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Molecular Determinants of Microbial Resistance to Thiopeptide Antibiotics

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Abstract: Ribosomally produced thiopeptide antibiotics are highly promising lead compounds targeting the GTPase-associated region (GAR) of the bacterial ribosome. A representative panel of GAR mutants suspected to confer resistance against thiopeptide antibiotics was reconstituted *in vitro* and quantitatively studied with fluorescent probes. It was found that single-site mutations of the ribosomal 23S rRNA binding site region directly affect thiopeptide affinity. Quantitative equilibrium binding data clearly identified A1067 as the base contributing most strongly to the binding environment. The P25 residue on the ribosomal protein L11 was essential for binding of the monocyclic thiopeptides micrococcin and promothiocin B, confirming that the mutation of this residue in the producer organism confers self-resistance. For the bicyclic thiopeptides thiostrepton and nosiheptide, all studied single-site resistance mutations on the L11 protein were still fully capable of ligand binding in the upper pM range, both in the RNA−protein complex and in isolated 70S ribosomes. These single-site mutants were then specifically reconstituted in *Bacillus subtilis*, confirming their efficacy as resistance-conferring. It is thus reasoned that, in contrast to modifications of the 23S rRNA in the GAR, mutations of the L11 protein do not counteract binding of bicyclic thiopeptides, but allow the ribosome to bypass the protein biosynthesis blockade enforced by these antibiotics in the wild type.

Introduction

The resistance of pathogenic bacteria to standard antibiotics is strongly increasing, in both nosocomial and community-acquired infections.[1] To combat the bacterial pathogens involved, new strategies and molecules with unused modes of action are intensively investigated.[2] Recurrent promise in the area is contributed by thiopeptide antibiotic scaffolds (Figure 1), among them thiostrepton (**1**) and micrococcin (**2**),[3] which were originally identified more than 50 years ago. Recent research has conclusively demonstrated that these highly complex polyheterocyclic molecules are ribosomally synthesized in *Streptomyces*, *Bacillus*, and *Micrococcus* strains,[4] and that thiostrepton (**1**) is configurationally labile.[5]

In bacteria, thiopeptide antibiotics target the ribosome or ribosome-associated factors.[3] By virtue of strongly inhibiting protein synthesis, they repress Gram-positive species with astonishing efficacy *in vitro*. Very high activity against clinically relevant methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecium* (MREF) as well as penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vancomycin-resistant enterococci (VRE) were found for thiopeptide antibiotics or derivatives.[3,6,7] Additionally, in recent screening efforts thiopstrepton **1** was identified as a promising candidate for further investigation against infections caused by *Mycobacterium tuberculosis*.[8] Thiopeptide antibiotics were introduced as feed

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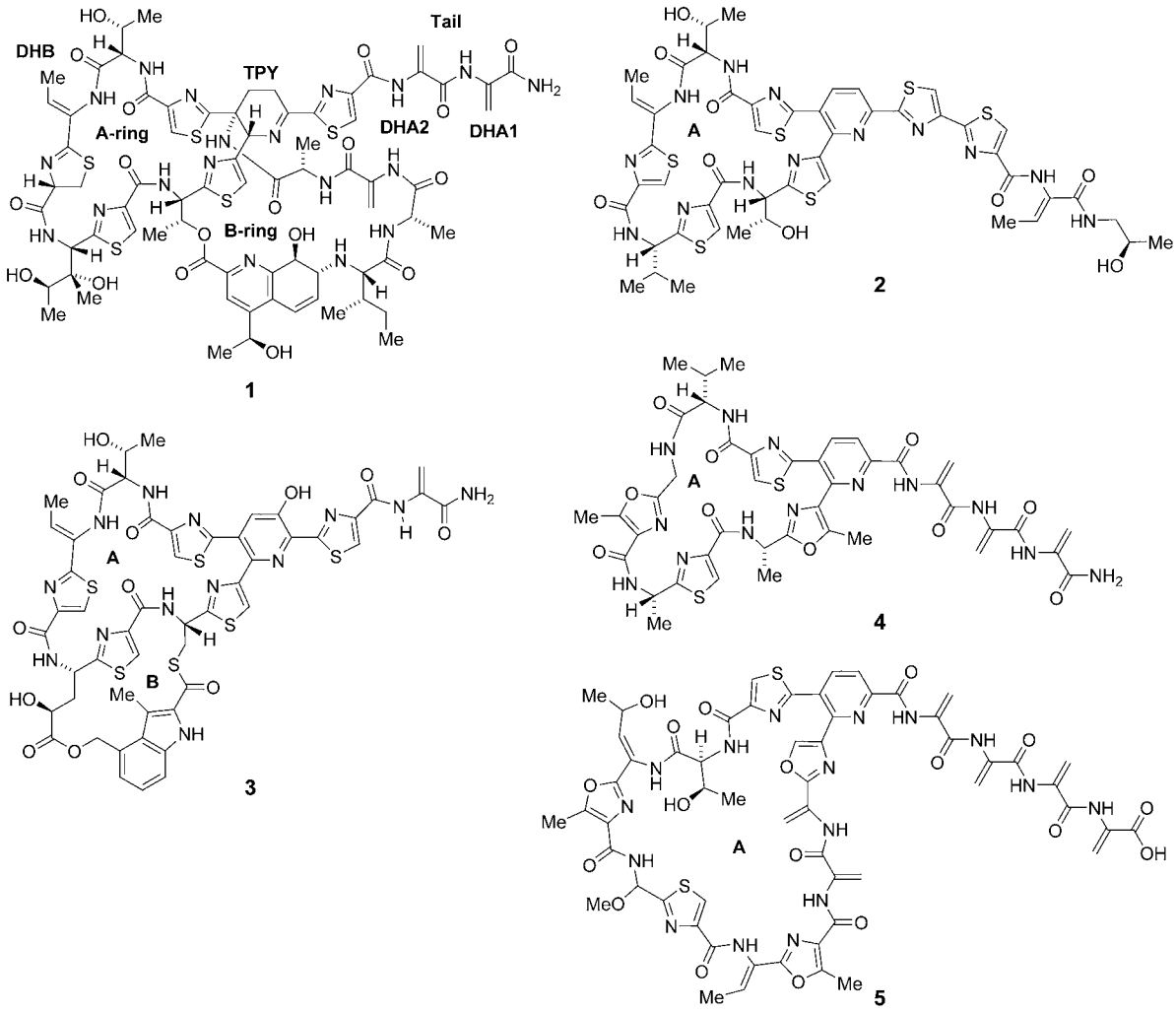

Figure 1. Molecular structures of the thiopeptide natural products thiostrepton (**1**), micrococcin (**2**), nosiheptide (**3**), promothiocin B (**4**), and promoinducin (**5**). Crucial residues of **1** and similar rings are labeled.

