

Thiostrepton-resistant mutants of *Thermus thermophilus*

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ABSTRACT

Ribosomal protein L11 and its associated binding site on 23S rRNA together comprise one of the principle components that mediate interactions of translation factors with the ribosome. This site is also the target of the antibiotic thiostrepton, which has been proposed to act by preventing important structural transitions that occur in this region of the ribosome during protein synthesis. Here, we describe the isolation and characterization of spontaneous thiostrepton-resistant mutants of the extreme thermophile, *Thermus thermophilus*. All mutations were found at conserved positions in the flexible N-terminal domain of L11 or at conserved positions in the L11-binding site of 23S rRNA. A number of the mutant ribosomes were affected in *in vitro* EF-G-dependent GTP hydrolysis but all showed resistance to thiostrepton at levels ranging from high to moderate. Structure probing revealed that some of the mutations in L11 result in enhanced reactivity of adjacent rRNA bases to chemical probes, suggesting a more open conformation of this region. These data suggest that increased flexibility of the factor binding site results in resistance to thiostrepton by counteracting the conformation-stabilizing effect of the antibiotic.

INTRODUCTION

Ribosomal protein L11 and the rRNA region to which it binds on the large ribosomal subunit form part of the primary site at which translation factors interact with the ribosome. The X-ray crystal structure of L11 bound to its associated rRNA revealed that the protein has two domains, with the C-terminal domain (CTD) anchoring the protein onto the ribosome via interactions with the highly compact 1050–1100 region of 23S rRNA (1) and the N-terminal domain (NTD) making only limited contacts with rRNA (2,3). The resemblance of the L11–rRNA complex in the intact 50S subunit observed by cryo-electron

microscopy (cryo-EM) (3) to that observed by X-ray crystallography of the isolated complex indicates that this region of the ribosome forms an autonomous structure in the context of the ribosome. Cryo-EM reconstructions of ribosome-elongation factor G (EF-G) complexes indicate that L11 undergoes a conformational change to form an ‘arc-like’ connection of the NTD with the G' domain of EF-G (3). A similar connection with elongation factor Tu (EF-Tu) has also been observed (4).

Thiostrepton, a thiazole-containing cyclic peptide antibiotic, binds to 23S rRNA in the 1067 and 1095 loops of the L11 binding site (5,6) and has been proposed to inhibit EF-G-dependent translocation by blocking essential conformational changes in this region (7,8). Resistance to thiostrepton in the producing organism is conferred by 2'-O-ribose methylation of residue A1067 (9), but can also occur in other bacteria as a result of mutations in this region of 23S rRNA (10,11) or by mutation or deletion of ribosomal protein L11 (8,12,13; Table 1). Some of those rRNA mutations that have been investigated were also shown to impair the functions of EF-G (10), EF-Tu (14) and initiation factor 2 (15). The effects of mutations in L11 on the higher-order structure of the factor-binding site have implications for both the mode of action of thiostrepton and for the function of the L11 region of the ribosome.

Here, we describe the isolation of thiostrepton-resistant (thiostrepton^R) mutants of *Thermus thermophilus* HB8 and the examination of their effects on local rRNA conformation by chemical modification with base-specific reagents. Additionally, we assessed the effects of some of the more deleterious mutations on EF-G function *in vitro*, together with an estimation of the level of thiostrepton resistance, relative to wild-type, conferred by the mutation. Ribosomes from this organism are suitable for X-ray crystallographic analysis (16,17), potentially allowing the structural consequences of mutations to be examined directly. Mutations in L11 at several conserved proline residues resulted in the appearance of novel chemical modifications, indicating a more solvent-accessible conformation of the L11 region. We infer from these data that thiostrepton acts by stabilizing a more compact conformation and that resistance to thiostrepton can be conferred by mutations which favor an open conformation of the L11 region of the ribosome.

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Table 1. Mutations identified in this study in the *rplK* (L11) and *rrl* (23S rRNA) genes of *T.thermophilus* HB8 that confer thiostrepton resistance are in bold face

Gene	Mutation	Frequency	Thiostrepton (µg/ml)	Doubling time (min)	Organism ^a
<i>rplK</i> (L11)	Wild-type			39 ± 2	
	P21H (ccc to cac)	5	0.1, 1.0	42 ± 3	Hha, Eco ^b , Bme (8,13)
	P21S (ccc to tcc)	5	0.1, 1.0	43 ± 2	
	P21L (ccc to ctc)	3	0.1	46 ± 1	
	P21T				Hha, Eco ^b (8)
	P22L (ccg to ctg)	4	0.1	47 ± 2	Bme (13)
	P22S (ccg to tcg)	5	0.1	41 ± 1	
	P22T (ccg to acg)	1	0.1	41 ± 3	
	G24V				Bme (13)
	P25R (ccg to cgg)	3	0.1	49 ± 2	
	P25H ^c				Bme (13)
	P25L				Bme (13)
	G31C				Bme (13)
	P55H (ccc to cac)	1	0.1	42 ± 2	
	ΔA20-P21	1	1.0	63 ± 3	
<i>rrlA</i> or <i>rrlB</i> (23S rRNA)	ΔL11 ^d				
	A1095G	17	0.1, 1.0	47 ± 2	Eco (6)
	A1095C	6	0.1, 1.0	48 ± 2	Eco (6)
	A1095U				Eco (6)
	A1067G	2	0.1, 1.0	43 ± 1	Eco, Hha, Hcu (10,11)
	A1067U	2	1.0	50 ± 2	Eco, Hha, Hcu (10,11)
	A1067C				Eco (6,10)
	+G1067.1 ^e	1	0.1	57 ± 2	

