of each plasmids was confirmed by restriction endonuclease analysis and by sequencing the mutagenised regions. The enzymes used for DNA manipulations were all purchased from Boehringer Mannheim, except for Sequenase (US Biochemical Cooperation), and were used according to the suppliers' recommendations.

### Isolation of ribosomes and rRNA

DH1 cells harbouring plasmids were harvested at an $A_{450}$ of 0.4, cooled on ice, lysed by sonication, and ribosomes were isolated by centrifugation (17). rRNA was prepared by extracting ribosomes three times with an equal volume of water-saturated phenol/chloroform followed by extraction with chloroform. RNA was precipitated from 0.3 M sodium acetate with 2.5 vol ethanol and was resuspended in water.

### Antibiotic binding and rRNA probing

Ribosomes (at 0.08 μM) were incubated in 100 μl binding buffer [50 mM Hepes–KOH pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT and 0.2 unit RNasin (Promega)] for 20 min at 30°C with 1–1000 molar equivalents of the antibiotics thiostrepton or micrococcin. Ribosomes were probed at 37°C with dimethylsulphate (DMS, Merck) (2 μl of a 1:6 dilution in 96% ethanol) for 8 min, or with 0.1 unit cobra venom V1 RNase (Pharmacia) for 3 min, or with 1 unit RNase T1 or T2 (Sigma) for 50 min. The reactions were stopped, and rRNA was extracted and precipitated as described (12,19). Probing of naked rRNA and protein–rRNA complexes, and estimations of bound protein:rRNA stoichiometry, were performed as described by Egebjerg *et al.* (12).

### Primer extension

Primer extension of rRNA using reverse transcriptase (AMV, Life Sciences) (19) was carried out with 5′-[$^{32}$P]-labelled deoxyoligonucleotide primers. The primer, 5′-CCCAACAA-CGCATAAGCGT, complementary to 23S nucleotides 1169–1187 was used to screen chromosomal-coded 23S rRNA, while plasmid-coded 23S rRNA was screened with the primer, 5′-CCCAACAGTGCACAAGCAC, complementary to the specific priming site in plasmid encoded 23S rRNA (16). Extension products were run on 6% acrylamide/7 M urea gels, and band intensities were estimated by scanning with an LKB Ultrascan XL enhanced laser densitometer.

## RESULTS

### Phenotypes of cells with mutations at 23S rRNA positions 1067 and 1095

Mutant rRNAs were expressed *in vivo* to determine changes in cell phenotype and ribosome function. Growth rates of cells with mutations at 23S rRNA positions 1067 and 1095 were estimated on plates containing ampicillin alone or together with erythromycin. The concentration of erythromycin used here is about twice that required to inhibit wild-type cell growth (14), thus only ribosomes containing plasmid-coded 23S rRNA (with