additives in animal farming early on, and thiostrepton is currently used in combination therapy ointments to treat dermatological indications in domestic animals (Panalog, Animax, Solvaderm, Derma-4, etc.). The rather unfavorable physicochemical properties of thiopeptide antibiotics, namely, their considerable molecular weight and rather low aqueous solubility, are customarily believed to limit further development as drugs for systemic application in humans, but for some derivatives promising activities have been reported in animal models.[7]

The major molecular target of thiopeptide inhibition is the complex of the 23S rRNA and the ribosomal L11 protein, situated at the so-called "stalk base" of the GTPase-associated region (GAR).[3] This target remains unused for human therapy thus far. No cross-resistance with common antibiotics has been found for it, which should render the thiopeptide binding region per se highly promising for antibiotic development.[4f,6,7,9] Unlike most other antibiotics targeting the ribosome, which mainly bind to the rRNA,[10] thiopeptides block a cooperative binding region formed by the rRNA and a ribosomal protein. Over time, considerable biochemical evidence[11,12] and structural studies[13] have accumulated, which clarified that most thiopeptides interact with nucleobases A1067 and A1095 (*E. coli* numbering) at the tips of helices 43 and 44 of the 23S rRNA (Figure 2), as well as a proline-rich helix in the L11 protein N-terminus (21-**PP**VG**P**ALQQH-30, *Thermus thermophilus* sequence and numbering). Owing to the switch-like, multiconformational quality of the GAR[13g] and the different structure determination techniques and preparation conditions, several models for thiopeptide binding have been proposed.[12d,13c–f] Recent X-ray crystal structures of thiopeptides soaked into the 50S subunit of *Deinococcus radiodurans* could be refined to 3.3 Å resolution for thiostrepton (**1**) and assigned the binding site of these

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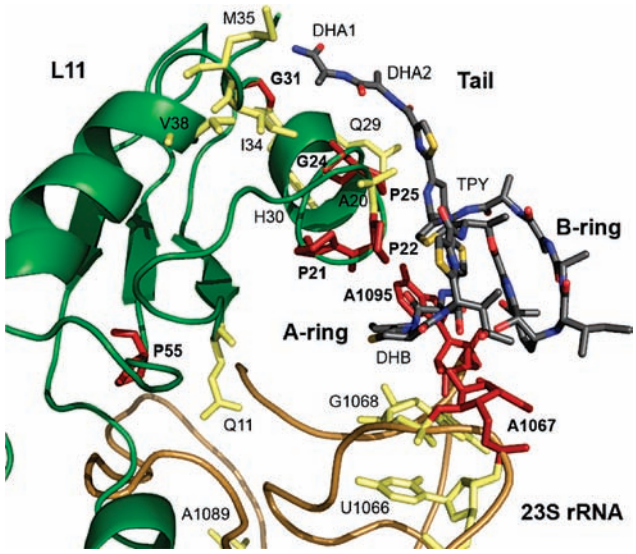


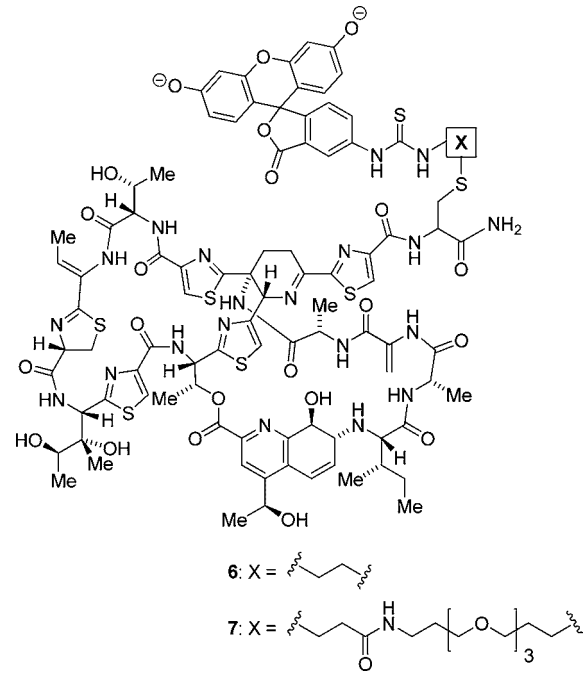
Figure 2. Mutations of the GAR studied in this work mapped on the thiostrepton crystal structure data at the ribosome (PDB 3cf5).[13f] Sites previously reported to lead to reduced sensitivity when mutated are colored red;[13] other residues investigated in this study are colored yellow. Thiostrepton is labeled similar to Figure 1.

antibiotics to a cleft-like cavity between the L11 protein and the 23S rRNA,[13f] in line with the cooperative binding event and much of the biochemical evidence (Figure 2). Early filter binding studies and recent investigations with fluorescent labels on RNA and on thiopeptide ligands have successively detailed that thiopeptides bind to the respective, reconstituted RNA−protein complex with affinities in the lower nanomolar to picomolar range.[6,14,15]

Resistance development of bacterial pathogens to thiopeptide antibiotics has not been documented *in vivo*. However, for several bacterial species spontaneous resistance-conferring mutations have been described to occur at low rates after challenging the bacteria *in vitro* with thiopeptides for selection.[7,16−18] Mutations found on the L11 protein reached from single amino acid exchange to complete loss of the full protein or its N-terminal ligand-binding domain.[7,16] On the rRNA only mutations of nucleobases A1067 and A1095 were reported to contribute to thiopeptide resistance.[5,18] Furthermore, thiostrepton (**1**)-producing streptomycetes express a specific methyltransferase to catalyze the 2′*O*-methylation of A1067, which renders the organism self-resistant.[17] The respective gene is frequently applied as a tool in *Streptomyces* biotechnology for strain selection.[17e,f] However, beyond these data the molecular basis of resistance against thiopeptide antibiotics remains unclear. In order to fathom the molecular recognition events involved in these diverging resistance mechanisms, we investigated a collection of single-site modifications on rRNA and L11 protein on the molecular level by determining quantitative equilibrium binding data with different key thiopeptide antibiotics. Critical cases were reconstituted to study their influence in live cells. Figure 2 compiles the investigated residues on the ribosomal crystal structure data obtained for thiostrepton bound to the 50S subunit of *D. radiodurans*.[13f]

Results

In order to quantitatively assess thiopeptide binding to the mutated GAR, we employed fluorescence anisotropy titrations using H43/H44 wt RNA fragments (58nt, *E. coli* sequence positions 1051−1109) and *T. thermophilus* L11 (*Tth*L11) wt protein. Attachment of a fluorescent dye to the terminal dehydroalanine residue of truncated thiostrepton had been found to result in high-affinity binders for the complex formed by RNA and protein.[6] We have now investigated other linkers between the thiostrepton core and the fluoresceine dye and found probe **6** superior to the PEG-linked variant **7** with respect to ease of synthesis, purification, and long-term stability. Using probe **6**, we found a K_D of 0.33 nM to the RNA/protein complex, 2-fold less than for the previously used probe **7** (0.14 nM) but still comparable to the parent compound **1** (0.20 nM).[6] Displacement titrations with unlabeled thiopeptides gave similar results for both probes **6** and **7**.