Amino acid and nucleic acid residues are numbered according to the equivalent *E.coli* positions. The thiostrepton concentrations at which the mutants were selected are indicated. Doubling times for the thiostrepton^R isolates were measured in the absence of drug. Previously described thiostrepton- or micrococcin-resistance mutations are indicated by the organism in which they were identified or constructed.

^aHha, *Halobacterium halobium*; Eco, *Escherichia coli*; Bme, *Bacillus megaterium*; Hcu, *Halobacterium cutirubrum*.

^bConstructed as a fusion protein with glutathione-S-transferase, and examined *in vitro*.

^cThe cct to cat mutation that was isolated was incorrectly reported as causing a P to I substitution.

^dL11-minus phenotypes resulted from a number of different deletions and mutations in *B.megaterium* (31), *B.subtilis* (32), *Streptomyces antibioticus* (33), *S.lavendulae* (33), *S.griseoflavus* (33), *S.griseus* (33), *S.coelicolor* (34) and *Chlamydomonas reinhardtii* chloroplast (12).

^eInsertion of a G residue between position 1067 and 1068.

MATERIALS AND METHODS

Bacterial strains and media

Thermus thermophilus strain HB8 (18) (ATCC 27624) was obtained from the American Type Culture Collection (ATCC). All *T.thermophilus* cultures were grown in ATCC medium 1598 (Thermus Enhanced Medium, TEM). For plates, TEM was solidified with 2.8% (w/v) Difco agar. Doubling times were determined by monitoring exponential growth in TEM at 72°C using a Klett–Summerson photometer.

Selection and isolation of thiostrepton^R mutants

Spontaneous thiostrepton^R mutants of *T.thermophilus* strain HB8 were obtained by growing overnight cultures in liquid TEM and plating ~10⁹ cells on solid TEM containing either 0.1 or 1.0 µg/ml thiostrepton [added from 5 mg/ml stock solution in dimethyl sulfoxide (DMSO)]. Plates were incubated for 72 h at 72°C to allow colony growth, then individual thiostrepton^R mutants were purified by streaking onto solid TEM containing thiostrepton (at the concentrations of the original selections), then onto solid TEM (antibiotic-free).

Sequencing of mutants

All thiostrepton^R isolates were examined for mutations in *rplK* (the gene encoding ribosomal protein L11) and in the *rrlA* and *rrlB* genes (encoding 23S rRNA). DNA fragments containing either the coding sequence for *rplK*, or a 618 bp fragment of

the 23S rRNA genes encompassing the coding sequence for the L11-binding region, were amplified by PCR using *Taq* DNA polymerase (Promega). The DNA fragment containing the *rplK* coding sequence was amplified using primers Tth-rplK1 (5'-CCTAACTTGGGGGGGAGCCTAGGAGGG-3') and Tth-rplK2 (5'-CTGGATGATCTCCTCGCCGCCACG-3'), and the fragment containing part of the 23S rRNA coding sequence was amplified using primers Tth-23SB (5'-GCCGTGGACCCGAAACCGGGCGAG-3') and Tth-23SC (5'-CCCTCTTTA-TCGTTACTCATGCCG-3'). PCR products were sequenced using either primer Tth-rplK1 or Tth-23SK (5'-CCCCA-GACCCGCAGCTTCGGCGCTGG-3').

Primer extension analysis of 23S rRNA

Primer extension reactions were carried out using rRNA extracted from purified ribosomes. Primers used for extension analysis were Tth1067RT (5'-CGCACTCTTTAAAGGATG-GCTGCTTC-3') for 23S rRNA mutations at position 1067 and the +G1067.1 mutant, and Tth1095RT (5'-CGCGGCGC-CACTCGACCAGTGAGC-3') for 23S rRNA mutations at position 1095.

Preparation and chemical modification of ribosomes

Ribosomes were prepared essentially as described (19). Modification of rRNA in 70S ribosomes with dimethyl sulfate (DMS) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-ρ-toluene sulfonate (CMCT) was carried out as described

previously (20). Ribosomes were incubated at 50°C for 10 min prior to modification and, for all analyses, at least two independent ribosome preparations were examined. The L11-binding region of 23S rRNA was scanned using ³²P end-labeled oligonucleotide primer Tth23S-X (5'-GCCTGGGGTCATC-CCCCAGACCCGACG).