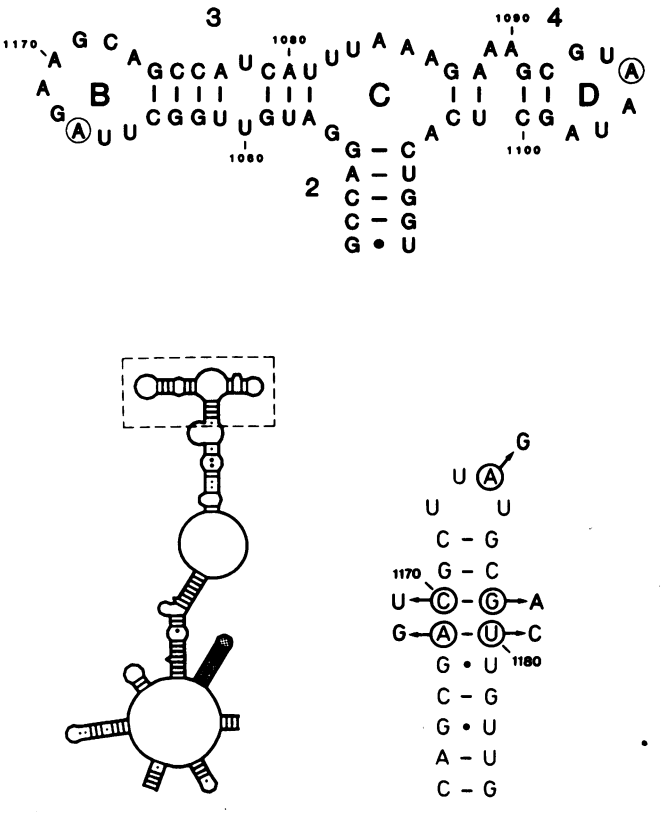


**Figure 1.** Outline of a portion of the 23S rRNA domain II secondary structure (lower left) (34) showing the relative positions of the GTPase region (boxed) and the priming site (shaded helix). The sequence of the GTPase region is shown (top) with nucleotides 1067A and 1095A encircled. The bases changed around position 1170 to facilitate specific priming are indicated (lower right).

the 2058G mutation) are translationally active under these conditions. Each of the transversion mutations (1067C, 1067U, 1095C and 1095U) reduced the growth rate of cells on ampicillin at 37°C, and this effect was more pronounced on erythromycin at 30°C, 37°C and 42°C where the growth rate was reduced by up to 50% (Figure 2). The more conservative change to 1067G had no effect on cell growth, while the 1095G mutation reduced the growth rate on erythromycin at 30°C, and 37°C but did not affect cell growth on erythromycin at 42°C or at 37°C in the absence of this drug.

### Structural probing and r-protein binding to mutant rRNA

As rRNA was transcribed and assembled into ribosomes *in vivo*, approximately half the ribosomes contained wild-type 23S rRNA encoded by the chromosomal *rrn* operons, and the other half contained mutagenised, plasmid-coded 23S rRNA (16). All the rRNA probing experiments with DMS, and RNases V1, T1 and T2 were carried out on this heterogeneous mixture. Nucleotide accessibilities were analysed by reverse transcriptase primer extension from the specific allele in the 1170 region (Figure 1) which makes it possible to differentiate between wild-type and mutant rRNAs (16). Probing the structure of the GTPase region in rRNA in the free state and in ribosomes showed no change in the accessibility of nucleotides except at the mutated positions (data not shown).

To test whether the introduction of mutations at 1067A or 1095A influenced the binding of the r-proteins to the GTPase region, naked rRNA was digested with RNase $V_1$ before and after binding of r-proteins. The mutant rRNAs showed identical footprints on binding of r-protein L11, with enhanced hydrolysis at positions 1031G, 1036G, 1039A and protection at 1061U, 1077A to 1081U and 1088A to 1091G (data not shown), as

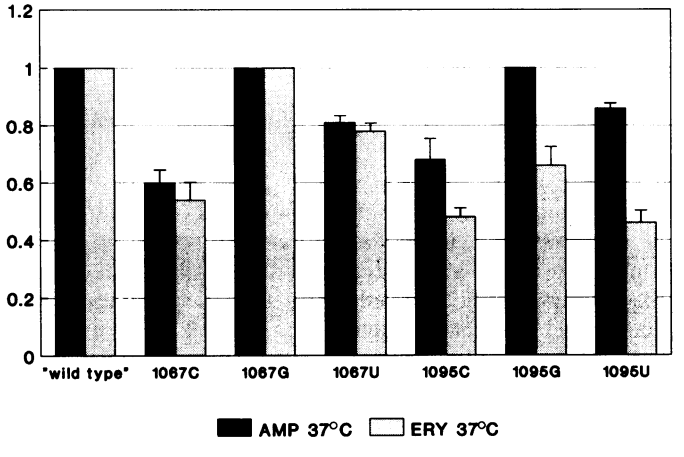


**Figure 2.** The effects of plasmid-coded mutations at 23S rRNA positions 1067 and 1095 on the growth rates of cells cultured at 37°C on ampicillin (amp) or on ampicillin plus erythromycin (ery). Growth rates are expressed relative to cells harbouring plasmid pGK1067A without mutations in the GTPase region ('wild type'), which formed colonies 1 mm in size after 16 h (amp) and 18 h (ery). Bars represent standard errors of the means of at least three experiments. All plasmid-coded 23S rRNA genes contain the new priming site at 1170 (which does not affect growth) and the 2058A→G mutation that confers erythromycin resistance. Cells without this latter mutation do not grow on erythromycin. The growth on erythromycin depicted here was therefore dependent on the translational efficiency of the ribosomes with plasmid-coded mutant rRNA. Relative growth rates at 30°C and 42°C followed the same pattern as those above, with the exception that the 1095G mutation grew as fast as the control at 42°C on erythromycin.