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The unperturbed binding and moderate influence of linker type and length or the negatively charged dye on the probe's properties indicated that the linker attachment point is benign and exposed to the aqueous solvent, in line with previous studies.[12d,13] However, the presence of the L11 protein was absolutely indispensable for observing stable binding events of the labeled probes under these homogeneous equilibrium binding conditions.[6,12c,15] We could not observe any binding of the labeled probe **6** or **7** to the respective RNA fragment up to concentrations of 10 μM in the absence of L11 (see Supporting Information). The independence of the binding data from linker length and the displacement titration data (*vide infra*) suggests that the unlabeled ligands perform similarly. It cannot be excluded that the wt RNA fragment or its frequently used mutant U1061A[19] with its artificially stabilized secondary structure displays weak residual affinity to unlabeled thiopeptides.[20] However, in either case ligand affinity weakened by at least 3−4 orders of magnitude is highly unlikely to have consequences in cells when considering the modest solubility of thiopeptide antibiotics in aqueous medium (low μM range).[6]

The H43/H44 wt RNA fragment had been reported before to be dependent on Mg^{2+} for reaching a fold competent for ligand binding.[21] In line with these reports, we detected no ternary complex formation of probe **6**, L11, and wt RNA in the absence of Mg^{2+} at neutral pH (7.4). An apparent $EC_{50}(Mg^{2+}) = 0.05$ mM and complete saturation at 1 mM Mg^{2+} were determined for this interaction by titration (Supporting Information), nicely corresponding to physiological Mg^{2+} levels. Under Mg^{2+}-free/high K^+ conditions and slightly acidic pH (6.1), which had been applied for solution structure determination of the *Thermotoga maritima* L11 protein in complex with RNA,[13d] probe binding was apparent, but we found the affinity 100-fold reduced to 34 ± 3 nM (Supporting Information).

We then screened for the influence of single-point RNA mutants on the binding affinity of the fluorescent probes. Fragments of the *E. coli* 23S rRNA (58nt, positions 1051−1109) carrying all possible single-site mutations of A1067 and A1095 were synthesized by T7-mediated *in vitro* transcription. Mutations proximal (U1066, G1068) and distal (A1089) were investigated as well, together with the 58 nt fragment incorporating a 2′*O*-methylated nucleotide at A1067, which was obtained by total chemical synthesis.

The 2′*O*-methylated A1067 RNA derivative showed a drastic affinity decrease (~3000-fold) to probe **6**, reporting the effectiveness of the resistance mechanism of the thiostrepton producer *Streptomyces azureus*.[16] High affinity losses were also found for the transversion mutants A1067C and A1067U (~1000-fold), but not for the transition A1067G, which performed comparably to the wt RNA. This indicated that thiopeptide affinity to residue 1067 is mainly triggered by the purine or pyrimidine nature of the base heterocycle. In fact, previous studies[11] on the A1067G mutation showed that this modification had only very weak impact on thiostrepton activity in bacteria.[16,18,20]

Interestingly, mutations of residues directly adjacent to A1067 had no effect on probe binding and are hence not expected to be involved in thiopeptide recognition (Figure 3, Table 1). The same was true for A1089G far away from the proposed thiopeptide binding site. Analysis of position 1095 revealed that each mutation led to a fair decrease of the probe affinity (7- to 15-fold), in line with the increased thiopeptide tolerance described. Taken together, these results show that resistance-conferring mutations on the RNA level implicate a reduction of thiopeptide affinity dependent on RNA structure. The impact of mutations at the A1095 site was much less pronounced than at A1067, indicating this nucleobase as the main element of RNA recognition by **1** (Figure 3).

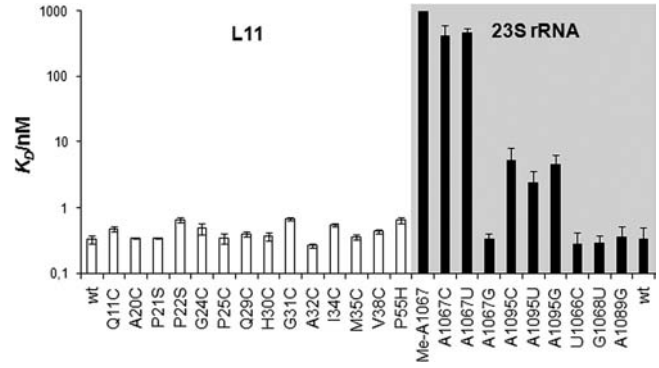

Figure 3. Summary of quantitative binding data of fluorescently labeled thiostrepton to *Tth*L11 protein and 23S rRNA mutants. Note the logarithmic scale.

Table 1. Affinities of RNA Single-Point Mutant/wt *Tth*L11 Complexes to Thiostrepton Probe **6**

entry	RNA	K_D/nM
1	wild type (wt)	0.33 ± 0.17
2	A1067C	410 ± 168
3	A1067U	460 ± 90
4	A1067G	0.34 ± 0.07
5	A1095C	5.2 ± 2.8
6	A1095U	2.3 ± 1.2
7	A1095G	4.5 ± 1.9
8	U1066C	0.28 ± 0.15
9	G1068U	0.29 ± 0.09
10	A1089G	0.36 ± 0.17
11	2′-*O*-methyl A1067	970 ± 20

We then analyzed the impact of 14 different point mutations in the L11 N-terminus—including six thiopeptide-resistance mutations previously described[16] (Figure 2)—on their impact on the binding of fluorescently labeled **1**. All proteins were generated by site-directed mutagenesis of the *Tth*L11 wt protein rplK gene. Notably, none of them showed significantly altered binding affinities to the probe (Figure 3, Table 2), which varied only between 0.27 and 0.69 nM (Table 2). Alkylating cysteine residues in the binding region with iodoacetamide (where possible) did not influence the probe binding to a major extent either (data not shown).

To rule out that our results were skewed by the fluorescently labeled probe, we performed competition titrations with **1**. The apparent affinities thus determined were comparable (~0.2 nM, see Figure 5 and Supporting Information) for the three proteins tested (L11 wt, G24C, and P25C), demonstrating that the fluorescent label itself is benign and that **1** binds to these mutants with similar affinity. Similar results were obtained for the closely related bicyclic thiopeptide nosiheptide (**3**) (Figure 5 and Table 3). Hence binding of **1** and **3** to *Tth*L11/RNA complexes seemed almost unperturbed by mutations of the protein.