Cloning and purification of EF-G

Oligonucleotides were designed to be complimentary to up- and downstream regions of genomic DNA immediately flanking EF-G (accession number X16278), such that the protein produced from the clone would possess the wild-type sequence. An NdeI site was incorporated in the upstream oligonucleotide (5' GATATACATATGGCGGTC AAGGTA-GAGTACG 3') and an EcoRI site in the downstream oligonucleotide (5' GCTCGAATTCATTGACCCTTGATG-AGCTTCTCC 3'). PCR amplification from *T.thermophilus* genomic DNA was carried out, the product digested with NdeI and EcoRI and ligated into the similarly digested pET30b vector (Novagen). Competent *E.coli* DH5α cells were transformed with ligation mixes, then plasmids were recovered from transformants and insert DNA was analyzed by complete sequencing of both strands (Davis, CA). For EF-G purification, the plasmid was transformed into *E.coli* strain BL21 (DE3). Cultures were grown for ~2 h at 37°C, then EF-G production was induced by addition of IPTG (1 mM final concentration) and growth continued for 2–4 h. Cells were harvested and washed by centrifugation (Sorvall GSA rotor, 5000 rpm), cracked in a French pressure cell (20 000 psi) in 10 mM HEPES-KOH pH 7.6, 10 mM MgCl₂, 50 mM NH₄Cl, 5 mM β-mercaptoethanol, and cell debris removed by centrifugation (Beckman L8, Ty65 rotor, 30 000 rpm, 4°C). *E.coli* proteins were denatured by heating at 72°C for 30 min and removed by centrifugation (12 000 g, 10 min). EF-G was concentrated by centrifugation using a Centricon (Amicon) unit and protein concentration determined from the extinction coefficient (45 230 M⁻¹ cm⁻¹).

EF-G-dependent GTP hydrolysis assays

In vitro rates of [γ -³²P]GTP hydrolysis dependent upon EF-G were assayed as described previously (21) except that the reaction was carried out at 65°C. Where present, thiostrepton was included at a 3:1 molar excess over ribosomes [40 nM; 0.1% (v/v) DMSO final concentration] and was preincubated for 5 min at 65°C before initiating the reaction by the addition of EF-G.

RESULTS

Selection, characterization and growth of thiostrepton^R mutants

Thiostrepton^R mutants of *T.thermophilus* strain HB8 arose spontaneously in overnight cultures at frequencies of ~10⁻⁷ (for mutants selected on 1.0 μg/ml thiostrepton) and 10⁻⁶ (for mutants selected on 0.1 μg/ml thiostrepton). In total, several thousand thiostrepton^R isolates were generated and a subset of some 60 mutants was purified and chosen for sequencing.

Based on the locations of previously described thiostrepton-resistance mutations in other organisms, the 5' end of *rplK* (the gene encoding the large ribosomal subunit protein L11) and

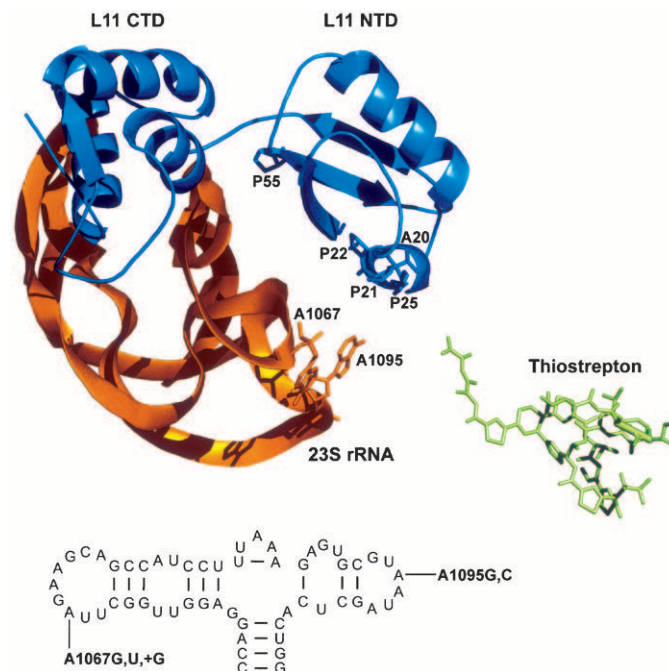


Figure 1. Location of residues that were mutated in this study in the tertiary structure (2) of ribosomal protein L11 (blue) and associated 23S rRNA (orange; PDB accession code 1MMS). The putative thiostrepton-binding pocket (35) formed between the N-terminal domain (NTD) of L11 and the 23S rRNA is sufficiently large to accommodate the drug (shown in green; PDB accession code 1E9W), although not necessarily in the orientation shown here. Figure rendered with PyMOL (36). Also shown is the corresponding 23S rRNA secondary structure for part of domain II from *T.thermophilus*, indicating the positions of mutations described in this study. (Source: Comparative RNA website, www.rna.icmb.utexas.edu).

part of the *rpl* genes that encode the L11-binding region of 23S rRNA were targeted for initial sequencing. Both these regions were sequenced in all isolates, and in all cases mutations within either of these two regions were detected (Table 1). A total of nine L11 mutations and five rRNA mutations were identified. All the L11 mutations occurred at positions in the NTD of the protein, near the cleft formed between this domain and the rRNA. The rRNA mutations occurred in the 1067 and 1095 loops, near the interface with the NTD of L11 (Figure 1).