previously observed for wild-type rRNA (12). The complete protection of bases in mutant and wild-type rRNAs indicated stoichiometric r-protein binding. The pentameric r-protein complex L10.$(L12)_4$ binds co-operatively with L11 to an adjacent site on the rRNA. This r-protein complex also gave identical footprints on wild-type and mutant rRNAs with enhanced hydrolysis at nucleotides 1030C, 1031G, and 1039A, and protection of 1043C and 1044C, consistent with previous data (12).

### Quantification of thiostrepton and micrococcin binding to ribosomes

Each antibiotic gives a characteristic DMS footprint pattern on 23S rRNA in the ribosome (8). Both drugs give moderate protection at 1070A, weak protection at 1073A and strongly protect 1095A. However, the drugs cause distinctly different effects at 1067A, the accessibility of which is enhanced by micrococcin but shielded by thiostrepton. The DMS footprint patterns were scanned within the region where drugs interact with the rRNA (Figure 3) to quantify the effects of the mutations on drug binding (Tables 1 and 2).

### Drug binding to ribosomes mutagenised at 23S rRNA position 1067

The accessibility of 1095A was used as an indicator of drug binding to ribosomes containing 23S rRNA mutagenised at position 1067 (Table 1). The degree of protection at 1095A is taken to correspond to the proportion of ribosomes binding the drugs. Binding of thiostrepton to mutant ribosomes was lower compared to the wild-type ribosomes present in the same sample (Table 1). This effect is particularly marked at the lowest thiostrepton concentration (equimolar drug and ribosomes) where the reduced interaction with mutant ribosomes provided more free drug to bind to wild-type ribosomes which consequently showed a correspondingly higher rRNA protection. At higher drug concentrations, 1067G mutant ribosomes bound thiostrepton essentially as well as wild-type ribosomes, although there was still a clear reduction in binding to the 1067C and 1067U mutant ribosomes. Binding to the 1067C ribosomes was followed up to a 1000-fold molar excess of drug, where the level of drug binding began to approach that of wild-type ribosomes (Figure 4).

The 1067 mutations cause an even greater reduction in the protection afforded by micrococcin. In this case, the effect of the 1067G mutation was almost as marked as that of 1067C and 1067U (Table 1). Interaction of the 1067C mutant ribosomes were monitored with up to 1000-fold molar excess of micrococcin, and even at this level only approximately 25% of the mutant ribosomes bound the drug (Figure 4).

### Drug binding to ribosomes mutagenised at 23S rRNA position 1095

Ribosomes with mutations at position 1095 were assayed for drug binding in a similar way to that described above, using protection against DMS at 1067A as an indicator of thiostrepton binding (Table 2). All the mutations significantly reduce binding at the lowest drug concentration. This effect becomes less obvious with increasing thiostrepton concentrations, and at a 10-fold molar excess of the drug, there was no difference in the degree of drug binding to mutant or wild-type ribosomes.