In order to find out if a different binding profile resulted from the GAR in the native ribosomal environment, we investigated intact 70S ribosomes from *E. coli*. In the past, fluorescently

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Table 2. Affinities of *Tth*L11 Single-Point Mutant/wt RNA Complexes to Thiostrepton Probe **6**

entry	L11	K_D/nM
1	wild type (wt)	0.33 ± 0.05
2	Q11C	0.48 ± 0.04
3	A20C	0.35 ± 0.01
4	P21S	0.34 ± 0.01
5	P22S	0.66 ± 0.06
6	G24C	0.49 ± 0.09
7	P25C	0.35 ± 0.06
8	Q29C	0.41 ± 0.03
9	H30C	0.37 ± 0.06
10	G31C	0.69 ± 0.05
11	A32C	0.27 ± 0.02
12	I34C	0.56 ± 0.03
13	M35C	0.36 ± 0.03
14	V38C	0.44 ± 0.03
15	P55H	0.65 ± 0.07

labeled erythromycin derivatives have been successfully used for investigation and screening of the macrolide binding site on *E. coli* ribosomes.[22] To make the GAR amenable for such a study, ribosome chimeras featuring the *Tth*L11 protein and its respective mutants were generated using a described L11 knockout strain[23] with mild overexpression of His_6-tagged *Tth*L11 protein mutants. To isolate the ribosomes efficiently, we utilized a mild affinity-based chromatography protocol.[24] The 23S rRNA remains highly intact using this procedure,[24b] which is crucial considering the exposed positioning of the GAR on the 70S ribosome's rim. Correct incorporation of the tagged L11 in the isolated ribosome fractions was confirmed by SDS-PAGE analysis and immunoblotting using anti-His_6 antibodies (Figure 4).

Titration of probe **6** with the purified ribosome chimeras *in vitro* led to sigmoidal-shaped binding curves, which showed apparent affinities in the 0.5−2 nM range (Figure 4) irrespective of the protein mutant investigated. Given the finite homogeneity of any ribosome preparation and the biophysical limitations of this 1:1 binding study (nM concentrations of labeled probe **6** were necessary), we consider these data only approximating the upper limit for the binding constants of probe **6** to full-size ribosomes, and in extension thiostrepton (**1**) itself. The "true" affinities may be much higher. However, if the mutations strongly reduced ligand binding to a level affecting function in cells (higher nM to μM range), they should become detected in this assay.

L11-deficient ribosomes did not show stable probe binding (Figure 4) in the presence of detergent (NP-40). In the absence of detergent, an ambiguous increase of anisotropy (Figure 4) and brightness (not shown) was observed for L11-deficient ribosomes, likely indicating nonspecific binding or aggregation. Indeed, anisotropy measurements with L11-carrying ribosomes (wt or mutant) were not affected by detergent, and fluorescence lifetime measurements showed a similar trend when excess wt ribosomes (100 nM, $\tau = 3.72 \pm 0.11$ ns) or L11-deficient ribosomes ($\tau = 3.87 \pm 0.12$ ns) were added to free probe **6** ($\tau = 3.34 \pm 0.08$ ns). We conclude that L11 is indispensable for high-affinity thiopeptide binding on fully assembled ribosomes, just as on the isolated RNA/protein complex, and that binding to ribosomes has to be expected for all the L11 mutants studied at least in the low nM range.

These results prompted us to analyze if these L11 point mutations could be solely responsible for rendering bacterial cells refractory to thiopeptide action, as surmised earlier.[16] In fact, in the previous studies the respective protein mutations have been identified by sequencing of the resistant organism's rplK genes coding for the L11 protein, but had not been specifically reintroduced into cells. As thiopeptide antibiotics do not penetrate the outer membrane of Gram-negative bacteria (such as *T. thermophilus* or *E. coli*),[25] we conducted studies in Gram-positive *B. subtilis* where an L11-deficient knockout strain has been described (Bsu *rplK*$^-$).[26] We found this strain heavily impaired compared to the wild type, as indicated by strongly reduced growth. Under our conditions we observed a doubling time of 20 ± 1 min for *Bsu* wt, whereas for *Bsu* rplK$^-$ we found a 6-fold increase (116 ± 17 min).[26] This underlines the importance of the L11 protein for bacterial viability[27] and suggests that resistance by loss of L11 should strongly suspend bacterial pathogenicity.

*Tth*L11 wt protein as well as the single-point exchange mutants P21S, P22S, G24C, and P25C were then transiently overexpressed in Bsu *rplK*$^-$ and compared to the empty expression vector[28] as control. The resulting cultures were screened for thiopeptide resistance. With the application of thiostrepton (**1**, 0.5 μM, Figure 5), *B. subtilis* wt showed no growth at all, whereas the L11-deficient strain was tolerant to **1**, as expected.[26] Importantly, the transient expression of *Tth*L11 wt protein in the L11-deficient strain restored the sensitivity to **1**, as evidenced by a significantly reduced growth rate (−60%). All other L11 mutants tested (P22S, G24C, P25C) showed no response to treatment with **1** in this assay, indicating that they were fully insensitive to the application of thiostrepton within the limits of this experiment. Taken together, these results confirmed the relevance of the protein mutations[9] in cells and showed that inhibitor binding does not significantly impact cell viability in the mutant cells studied.

In order to further clarify the interplay of binding and resistance, we investigated the monocyclic thiopeptide natural

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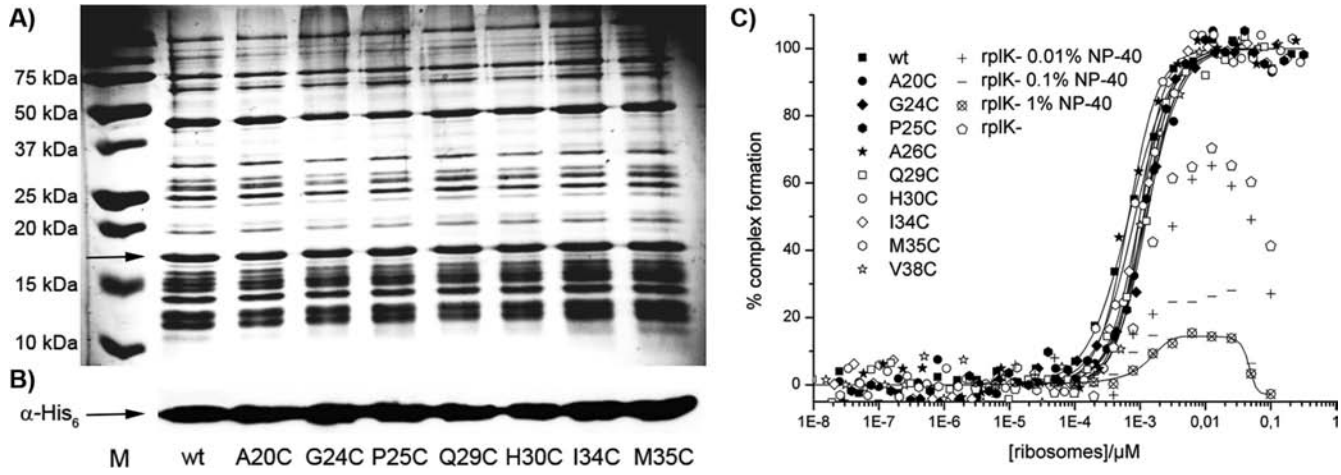