T.thermophilus is naturally transformable with either chromosomal or plasmid DNA (22,23). To confirm that the mutation identified in each mutant was solely responsible for the thiostrepton-resistance phenotype, wild-type HB8 was transformed with genomic DNA from a representative isolate of each mutant and transformants were selected on plates containing thiostrepton. In all cases, the transformants were found to contain the original mutation. The high efficiency of transformation indicated that a single mutation is responsible for the thiostrepton-resistance phenotype, and as the transformation efficiency is several orders of magnitude greater than the frequency of spontaneous mutation, the recovery of the original mutations almost certainly resulted from transformation with donor genomic DNA.

Alignment of the amino acid sequences of L11 from a range of organisms (using the ClustalW multiple sequence alignment program at www.ebi.ac.uk/clustalw/) reveals the high degree of conservation at positions where the thiostrepton-resistance

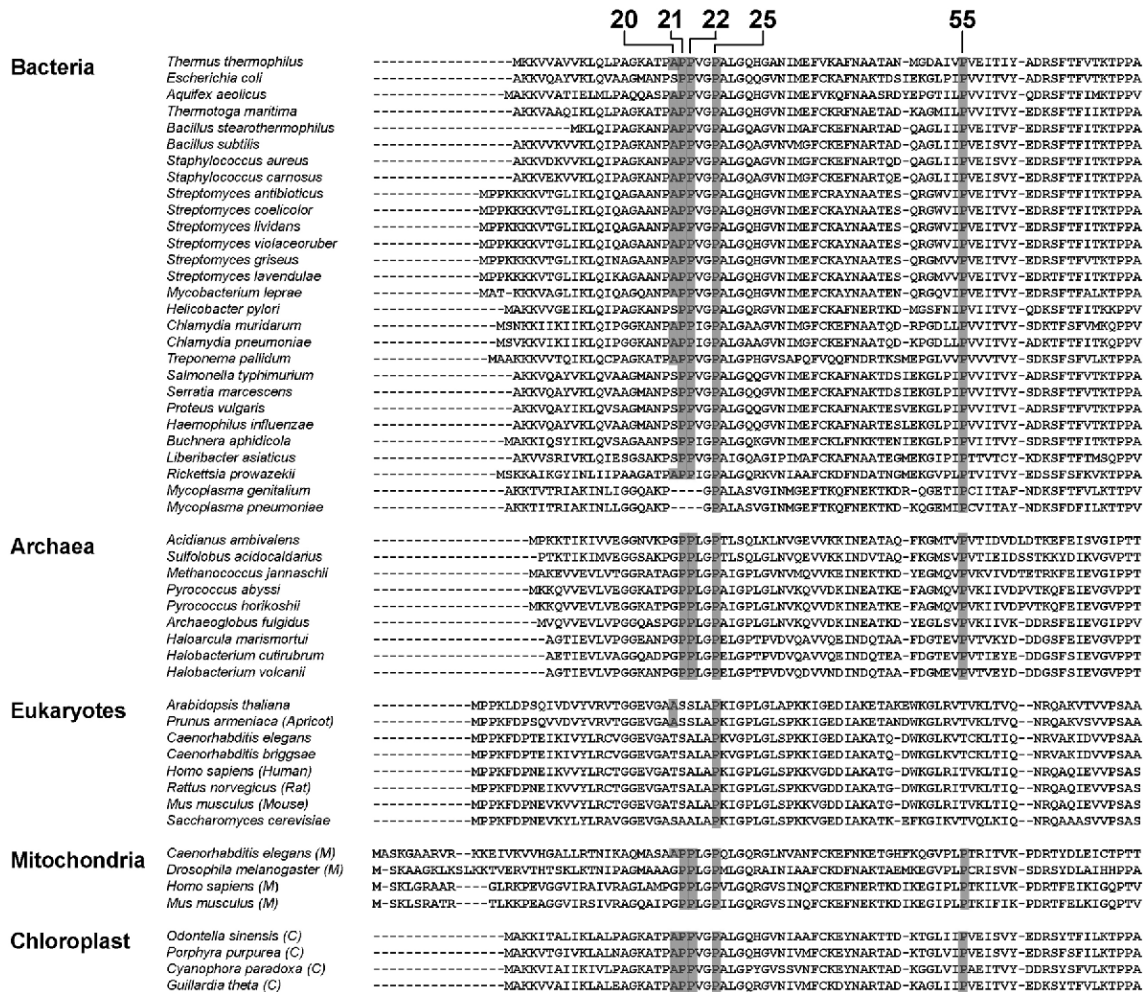


Figure 2. Alignment of amino acid sequences of the L11 NTD from various organisms across all three domains of life and including mitochondrial and chloroplast L11 sequences. Positions mutated in *T.thermophilus* are numbered according to the *E.coli* sequence, and the level of conservation at these positions indicated by highlighting.

mutations were found in this study (Figure 2). In particular, P25 is essentially completely conserved in all organisms for which the L11 sequence is available, across all three domains of life and including mitochondrial and chloroplast sequences (146 sequences in total, 53 of which are shown in Figure 2). Other positions within L11 where mutations occurred are also very highly conserved. Position 55 is a proline in all but eukaryotic nuclear-encoded ribosomes (which contain threonine at this position). Similarly, there is a high degree of conservation of P21 and P22 among bacteria, archaea, mitochondria and chloroplasts.