Under the conditions used here, stoichiometric binding of micrococcin to ribosomes causes an approximately 2-fold enhancement in the reactivity of 1067A towards DMS (Figure

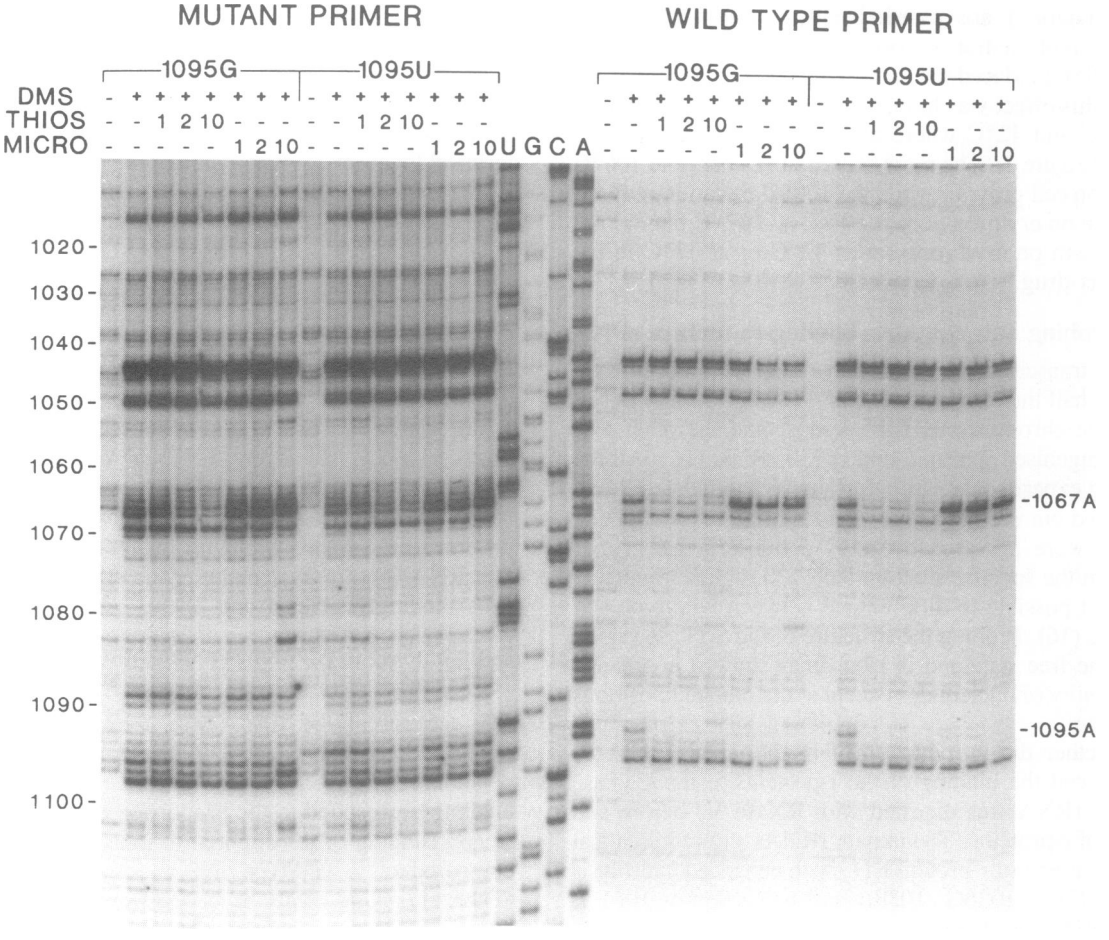


**Figure 3.** Autoradiographs showing drug interaction with wild-type and mutant 23S rRNAs. Thiostrepton (thios) and micrococcin (micro) were bound to heterogeneous mixtures of wild-type and mutant ribosomes. The molar ratio of the drugs relative to ribosomes were 1, 2 or 10, as indicated; − indicates no drug. Drug−ribosome complexes were modified with dimethyl sulphate (DMS) where shown (+). Ribosomal RNA was extracted and reverse transcribed from primers specific for plasmid-coded (mutant) or chromosome-coded (wild-type) 23S rRNAs. The transcripts were electrophoresed on two gels run under identical conditions. The footprint patterns on the left are of 23S rRNA containing the 1095G or 1095U mutation; the patterns on the right are of wild-type 23S rRNA present in the same reactions. Positions of altered nucleotide reactivity at 1067A and 1095A are indicated. Dideoxy sequencing reactions (U,G,C,A) were performed on an unmodified rRNA template.