Figure 4. Incorporation of mutant L11 protein in ribosome chimeras and binding study. (A) SDS-PAGE analysis of the ribosomal proteins from isolated ribosomes. *M* = molecular weight marker (Precision Plus, Bio-Rad, München, Germany); arrows indicate the L11 protein band. (B) Western blot analysis of His_6-tagged proteins from isolated 70S ribosomes. (C) Isothermal titration binding curves of probe **6** to isolated ribosome chimeras. Only on L11-deficient ribosomes (rplK$^-$) anisotropy measurements were strongly influenced by detergent.

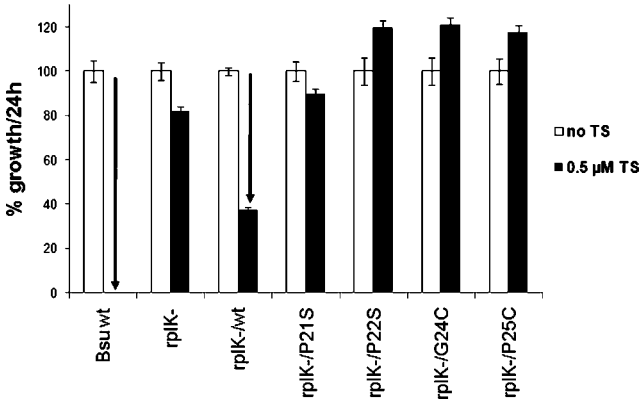

Figure 5. Reconstitution of L11 mutations in *B. subtilis* cells by complementing an L11-deficient strain with transiently expressed mutant *Tth*L11 proteins and screening for thiostrepton (TS) resistance.

Table 3. Affinities of Thiopeptide Natural Products **1**−**5** to Selected L11 Mutant/wt RNA Complexes

	K_D/nM				
*Tth*L11	**1**	**2**	**3**	**4**	**5**
wild type	0.23 ± 0.02	1.46 ± 0.11	0.11 ± 0.01	1040 ± 125	113 ± 13
G24C	0.24 ± 0.01	1.64 ± 0.20	0.11 ± 0.01	1304 ± 171	195 ± 21
P25C	0.30 ± 0.02	>10 000	0.07 ± 0.01	>10 000	139 ± 19

products micrococcin (**2**), promothiocin B (**4**), and promoinducin (**5**) in comparison to the bicyclic thiopeptides **1** and **3**. Micrococcin (**2**) binds to the GAR, but was reported to affect the GTPase activity of the elongation factors in a different fashion from the bicyclic compounds **1** and **3**.[29−33] Monocyclic

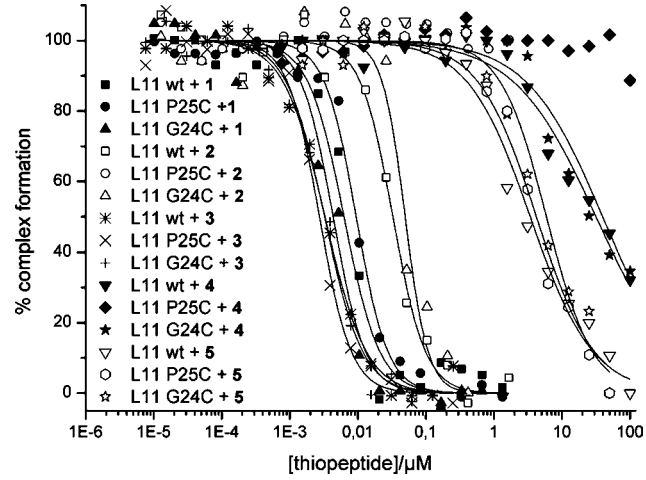

Figure 6. Displacement titrations of **1**−**5** on mutant L11/rRNA complexes. Stoichiometric amounts of RNA and fluorescent probe (5 nM each) were complexed with an excess of L11 (0.6 μM) and titrated with increasing amounts of the thiopeptide ligands.

thiopeptides with an enlarged A-ring—such as GE2270A or thiomuracin—directly target EF-TU.[4e,34]

Interestingly, when performing competition titrations using micrococcin (**2**), probe **6** could not be displaced from complexes of wt RNA and the *Tth*L11 mutant P25C (residue 26 in *E. coli* and *B. subtilis* numbering, Figure 6). Apparently, **2** is not able to bind to complexes carrying a mutation at P25 any more (Table 3), confirming indications from *Bacillus megaterium*.[12b] Notably, in the micrococcin producer *Bacillus cereus* additional copies of the rplK gene exist that code for an exchange Pro→Thr at this position.[4b] We thus confirm that mutation of this particular proline residue selectively abolishes micrococcin binding to the GAR and can hence be regarded as the natural resistance mechanism of the micrococcin producer.

Similar results were obtained for the structurally corresponding promothiocin B (**4**). In this case reduced affinity to P25 mutants was observed likewise, suggesting a binding mode similar to micrococcin. The monocyclic thiopeptide promoin-

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ducin (**5**), with a much larger A-ring (35 vs 26 atoms), did not discriminate L11 wt, G24C, and P25C, indicating an indifferent binding mode not crucially involving P25 (Table 3). These data imply that the repulsive interaction with P25 mutants depends on the size of the main macrocycle (A-ring) and can be masked by a constraining second macrocycle (B-ring) in the thiopeptide scaffold, which can be assumed to rigidify and tune the binding conformation.

Crystal structure data for **2**[13f] indicated a binding pose with direct involvement of the acyclic appendage ("tail") with the binding site, which was not present for **1**. Our data for **2** and the highly related promothiocin B (**4**) support this view. Compared to **2**, the dehydrobutyrine residue in the A-ring is missing in **4**, and the characteristically different tail structure of **4** features three dehydroalanines instead of catenated heterocycles. This results in a 5000-fold loss in affinity for **4**. The thiazole rings in the tail of **2** hence might indeed reinforce ligand binding of monocyclic thiopeptide antibiotics, potentially by a productive hydrophobic stacking interaction with A1095.[13f] The dehydrobutyrine residue is key for tight binding as well, as evidenced by a 10-fold affinity increase from **4** to promoinducin (**5**), which even features an unfavorably enlarged A-ring. Overall, the GAR seems to display affinity to a certain range of hydrophobic ligands, but crucial residues and conformational constraints select between at least two different high-affinity binding modes. These are nicely reported by the distinct resistance mechanisms for thiostrepton (**1**) and micrococcin (**2**).