Positions in the 23S rRNA at which mutations occurred also show a very high degree of conservation. The most highly conserved of these is A1095, which occurs in all bacteria and archaea, as well as in the vast majority of chloroplasts and nuclear-encoded eukaryotic rRNAs. Residue 1067 is an A in virtually all bacterial, archaeal and chloroplast rRNAs, whereas a G is found in most nuclear-encoded eukaryotic rRNAs (24).

The thioestrepton^R mutants of *T.thermophilus* isolated in this study contain both novel mutations as well as mutations described previously in other organisms (Table 1). Because several additional thioestrepton-resistance mutations in this

region have been identified in other organisms, and since some of the mutations reported here were isolated only once, it is unlikely that this study represents an exhaustive search of all possible *T.thermophilus* thioestrepton-resistance mutations.

The doubling times for the isolates ranged from 41 min for the P22S and P22T mutants (a nearly wild-type growth rate) to 63 min for the ΔA20-P21 mutant in TEM (without thioestrepton) at 72°C (Table 1). Most of the mutants showed only moderate growth defects. The slowest growing mutants were those for which the largest changes occurred, i.e. the deletion of two amino acids from the NTD of L11, ΔA20-P21, and the insertion of a G residue at position 1067 (*E.coli* numbering) of the 23S rRNA (+G1067.1).

Pure populations of ribosomes bearing rRNA mutations due to gene conversion

T.thermophilus contains a single copy of each ribosomal protein gene, but carries two copies of the 5S, 16S and 23S rRNA genes (25). Thus, the rRNA mutants were potentially either homozygous or heterozygous. However, the presence of only two copies of each rRNA gene in *T.thermophilus* (compared

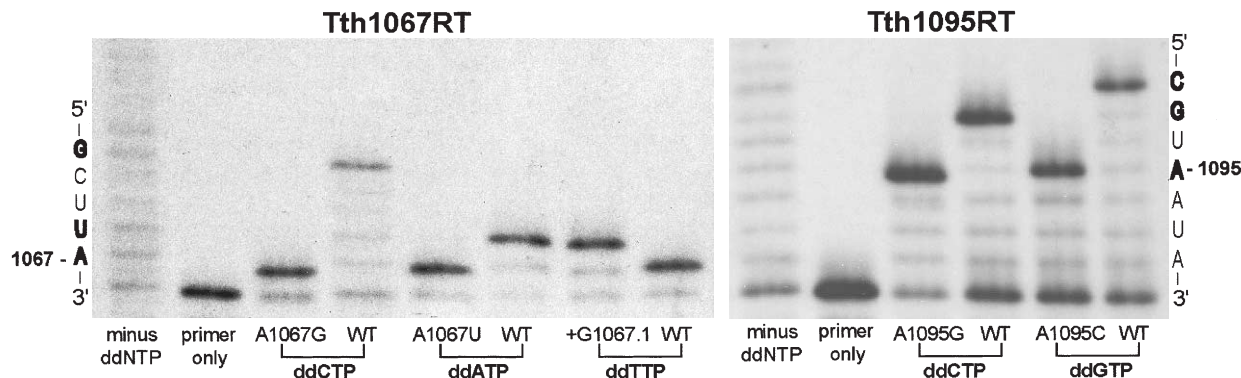


Figure 3. Pure populations of mutant rRNA as determined by primer extension analysis of 23S rRNA from thiostrepton^R mutants of *T. thermophilus* using primers Tth1067RT (to examine mutations at position 1067 and the +G1068.1 insertion) and Tth1095RT (to examine mutations at position 1095). The wild-type sequence at each extension position is indicated on the vertical axis, with stop positions indicated in bold face. 'Minus ddNTP' extensions were carried out on wild-type template rRNA using all four deoxynucleotides, with no dideoxynucleotides, to reveal natural extension terminations at each position. 'Primer only' extensions were carried out without template rRNA, using dideoxythymidine-5'-triphosphate (ddTTP). The dideoxynucleotide termination mix used in each extension is indicated in bold face (ddCTP, dideoxycytidine-5'-triphosphate; ddATP, dideoxyadenosine-5'-triphosphate; ddTTP, dideoxythymidine-5'-triphosphate; and ddGTP, dideoxyguanosine-5'-triphosphate). The template rRNA used in each extension is indicated beneath each lane (WT, wild-type).

with seven rRNA operons in *E. coli*), combined with the high efficiency of homologous recombination in this organism (23) usually resulted in pure populations of mutant ribosomes, presumably due to gene conversion involving a double-strand break repair mechanism (26).