3 and Table 2). This effect is abolished by the mutations at 1095. Micrococcin affects the accessibility of other bases within this rRNA region towards DMS and the T1 and V1 RNases (8). Concomitant loss of these effects (data not shown) indicates that the changes in the enhancement at 1067A correlate directly with lowered drug binding.

## DISCUSSION

### Reduction in antibiotic binding

We have shown by use of a footprinting assay that mutagenesis of 1067A and 1095A in 23S rRNA reduces binding of the antibiotics thiostrepton and micrococcin to ribosomes. Tranversion mutations produce the largest effects as can be seen in the case of thiostrepton binding to ribosomes with the 1067 mutations. The 1067C substitution caused at least a 1000-fold reduction in thiostrepton binding (Figure 4), and the effect of 1067U was similar; 1067G, however, had a more minor effect on drug binding. Thompson *et al.* studied thiostrepton binding to ribosomes with the 1067 mutations using charcoal-adsorption and GTP hydrolysis assays, and obtained compatible results (11). Footprinting has previously been shown to be a reliable means of estimating the binding affinities of drugs to the peptidyl transferase region in wild-type and mutant ribosomes (20). As the drug concentrations used in this study lie several orders of magnitude higher than the dissociation constant for the interaction of thiostrepton with wild-type ribosomes (21), the effects of the mutations represent an appreciable reduction in binding affinity.

The 1067 mutations cause a more marked fall in the level of micrococcin binding than that observed for thiostrepton. In this case, the 1067G mutation also clearly lowers micrococcin binding even at a 10-fold molar excess of the drug. The mutations at position 1095 have an even more drastic effect on micrococcin interaction, and there was no detectable binding to ribosomes with 1095C at a 10-fold molar excess of drug. Mutations at position 1095 also reduce thiostrepton binding although these effects are relatively small, with reduced drug binding to 1095G ribosomes only evident at the lowest thiostrepton concentration. Such small changes in drug affinity are, however, readily detected as binding reactions are carried out on a mixture of wild-type and mutant ribosomes which compete for a limited amount of drug.

**Table 1.** The effects of mutations at 23S rRNA position 1067 on drug binding to ribosomes

| Molar ratio drug:ribosome | "Wild type" Mutant primer | "Wild type" Wt primer | "Wild type" Difference | 1067C Mutant primer | 1067C Wt primer | 1067C Difference | 1067G Mutant primer | 1067G Wt primer | 1067G Difference | 1067T Mutant primer | 1067T Wt primer | 1067T Difference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NO DRUG | 1 | 1 | | 1 | 1 | | 1 | 1 | | 1 | 1 | |
| 1X Thio | 0.54 | 0.45 | 0.09 ±0.06 | 0.92 | 0.22 | 0.70 ±0.06 | 0.86 | 0.26 | 0.60 ±0.10 | 1.10 | 0.23 | 0.87 ±0.03 |
| 2X Thio | 0.20 | 0.21 | 0.01 ±0.03 | 0.81 | 0.21 | 0.61 ±0.08 | 0.39 | 0.19 | 0.20 ±0.16 | 0.77 | 0.17 | 0.60 ±0.05 |
| 10X Thio | 0.20 | 0.15 | 0.05 ±0.05 | 0.59 | 0.23 | 0.36 ±0.05 | 0.25 | 0.12 | 0.13 ±0.06 | 0.55 | 0.16 | 0.39 ±0.10 |
| NO DRUG | 1 | 1 | | 1 | 1 | | 1 | 1 | | 1 | 1 | |
| 1X Micro | 0.51 | 0.40 | 0.11 ±0.02 | 0.89 | 0.20 | 0.69 ±0.05 | 0.90 | 0.24 | 0.66 ±0.13 | 0.91 | 0.27 | 0.64 ±0.05 |
| 2X Micro | 0.25 | 0.19 | 0.06 ±0.01 | 0.87 | 0.23 | 0.64 ±0.07 | 0.82 | 0.20 | 0.62 ±0.05 | 0.95 | 0.21 | 0.74 ±0.01 |
| 10X Micro | 0.17 | 0.09 | 0.08 ±0.01 | 0.77 | 0.14 | 0.63 ±0.05 | 0.55 | 0.12 | 0.43 ±0.14 | 0.90 | 0.20 | 0.70 ±0.04 |