Discussion

On the molecular level, the binding of the L11/RNA complex in the GAR by bicyclic thiopeptides carries a bipartite character. The molecular recognition of the composite RNA/protein target complex by thiopeptide antibiotics seems to be highly dependent on local RNA structure, in particular on the two crucial residues A1067 and A1095. Interestingly, the adaptability of the GAR and especially of residue A1067 have previously been identified as key factors in the processing of aminoacyl-tRNA during decoding and tRNA accommodation,[13g] which suggests that this region is a hot spot for binding and recognition events. Resistance mutations on the rRNA lead to reduced affinity for thiopeptides, and the *S. azureus* resistance gene responsible for 2′*O*-methylation of A1067 capitalizes on this property as well. Hence bicyclic thiopeptide antibiotics such as **1** primarily recognize the rRNA portion of the GAR, although the L11 protein is absolutely indispensable for high affinity binding.

Resistance to the monocyclic thiopeptide antibiotic micrococcin (**2**) can arise from a point mutation in the L11 protein, which does abrogate inhibitor affinity, presumably by affecting a key binding interaction with P25. Mutations on the L11 protein likewise confer indifference to the bicyclic thiopeptide antibiotics **1** and **3**, as clearly shown by specific reconstitution of individual mutants in *B. subtilis*. However, thiostrepton (**1**) and nosiheptide (**3**) bind tightly to the target structure, both in the isolated L11/RNA complex and in full-size 70S ribosomes. Hence resistance does not go in line with reduced binding in these cases.

Resistance mutations that do bypass stable inhibitor binding are not very common but have been described, for example for HIV-1 reverse transcriptase inhibitors[35] or for macrolide antibiotics binding the 50S ribosomal subunit.[36] At the GAR, this functional dichotomy cannot easily be explained by changes in the topography of a static binding site environment, which would accommodate the binding inhibitors in a "lock-and-key" fashion. Most of the relevant mutations on L11 involve Pro residues. An increase in conformational flexibility of the proline-rich helix in the L11 N-terminal domain can hence be anticipated in the resistance mutants. This might allow adaptation of this ribosomal region to the obvious blockade occurring when an inhibitor is bound to the wt GAR. The well-studied dissimilar action of micrococcin (**2**) and thiostrepton (**1**) at the GAR of wt ribosomes provides circumstantial support for this notion. **2** was reported to promote GTP hydrolysis from EF-G *in vitro*,[29,30] but **1** inhibits GTP turnover.[30−33] Such differences imply that binding of the different ligands is functionally distinct and that it is not mutually exclusive with elongation factor (EF) binding and hence translational processing. Furthermore, recent crystal structure data indicate conformational changes in this region upon binding of EF-G and EF-TU to the GAR, in particular movements of the L11 N-terminal domain with respect to the 23S rRNA.[37] Resistance mutations of crucial residues of L11 could allow overcoming the conformational blockade exerted by the molecular "glue" of a bound ligand, which apparently rigidifies the respective orientations of 23S rRNA and L11 protein by tightly inserting into the dynamic cleft separating the two biomacromolecules.[13d,e,g,37,38] This added flexibility might then rescue translation by re-enabling EF-G catalysis, which stalls in the wt,[38] although alternative explanations for co-occupancy of inhibitor and EFs cannot be excluded at this stage.

Some experimental data on thiostrepton competition with EF-G and/or stabilization of a ternary ribosome/thiostrepton/EF-G complex have been reported.[30,31,39,40] Additionally, IF-2 was shown to be affected by **1** in a similar fashion to EF-G.[40] Hence, the resistance mutations studied on the L11 protein might also facilitate intermittent displacement of the strongly bound bicyclic thiopeptide inhibitors by ribosomal factors, potentially promoted by phosphate release from their GTP-loaded states. Further substantiation of this property must await investigations of structure and dynamics of this ribosomal region when the L11 protein is mutated. Nonetheless, on the 23S rRNA resistance mutations or modifications of crucial residues in the GAR were found to directly compromise molecular recognition properties for thiopeptide antibiotics, which reduce ligand affinity to levels below physiological consequence.

Conclusion

The GTPase-associated region (GAR) is a highly promising target on the bacterial ribosome for the development of new anti-infective lead compounds. We have characterized the individual molecular contributions of thiopeptide binding to the reconstituted complex from *T. thermophilus* L11 protein and rRNA in a quantitative fashion. We found that in the cooperative binding event the molecular recognition of rRNA by thiostrepton

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and nosiheptide conforms to a lock-and-key pattern, whereas binding is much less influenced by resistance mutations of the L11 protein, as conclusively shown by full-size ribosome studies and in-cell reconstitution. Notably, micrococcin was found to sense a resistance mutation in the L11 protein, in line with genetic data from *B. cereus*.

The L11 protein was indispensable for high affinity binding in all cases, suggesting that it acts as a hydrophobic complement of the RNA binding/recognition event of thiopeptide ligands, which conformationally lock the orientation of 23S rRNA and the L11 protein. Resistance mutations on the L11 protein enable overcoming this conformational blockade, likely by increasing conformational flexibility and restoring EF-G function. These insights are expected to open up new avenues for making thiopeptides and the GAR applicable for combating bacterial pathogens and should stimulate new compound designs and screening formats for research on anti-infectives.

Experimental Section

RNA Synthesis and Purification. Canonical RNAs were synthesized by T7-polymerase-mediated *in vitro* transcription from cDNA oligonucleotides, coding for the sequence of a wild type or mutant 58nt fragment of *Escherichia coli* 23s rRNA (1051−1109) flanked on the 5′-end by the sequence of the T7 promotor as well as *Eco*RI and *Xba*I restriction sites.[12d] A list of the sequences used can be found in the Supporting Information. To ensure homogeneity and to allow sequence validation, the complementary oligonucleotides were annealed and ligated into an *Eco*RI/*Xba*I predigested pUC19 vector. Each resulting plasmid was transformed into *E. coli* XL1 blue cells by electroporation, amplified, isolated, and independently sequenced. The purified plasmids were linearized with *Xba*I to determine the 3′-ends of RNA transcripts and then used as templates for *in vitro* transcription (MEGAscript T7 High Yield Transcription Kit, Ambion). The RNA transcripts were directly purified by preparative native 10% PAGE (10 mM Tris, 10 mM boric acid, 2.5 mM EDTA, pH 8.3), eluted with H_2O, desalted with the aid of NAP5 columns (GE Healthcare), and stored in H_2O at −20 °C. RNA purity was confirmed by native 10% PAGE, UV spectroscopy, and CD spectroscopy (see Supporting Information).