DNA sequencing of homozygous mutants showed only a single termination product at the position of the mutation, whereas those from heterozygous mutants resulted in mixed termination products at the site of the mutation. Of the rRNA mutations obtained, A1095C and A1095G were isolated in both the heterozygous and the homozygous states, whereas all the other rRNA mutations occurred in the homozygous state. Heterozygotes, however, arose only on medium containing the lower thiostrepton concentration (0.1 $\mu\text{g/ml}$). Only homozygotes were used for further study, and the presence of pure populations of mutant ribosomes was confirmed by primer extension analysis (Figure 3).

Effects of mutations on EF-G-dependent GTP hydrolysis and its inhibition by thiostrepton

Ribosomes from wild-type *T. thermophilus* HB8 and a number of the thiostrepton^R mutants were examined for their sensitivity to thiostrepton in EF-G-dependent GTP hydrolysis *in vitro*. This assay is an indirect measure of the affinity of EF-G for the ribosome and has long been known to be exquisitely sensitive to antibiotics of the thiostrepton family (27). The rRNA mutants, A1067G and A1067U, were selected as their relative activities and sensitivities to thiostrepton in the assay were known from an earlier study in *E. coli* (10) and it was of interest to determine whether these were maintained in *T. thermophilus*. The mutant carrying the additional G residue inserted 5' to A1067 was of obvious interest as this mutation has not been reported previously. The protein mutants chosen were those for which the substitution at each position conferred the strongest growth defect (i.e. P21L, P22L, P25R and P55H). The double-deletion mutant ($\Delta\text{A20-P21}$) was again an obvious choice as this too is a novel mutation and, additionally, was the slowest growing of the mutants characterized.

Table 2. Effect of thiostrepton on EF-G-dependent GTP hydrolysis on wild-type *T. thermophilus* and some of the thiostrepton^R mutant ribosomes

Mutation	EF-G-dependent GTP hydrolysis (pmol GTP/ pmol 70S/min)		Inhibition (%)
	-Thiostrepton	+Thiostrepton	
None	1589 \pm 15	638 \pm 13	60
P55H	1109 \pm 10	643 \pm 8	42
P21L	1451 \pm 23	1088 \pm 14	25
P22L	1405 \pm 37	1208 \pm 3	14
P25R	1012 \pm 13	929 \pm 1	8
$\Delta\text{A20-P21}$	1108 \pm 18	1039 \pm 2	6
A1067G	1508 \pm 14	1191 \pm 5	21
A1067U	1689 \pm 38	1691 \pm 24	0
+G1067.1	1097 \pm 21	954 \pm 5	13

At a 3-fold molar excess of thiostrepton, wild-type ribosomes were substantially, but not totally, inhibited (Table 2). Curiously, at this concentration, complete abolition of hydrolysis has been observed for *E. coli* and *Bacillus megaterium* ribosomes. We have no explanation for this discrepancy, but differences relating to the necessary thermal stability of the *T. thermophilus* components may be of relevance. Only the A1067U mutant showed complete resistance to the drug at this input and, as in *E. coli*, the A1067G mutant was more thiostrepton sensitive than the A1067U mutant. The insertion mutant was more thiostrepton resistant than the A1067G mutant, but was considerably compromised in hydrolysis rate, consistent with its slow growth (Table 1). Among the protein mutants, a range of resistance levels was exhibited, from very weak (P55H) to high (P25R, $\Delta\text{A20-P21}$). Interestingly, there was no correlation between resistance levels and hydrolysis rates. For example, P55H and $\Delta\text{A20-P21}$ both showed reduced hydrolysis rates but were at opposite ends of the resistance spectrum. There is also no simple correlation between *in vitro* hydrolysis rates and growth rates, underscoring the sensitivity of this region to structural changes.