The data represent the relative accessibility of nucleotide 1095A to dimethyl sulphate modification after incubating ribosomes with 1-, 2- or 10-fold molar equivalents of thiostrepton (thio) or micrococcin (micro). Band intensities were measured by densitometry of autoradiographs, and were normalized relative to 1098A for each gel lane. The differences (with standard errors) between pairs of ribosome samples containing plasmid-coded 23S rRNA (screened with the mutant primer) and chromosome-coded wild-type 23S rRNA (wt primer) are shown. In the 'wild type' sample, the plasmid-coded rRNA has the wild-type sequence within the drug binding site. The 1067C mutation, which produced the greatest reduction in binding, was studied at higher drug concentrations (Figure 4).

**Table 2.** The effects of mutations at 23S rRNA position 1095 on drug binding to ribosomes

| Molar ratio drug:ribosome | "Wild type" Mutant primer | "Wild type" Wt primer | "Wild type" Difference | 1095C Mutant primer | 1095C Wt primer | 1095C Difference | 1095G Mutant primer | 1095G Wt primer | 1095G Difference | 1095T Mutant primer | 1095T Wt primer | 1095T Difference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NO DRUG | 1 | 1 | | 1 | 1 | | 1 | 1 | | 1 | 1 | |
| 1X Thio | 0.62 | 0.60 | 0.02 ±0.05 | 0.63 | 0.37 | 0.26 ±0.14 | 0.55 | 0.34 | 0.21 ±0.01 | 0.59 | 0.36 | 0.23 ±0.05 |
| 2X Thio | 0.41 | 0.38 | 0.03 ±0.05 | 0.48 | 0.36 | 0.12 ±0.04 | 0.35 | 0.35 | 0.00 ±0.00 | 0.46 | 0.31 | 0.15 ±0.05 |
| 10X Thio | 0.41 | 0.38 | 0.03 ±0.05 | 0.37 | 0.37 | 0.00 ±0.00 | 0.33 | 0.33 | 0.00 ±0.00 | 0.45 | 0.35 | 0.10 ±0.04 |
| NO DRUG | 1 | 1 | | 1 | 1 | | 1 | 1 | | 1 | 1 | |
| 1X Micro | 1.79 | 1.82 | 0.03 ±0.03 | 0.95 | 2.34 | 1.39 ±0.47 | 1.03 | 2.19 | 1.16 ±0.38 | 1.16 | 2.45 | 1.29 ±0.41 |
| 2X Micro | 1.85 | 2.07 | 0.22 ±0.09 | 0.95 | 2.69 | 1.74 ±0.38 | 0.95 | 2.17 | 1.23 ±0.07 | 1.08 | 2.37 | 1.29 ±0.41 |
| 10X Micro | 1.74 | 1.89 | 0.15 ±0.09 | 0.99 | 2.35 | 1.36 ±0.05 | 1.12 | 2.49 | 1.37 ±0.34 | 1.20 | 2.26 | 1.06 ±0.11 |

The data represent the relative accessibility of nucleotide 1067A to dimethyl sulphate modification after incubating ribosomes with 1-, 2- or 10-fold molar equivalents of thiostrepton (thio) or micrococcin (micro). The data were derived as described for Table 1; band intensities at 1067A were normalized relative to 1069A for each gel lane.

## Antibiotic resistance

Assuming that reduced drug binding correlates with resistance, as demonstrated for the 1067 mutations with thiostrepton (11), it is predicted that the 1095 mutations will give high levels of micrococcin resistance (especially in the case of 1095C) and slight thiostrepton resistance. *E.coli* is impermeable to these drugs, so confirmation of resistance is dependent on *in vitro* investigations with *E.coli* ribosomes (11) or studies on other organisms. Hummel and Böck (22) have shown by random mutagenesis that mutation of 1067A also confers thiostrepton resistance in halobacteria. In contrast to the data described above, Hummel and Böck also reported that 1067G conferred higher resistance than 1067U. It still remains, however, to be shown whether additional mutations (such as at position 1095) occurred in the halobacterial rRNA.