The 58nt 2′OMe-A1067 RNA derivative was obtained from Dharmacon (ABgene Limited, Epsom, UK) in fully 2′OH-ACE protected form.[41] For deprotection, the protected RNA was dissolved in 400 μL of 2′-deprotection buffer (100 mM acetic acid, adjusted to pH 3.8 with TEMED) and heated to 60 °C for 30 min. The deprotected RNA was freeze-dried, redissolved in H_2O, desalted with the aid of NAP5 columns, and stored at −20 °C. The 2′OMe-RNA was assessed by native 10% PAGE and UV and CD spectroscopy and found to be of similar purity compared to the samples synthesized by *in vitro* transcription. All RNA was quantified by UV spectroscopy, extinction coefficients wt: ε = 640 400 cm^{-1} M^{-1}; 2′O-Me-A1067: ε = 640 400 cm^{-1} M^{-1}; A1067U: ε = 635 900 cm^{-1} M^{-1}; A1067G: ε = 636 300 cm^{-1} M^{-1}; A1067C: ε = 634 000 cm^{-1} M^{-1}; A1095U: ε = 638 100 cm^{-1} M^{-1}; A1095G: ε = 637 500 cm^{-1} M^{-1}; A1095C: ε = 634 800 cm^{-1} M^{-1}; U1066C: ε = 637 100 cm^{-1} M^{-1}; G1068U: ε = 640 400 cm^{-1} M^{-1}; A1089G: ε = 639 700 cm^{-1} M^{-1}.

Protein Synthesis and Purification. The *T. thermophilus* (*Tth*) rplK gene coding for the L11 protein was cloned from *T. thermophilus* HB8 genomic DNA and ligated into the pQE-30 *Xa* vector (Qiagen) using a *Stu*I/*Bam*HI strategy.[12d] L11 mutants were produced using the "site-directed mutagenesis by whole plasmid synthesis" methodology[42] and confirmed by sequencing of the isolated plasmids. Primer sequences can be found in the Supporting Information. The wild type and mutant L11 proteins were expressed from the pQE-30 *Xa* vectors as N-terminally His_6-tagged fusion proteins in *E. coli* XL1 blue cells and purified by Ni^{2+}-NTA-affinity chromatography.[12d] Homogeneity was assessed by SDS-PAGE (>95% pure in all cases), and MALDI-TOF mass spectrometry was used to confirm the molecular weight of the mutant proteins. Correct and conforming folding was verified by CD spectroscopy, as described.[12d] Extinction coefficients wt: ε = 8250 cm^{-1} M^{-1}; P55H: ε = 8250 cm^{-1} M^{-1}, all other mutants: ε = 8370 cm^{-1} M^{-1}.

Thiopeptide Ligands. Thiostrepton (**1**) was obtained from Calbiochem. Micrococcin (**2**), promothiocin B (**4**), and promoinducin (**5**) were isolated from natural sources as described.[43−45] Nosiheptide was a gift from H. G. Floss (U Washington) and T. Mahmud (Oregon State U). All thiopeptide ligands were more than 95% pure by ^{1}H NMR, HPLC, MALDI-TOF, and LC-ESI MS. Stock solutions were prepared in trifluoroethanol (TFE) and quantified by UV spectroscopy. Thiostrepton (**1**):[6] ε_{280} = 0.027 cm^{-1} μM^{-1}; micrococcin (**2**):[6] ε_{280} = 0.034 cm^{-1} μM^{-1}; nosiheptide (**3**):[6] ε_{280} = 0.039 cm^{-1} μM^{-1}; promothiocin B (**4**):[45] ε_{313} = 0.010 cm^{-1} μM^{-1}; promoinducin (**5**):[44] ε_{315} = 0.015 cm^{-1} μM^{-1}.

Binding Experiments Using Fluorescence Anisotropy. Fluorescence anisotropy measurements were performed using a Tecan Safire II plate reader in 384-well plates (Optiplate-384 F, PerkinElmer) using an excitation wavelength of 470 nm, emission wavelength of 520 nm, emission bandwidth of 5 nm, and 50 scans per well at 22 °C. Affinity determination experiments were performed and evaluated following the protocol described.[6] The fluoresceine-tagged thiostrepton derivative **6** was used for all measurements. For the analysis of probe binding to wt/mutant RNA complexes, constant concentrations of the L11 wt protein (0.6 μM) and fluorescent probe **6** (5 nM) were titrated with varying concentrations of the respective wt/mutant RNA in measurement buffer (50 mM MOPS, 5 mM $MgCl_2$, 50 mM KCl, 2 mM TCEP, 5% (v/v) TFE, pH 7.5) in 50 μL final analysis volume. For the analysis of direct probe binding to wt/mutant protein complexes, constant concentrations of the protein L11 wt/mutant protein (0.6 μM) and fluorescent probe (5 nM) were titrated with varying concentrations of wt RNA. All measurements were conducted after equilibration at 4 °C for 15 h. All data were analyzed using Origin 7.5 (OriginLab Corporation). Sigmoidal-shaped curves were fitted using the equations derived earlier,[6] yielding the apparent K_D values for the binding of the thiostrepton probe to the respective protein/RNA complex.

Displacement Titrations. For displacement titrations 5 nM fluorescent probe **6**, 5 nM RNA, and 0.6 μM L11 protein were titrated with varying concentrations of the analyte compounds. In the wells of a 384-well plate, to measurement buffer (see above) were added aliquots of protein, fluorescent probe, displacement analyte, and RNA, to give a final volume of 50 μL, and equilibrated for 15 h at 4 °C. Measurements were taken as described above. After plotting against the thiopeptide concentration, the data were fitted to Hills equation (Origin 7.5, OriginLab Corp.) for analysis to yield apparent IC_{50} values, which were converted into apparent K_D data using eq 1.

$$K_{app} = \frac{IC_{50}}{TS^*} \times k_2 \quad (1)$$

where K_{app} is the apparent K_D, TS* is the concentration of the probe (5 nM), and k_2 is the K_D of the probe (0.33 nM for **6**).

Heterologous Expression of *Tth*L11 in *B. subtilis*. *T. thermophilus* rplK wt and mutant genes were amplified by PCR using the

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pQE30 *Xa* constructs as template and ligated (*Bam*HI, *Xba*I) into the *E. coli/B. subtilis* shuttle vector pHT08 (MoBiTec, Göttingen, Germany)[28] to allow a chloramphenicol-dependent selection of positive *B. subtilis* transformants as well as an IPTG-triggered expression of L11 as His_8-tagged fusion protein in *B. subtilis*. pHT08-*Tth*L11 forward primer sequence: TAGGATCCATGAAGAAAGTCGTTGCGGTGGTC; reverse primer sequence: TATCTAGATTAGGCATCCTTCACCTCCGGAG. The plasmids were transformed into *E. coli* XL1 blue cells, which were grown on selection medium (amp^r) for amplification, and the plasmids were isolated and sequenced to confirm identity.