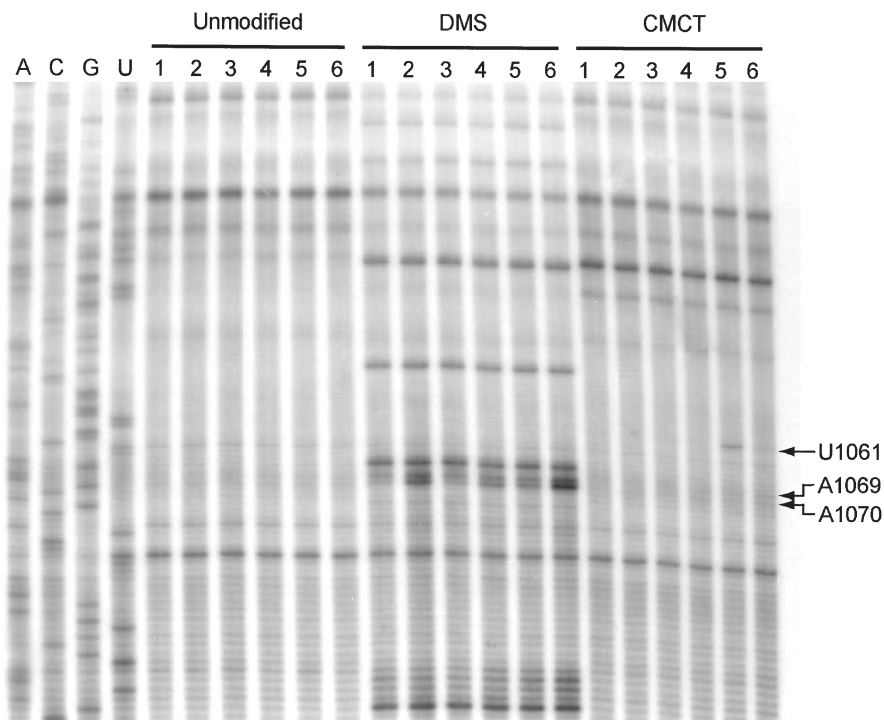


Figure 4. Structure probing gel of 1050–1100 region of 23S rRNA using an oligonucleotide primer complementary to positions 1157–1184. A, C, G and U, dideoxy sequencing lanes; lane 1, ribosomes from wild-type strain HB8; lane 2, ribosomes containing the L11 P21L substitution; lane 3, ribosomes containing the L11 P22L substitution; lane 4, ribosomes containing the L11 P25R substitution; lane 5, ribosomes containing the L11 P55H substitution; and lane 6, ribosomes containing the L11 mutation Δ A20-P21. Unmodified, DMS- or CMCT-modified ribosomes are indicated. The arrows indicate the positions of U1061, A1069 and A1070, where novel modifications were observed.

Effects of L11 mutations on the conformation of rRNA in the factor-binding region

The same protein mutants chosen for the *in vitro* EF-G hydrolysis assay were analyzed for their potential to cause local structural changes in rRNA at the L11-binding region by assessing the accessibility of adjacent 23S rRNA nucleotides to chemical modification with DMS or CMCT. Ribosomes containing a P21L substitution in L11 or Δ A20-P21 showed enhanced reactivity to DMS at nucleotides A1069 and A1070 (Figure 4, DMS lanes 2 and 6). Similarly, ribosomes containing a P55H substitution in L11 exhibited increased reactivity of U1061 to CMCT (Figure 4, CMCT lane 5). Positions 1061 and 1070 are located near the interface with the NTD of L11 (Figure 5), whereas A1069 faces away from the protein.

The increased reactivity of these bases to chemical probes in ribosomes containing mutations within L11 could be due to local structural changes that would occur if the mutated amino acids were in the immediate vicinity of the reactive base. Alternatively, these modifications may result from a more open conformation of the L11 region. Consistent with this, U1061 is located nearly 9 Å away from P55 in the X-ray crystal structure of the L11–rRNA complex, whereas A1070 and A1069 are more than 9 and 15 Å away from P21, respectively.

DISCUSSION

Examination of the positions of the mutated residues in the tertiary structure of the L11 binding region reveals that the

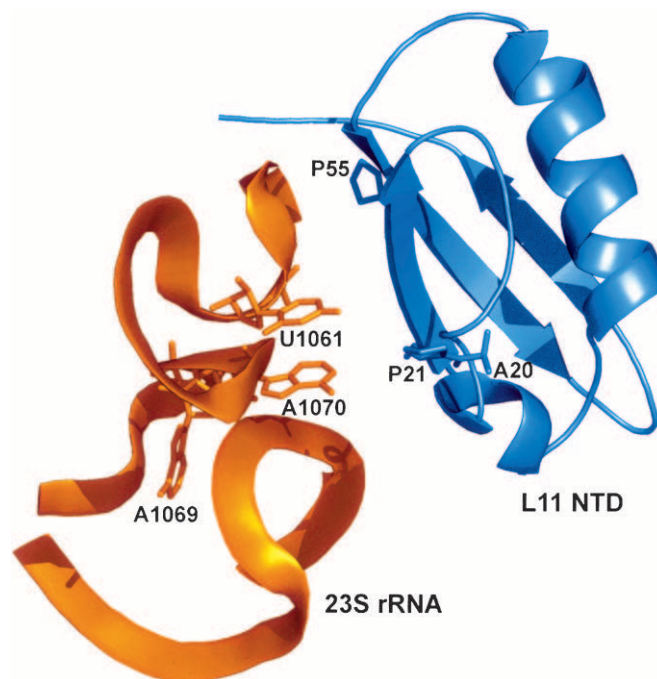


Figure 5. Location of 23S rRNA residues that exhibit novel reactivity to DMS (A1069, A1070) and CMCT (U1061) relative to the positions of the causative mutations in L11 in the tertiary structure (2) of the L11–rRNA complex (PDB accession code 1MMS). 23S rRNA is shown in orange; L11 is shown in blue. Figure rendered with PyMOL (36).