Although thiostrepton and micrococcin interact with the same region of rRNA, there are differences in the footprint patterns produced by the two drugs (8). The larger size of thiostrepton [molecular weight 1616 daltons (23) compared to 1143 daltons for micrococcin (24)] could facilitate more or different contacts within its binding site, making its interaction less sensitive to some of the single base alterations studied here. Other observations are also consistent with the requirements for binding of micrococcin being more fastidious than those of thiostrepton. Some archaeal ribosomes are resistant towards micrococcin but sensitive towards thiostrepton (25), and *Bacillus subtilis* with an altered r-protein BS-L3 is resistant to micrococcin but still sensitive towards thiostrepton (26).

## The antibiotic binding site

The mutations studied here lie within the binding region of the r-protein L11. Effective binding of the thiopeptide drugs is dependent on prior binding of L11 (5). Thus, it was important to establish whether the reduction in drug binding was an indirect consequence of disturbing the protein–rRNA interaction. This has been shown not to be the case. Ribosomes lacking L11 give a distinctive footprint pattern (9), and the footprint of the mutant

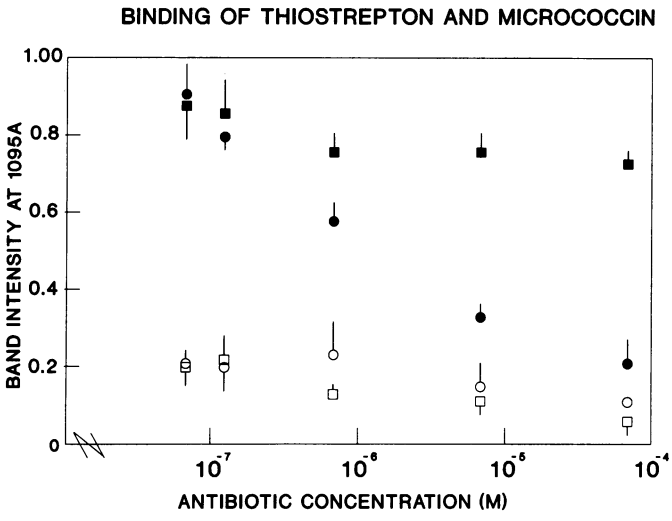


**Figure 4.** Effect of the 1067C mutation on drug binding to ribosomes. The protection by thiostrepton (circles) and micrococcin (squares) from DMS modification was measured up to a 1000-fold excess of drug. The relative binding to wild-type ribosomes (open symbols) and mutant ribosomes with 1067C (filled symbols) were determined as described for Table 1. Standard errors of the means of at least four experiments are shown for each point.

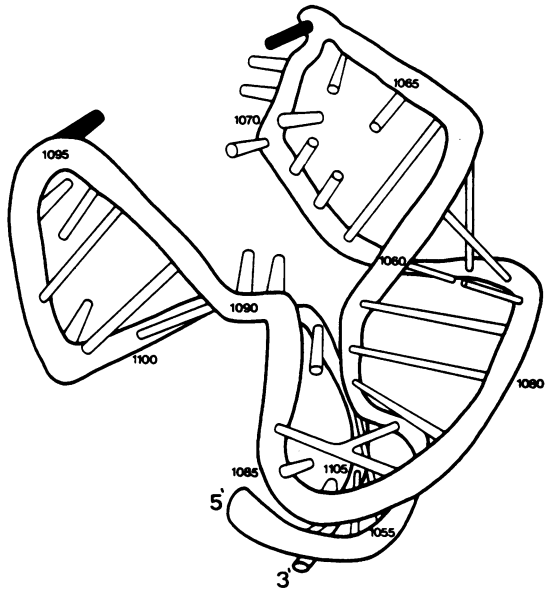


**Figure 5.** Tertiary structure proposed for the GTPase region of 23S rRNA (12). Bases at positions 1067 and 1095 are highlighted.

ribosomes investigated here indicates that L11 is bound in a 1:1 stoichiometry. Binding of L11 to purified 23S rRNA also showed that the mutations cause no detectable alterations in the bases protected by L11, or in the degree of protection at these bases. In addition, the data showed no evidence of higher order structural changes within this rRNA region. From this we conclude that the mutagenised bases are within the drug binding site. Thus, in wild-type ribosomes the drugs make direct contact with 1067A and 1095A.