For heterologus expression, *B. subtilis* wt (strain 1012) and *B. subtilis* $rplK^-$ were used.[26] Accounting for its slow growth, *B. subtilis* $rplK^-$ was routinely preselected by seeded growth on agar plates (37 °C overnight) containing 0.5 μM thiostrepton as selection marker to ensure strain homogeneity and the thiopeptide-resistance phenotype. Single thiostrepton-resistant colonies were picked from these selection plates and used to prepare transformation-competent cells of *B. subtilis* $rplK^-$, following a modified Spizizen's protocol.[26,46] Competent cells were transiently transformed with a set of the pHT08-*Tth*L11 wt/mutant constructs (1−2 μg of DNA, cam^r) following Spizizen's protocol.[46] For the selection of positive transformants, the cells were plated on chloramphenicol-containing selection plates and grown overnight at 37 °C. Transformants growing on these plates were used to inoculate individual cultures (4 mL of LB medium) for the thiopeptide-resistance scan.

B. subtilis wt (strain 1012) and *B. subtilis* $rplK^-$ expressing the His_8-tagged *T. thermophilus* L11 wt or mutant proteins were grown overnight at 37 °C in an orbital shaker (180 rpm) in 4 mL of LB medium supplemented with 1 mM IPTG (and chloramphenicol in the case of the transformants) to induce protein expression. On the next day, the cultures were diluted to an OD_{600} of 0.1 and used to inoculate triplicates of 2 × 4 mL of fresh LB medium (1:10) + 1 mM IPTG. The cells were grown for 2 h at 37 °C (180 rpm) and then supplemented with 0.5 μM thiostrepton (5 μL of a 100 μM solution in TFE) or 5 μL of TFE as control. The cells were further grown overnight at 37 °C in an orbital shaker (180 rpm), and the OD_{600} of each culture was determined after 24 h. The obtained OD_{600} data were normalized to the growth of the TFE controls per strain/mutant (= 100%).

Design and Synthesis of Ribosome Chimeras. *B. subtilis* ribosome chimeras were obtained from *B. subtilis* $rplK^-$ expressing the His_8-tagged *T. thermophilus* L11 wt as described above. For the production of *E. coli* ribosome chimeras the *E. coli* K12 W3110 rplK knockout strain (Keio collection, kan^r)[23] was transiently transformed with the previously constructed pQE30 *Xa*-derived *Tth*L11 mutant plasmids (amp^r). Positive clones were identified on kanamycin−ampicillin selection medium. For the ribosome preparation, transformants were grown in 100−300 mL of LB cultures (+ampicillin, +kanamycin). Instead of forcing strong overexpression of the His-tagged L11 fusion protein by the addition of IPTG, basal expression resulting from the leaky pQE *Xa* T5 promotor system was fully sufficient and simplified the purification process.

Purification of Bacterial Ribosomes. 70S ribosomes were purified following the affinity-based method of Maguire et al.[24a] For the preparation of the affinity matrix, 5 mL of SulfoLink matrix slurry (Pierce Protein Research Products, Rockford, IL) was equilibrated with 3 × 1 vol. of coupling buffer (50 mM Tris, 5 mM Na_2-EDTA, pH 8.5) and reacted with 1 vol. of 50 mM cysteine hydrochloride (in coupling buffer) for 1 h at room temperature. The matrix was loaded into an ÄKTA column (GE Healthcare) and washed with 10 volumes of H_2O. The column was either fitted to an automated ÄKTA FPLC system (GE Healthcare) or operated semimanually using a peristaltic pump (P1, Pharmacia).

For the purification of ribosomes, bacterial cells were harvested and lysed by ultrasonication as suspensions (0.5 g wet cells/mL) in ice-cold lysis buffer (20 mM Tris, 10.5 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 0.5 mM Na_2-EDTA, pH 7.5). Cell debris was removed by centrifugation, and the lysate was cleared by filtration through a 0.45 μm filter. The affinity column was equilibrated with 10 volumes of lysis buffer directly before use. The lysate was loaded with a rate of ~1 mL/min, and the column was eluted with 10 volumes of lysis buffer. Pure ribosomes were eluted with elution buffer (20 mM Tris, 10.5 mM $Mg(OAc)_2$, 300 mM NH_4Cl, 0.5 mM Na_2-EDTA, pH 7.5) in a gradient fashion. Ribosome concentration of appropriate fractions was determined by measuring the absorption at 260 nm and assuming an extinction coefficient of $\varepsilon_{260} = 4.2 \times 10^7\ M^{-1}\ cm^{-1}$.[47] The purity of ribosomes was checked by PAGE gels (for ribosomal proteins) and agarose gel electrophoresis (for rRNA) in comparison to a sample prepared by an ultracentrifugation protocol (courtesy of Dr. F. Seebeck, MPI Dortmund).

Immunoblotting for His_6-Tagged Ribosomal Protein Incorporation. Purified chimeric *E. coli* ribosomes were thermally denatured for 10 min at 95 °C in SDS-PAGE loading buffer and separated on an analytical 15% SDS-PAGE gel. The protein bands were electrophoretically transferred onto a PVDF membrane. After the transfer the blot membranes were blocked (3.8% (w/v) milk powder in TBS-T) for 1 h. For the detection of the His-tagged *Tth*L11 protein a directly HRP (horse radish peroxidase)-coupled α-His_5-antibody (Qiagen, Hilden, Germany) was used (1:1000 in 3.8% (w/v) milk powder in TBS-T; overnight, rt). The membranes were washed twice with TBS-T (10 min at rt), and the α-His_5−HRP conjugate was visualized using the SuperSignal West Pico luminol reaction kit (Pierce Protein Research Products, Rockford, IL).

Affinity Determination on Isolated Ribosomes. Apparent dissociation constants of probe **5** and purified ribosomes were determined by titrating 5 nM of the fluorescent probe with increasing concentrations of ribosome chimeras, incubating for 24 h to equilibrate, and monitoring for fluorescence anisotropy as described above. The influence of detergent was tested by adding 0.001 to 1% w/v of NP-40 to the titration buffer. Plotting of the resulting data against the ribosome concentration gave sigmoid curves, which were fitted using Hill's equation.

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Supporting Information Available: Oligonucleotide sequences, additional supporting figures, preparation of the labeled probe, and Mg^{2+}-dependency of the interaction. This information is available free of charge via the Internet at http://pubs.acs.org.

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