mutations occur at sites that encircle the putative binding pocket for thiostrepton Figure 1 (2). Previous studies have demonstrated that mutation of A1067 or A1095 of *E.coli* 23S rRNA reduce the affinity of thiostrepton for the ribosome by up to 1000-fold (5,6). Mutagenesis of RNA fragments corresponding to the L11 binding site of *E.coli* 23S rRNA revealed numerous positions where base substitutions reduced the binding of thiostrepton (28), with many of these changes also affecting, to varying degrees, the binding of L11. The only two positions at which changes dramatically reduced the binding of thiostrepton without significantly affecting L11 binding were A1067 and A1095. These two residues do not make direct contact with L11 but do form the entrance to the cleft where thiostrepton is presumed to bind (Figure 1). Therefore, it appears that our genetic selection strongly favors mutants which retain the ability to incorporate L11, although there are a number of L11-minus mutants that have been generated previously in a range of organisms by different deletions, mutations, and antibiotic resistance selection (Table 1). All show some degree of thiostrepton resistance but all the mutations result in extremely deleterious growth phenotypes, which may well hamper selection in *T.thermophilus*.

In contrast, serine or threonine substitutions at P21 of L11 from *Halobacterium halobium* and *E.coli* confer resistance, apparently without affecting thiostrepton binding (8). The ability of the L11 mutant ribosomes to function in the presence of bound thiostrepton implies that the drug stabilizes a non-productive conformation of the ribosome and that L11 mutations counteract this effect by destabilizing this region. The notion that P21 may be important for maintaining the native structure of L11 is also supported by the observation that both the *T.thermophilus* Δ A20-P21 and P21L mutants produced a similar modification profile in which A1069 and A1070 exhibit increased reactivity to DMS. In addition, substitutions at this position in recombinant *E.coli* L11 affect the thiostrepton-induced proteinase susceptibility of residues at the junction of the N- and C-terminal domains of the protein (8). The enhanced reactivity of A1069 to DMS indicates that these mutations not only affect the orientation of the NTD of L11, but must also perturb the conformation of the RNA, since the N1 of A1069 is not directly adjacent to the L11 NTD but instead is directed away from the protein. While none of the proline residues at which thiostrepton-resistance mutations were found in this study makes specific contacts with rRNA, the nearby Q29 may interact specifically with A1095 (2), and mutations disrupting this interaction could affect the equilibrium between different conformations of this region. In this context, it is noteworthy that the P21L mutation has only very mild consequences for the rate of EF-G-dependent GTP hydrolysis.

The P55H substitution produced conformational effects distinct from those of the P21 mutations, with U1061 becoming reactive to CMCT. This nucleotide forms a long-range stacking interaction with A1070, which is important for stabilizing the relative orientations of the A1067 and A1095 loops (2). In the wild-type ribosome, this residue may be protected from modification by the proximity of the NTD of L11, potentially via a stabilizing hydrogen bond interaction between K10 in the NTD and the RNA backbone at U1061. Unlike P21, P22 and P25, P55 of L11 is more remote from the putative thiostrepton-binding site and is located closer to the junction between the

N- and C-terminal domains (2,29). This proline could potentially interact with the hinge region between the two domains, thereby influencing the flexibility of the NTD. Possibly, the considerable effect this mutation has on the rate of EF-G-dependent GTP hydrolysis may be a reflection of an altered equilibrium in NTD conformations. Mutation of this residue might therefore disrupt the action of thiostrepton by adjusting the position of the NTD relative to the rRNA, although the resistance achieved is only modest, at best (Table 2). Indeed, examination of the crystal structure reveals that P55 is located $<4 \text{ \AA}$ away from the main chain of L71, a residue in the hinge region that specifically interacts with rRNA (2).

It should be noted that base-specific chemical reagents modify only a limited subset of base positions such that the conformational effects of these mutations may not be limited to the bases observed to undergo changes in reactivity. In addition, the chemical probing experiments presented here were performed well below the physiological temperature for *T.thermophilus*. It is possible that at physiological temperatures, a more pronounced conformational effect of these mutations would be manifested. Consistent with this notion, thiostrepton inhibition of EF-G-dependent GTP hydrolysis using *T.thermophilus* ribosomes and EF-G exhibits a strong temperature dependence (data not shown).

Two conformational states of the L11 region have thus far been observed by cryo-EM studies; an open conformation, which was seen in ribosome-EF-G-GMPPCP complexes, and a closed conformation, which was observed in fusidic acid-stabilized ribosome-EF-G complexes (3,4). These states presumably reflect the conformation of the region when EF-G is bound in the GTP and GDP forms, respectively. The transition from the closed to the open conformation involves movement of the L11 NTD by $\sim 5 \text{ \AA}$ away from the rRNA, and a rotation by $\sim 15^\circ$. It has been proposed that thiostrepton acts by blocking the transitions from pre- to post-translocation states in both directions (30). The L11 mutations described here produce two distinct open structures of the factor binding region, and it is tempting to speculate that they confer thiostrepton resistance by shifting the conformational equilibrium toward the native open conformation observed in wild-type, EF-G-GTP-bound ribosomes. Future cryo-EM, crystallographic and biochemical studies aimed at resolving these structural perturbations in greater detail and understanding their functional consequences further will illuminate the physiological relevance of the dynamics of the L11 region.

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