Thiostrepton binds remarkably tightly to ribosomes with a dissociation constant of below $10^{-9}$ M (21). This prompts the question as to how many other sites of direct ribosome–drug contact are required to achieve this. A recent study with reactive hydroxyl radicals indicates that drug–ribose interactions are limited to nucleotides 1067 to 1069, 1096 and 1097 (9). The drugs gave a rather more extensive footprint between positions 1067 and 1098 when investigated with relatively bulky chemical and ribonuclease probes (8), but it seems unlikely that all the protected nucleotides represent sites of direct drug–rRNA contact (8,9). Most data are consistent with the idea that the drugs make only few specific base contacts. Random mutagenesis indicates that mutations throughout the GTPase region show little or no direct effect on drug binding to ribosomes (our unpublished data). This fits well with a study in which this rRNA region from *Saccharomyces cerevisiae* was transplanted into *E.coli* 23S rRNA (27). The resultant rRNA has a guanine at position 1067 and an adenine at 1095, plus 19 other base substitutions relative to *E.coli* 23S rRNA. However, the level of thiostrepton resistance conferred is no greater than for the single 1067G substitution.

Thus the only drug–nucleotide contacts in the ribosome, that have been clearly identified, occur within loops B and D. These two loops are in close proximity in the tertiary structure model proposed for this region (Figure 5). The essential features of this model are supported by data from biochemical probing, covariance analysis (12), mutagenesis (28), and studies on rRNA fragments (29). The drugs appear to slot in between the loops to interact with nucleotides 1067A and 1095A. The existence of direct drug–protein interactions cannot be excluded and might well occur, but the lack of thiostrepton binding to isolated r-protein L11 (5) makes strong drug–protein interactions in the ribosome seem unlikely. The role of L11 in this context could be to stabilise a particular rRNA conformation favoured for thiostrepton binding. In the absence of r-proteins, thiostrepton binds to naked 23S rRNA (albeit with much lower affinity), and the drug also binds to an rRNA fragment encompassing the GTPase region (29). Drug binding to an isolated rRNA fragment containing this region was substantially reduced by numerous mutations (30).

## Functional importance of 23S rRNA nucleotides 1067A and 1095A

In this system, growing cells can be coerced to use exclusively plasmid-coded 23S rRNA for protein synthesis. Culturing of cells in erythromycin (which blocks protein synthesis by the wild-type ribosomes) showed that introduction of the 1067 and 1095 mutations into plasmid-coded 23S rRNA reduced the growth rate. This was particularly noticeable for the U and C substitutions at both 1067 and 1095. Presumably this is a direct result of these rRNAs being less efficient at protein synthesis. As these mutations cause no obvious changes in higher-order rRNA structure or r-protein binding, 1067A and 1095A apparently play a direct functional (although not strictly essential) role in translation.

Elongation factor G has been shown by cross-linking (31) and footprinting (32) to interact with 1067A, although it appears unlikely that the factor specifically recognises the adenine moiety of this nucleotide (11). Transfer RNA bound at the A-site interacts at 1068G (33), and its binding is possibly perturbed by the neighbouring mutations. Position 1095 has not yet been linked with a function in protein synthesis, but the importance of the adenine here is indicated by its conservation in all kingdoms.

Thiostrepton and micrococcin interact with both 1067A and 1095A, but perturb the accessibilities and functions of these nucleotides in different ways. These differences are apparent in the physiological effects of the drugs (7), in the interactions of the drugs with the N1 position of 1067A (8), and also in the different effects the mutations at 1095 have on drug binding (Table 2).

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