Characterization of two homologous 2′-O-methyltransferases showing different specificities for their tRNA substrates

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ABSTRACT

The 2′-O-methylation of the nucleoside at position 32 of tRNA is found in organisms belonging to the three domains of life. Unrelated enzymes catalyzing this modification in Bacteria (TrmJ) and Eukarya (Trm7) have already been identified, but until now, no information is available for the archaeal enzyme. In this work we have identified the methyltransferase of the archaeon Sulfolobus acidocaldarius responsible for the 2′-O-methylation at position 32. This enzyme is a homolog of the bacterial TrmJ. Remarkably, both enzymes have different specificities for the nature of the nucleoside at position 32. While the four canonical nucleosides are substrates of the Escherichia coli enzyme, the archaeal TrmJ can only methylate the ribose of a cytidine. Moreover, the two enzymes recognize their tRNA substrates in a different way. We have solved the crystal structure of the catalytic domain of both enzymes to gain better understanding of these differences at a molecular level.

Keywords: tRNA; modified nucleosides; methyltransferase; SPOUT

INTRODUCTION

The addition of methyl groups on the base or the ribose moiety of RNA is a common modification found in all three domains of life (Eukarya, Bacteria, and Archaea). These methylation reactions are catalyzed by methyltransferases (MTases), which can be divided in four distinct families based on their three-dimensional fold (Czerwoniec et al. 2009). Ribose methylations are catalyzed either by protein-only MTases or by ribonucleoproteic complexes in which a small C/D box RNA guides the MTase to the nucleotide to be modified. In Bacteria, exclusively the protein-only mechanism is present, while the two mechanisms are found in Eukarya and Archaea. In Eukarya, mainly ribosomal RNA (rRNA) and small nuclear RNA (snRNA) are modified by guided MTases (Clouet-d’Orval et al. 2005; Reichow et al. 2007). In Archaea, several 2′-O-MTases of rRNA and, to a limited extent, transfer RNA (tRNA) utilize the guided system (Ziesche et al. 2004; Renalier et al. 2005).

Since the methylation of the 2′-hydroxyl group favors the C3′ endo conformation of the ribose ring that enhances the local stacking and the RNA rigidity (Kawai et al. 1992), 2′-O-methylated nucleosides are frequently found in tRNA.

Those at positions 18, 32, and 34 are common to all domains of life (Juhling et al. 2009). In Bacteria, the formation of 2′-O-methylguanosine at position 18 (Gm18) is catalyzed by TrmH (Persson et al. 1997; Hori et al. 2002). In Eukarya, the homologous enzyme (called Trm3) is frequently fused to other domains whose functions are largely unknown (Cavaille et al. 1999). In contrast, it has been reported that Gm18 formation in the archaeon Sulfolobus solfataricus involves a guide RNA (Ziesche et al. 2004). Paralogs of TrmH are found in various bacteria (Anantharaman et al. 2002). These include TrmJ and TrmL, which catalyze the methylation of the ribose moiety of nucleosides on, respectively, positions 32 and 34 (Purta et al. 2006; Benitez-Paez et al. 2010). Surprisingly, in Eukarya, methylation of the ribose moiety of nucleosides 32 and 34 is carried out by the completely unrelated Trm7 enzyme, assisted by either the auxiliary protein Trm732 or Trm734 (Pintard et al. 2002; Guy et al. 2012).

Structurally, TrmH (formerly called Spou), TrmJ, and TrmL belong to the SPOUT class of MTases, so-called after its two founding members: SpoU and TrmD (Koonin and Rudd 1993; Anantharaman et al. 2002). Several crystal
structures of SPOUT MTases have been determined (Ahn et al. 2003; Elkins et al. 2003; Lim et al. 2003; Nureki et al. 2004; Liu et al. 2013; Shao et al. 2013). The SPOUT class MTases are characterized by an N-terminal Rossmanoidal α/β fold fused to a C-terminal topological knot. This knot forms the binding pocket for the substrate SAM, which functions as the methyl donor in the reaction. With the exception of the monomeric Trm10 (Shao et al. 2013), all known SPOUT proteins are dimers in which the active site is created by residues from both chains. The orientation of the separate protomers in the dimer allows for a further subdivision of the SPOUT MTases. In the TrmD family (containing among others TrmD and RlmH), the helices forming the dimerization site are oriented antiparallel to each other, while in the SpoU family (TrmH, TrmL, Nep1), these helices are perpendicular. Besides a few exceptions like TrmL and RlmH, most SPOUT proteins have N- and/or C-terminal extensions. Some of these consist out of a single helix on both termini of the protein (like in TrmH), while others have complete domains fused to the catalytic SPOUT fold (Tkaczuk et al. 2007). These extensions are often nucleic acid binding domains and, accordingly, are assumed to be involved in substrate (RNA) binding (Czerwoniec et al. 2009).

Extensive biochemical and structural information is available for TrmH of Thermus thermophilus (Hori et al. 2002; Nureki et al. 2004; Watanabe et al. 2005, 2006; Ochi et al. 2010, 2013). Recently, the crystal structure of TrmL of Escherichia coli was also published (Liu et al. 2013). In contrast, detailed biochemical and structural information on Trm1 is lacking. In this work, we report the characterization of the TrmJ proteins from E. coli (EcTrmJ) and the crenarchaeon Sulfolobus acidocaldarius (SaTrmJ) on a biochemical and structural level. We show that these TrmJ homologs recognize different elements in the tRNA and that EcTrmJ has expanded specificity toward the nucleoside at position 32 of the substrate tRNA compared with SaTrmJ. The crystal structures of the SPOUT domains of the two homologs were determined and used to investigate the structural basis underlying these differences in specificity.

RESULTS AND DISCUSSION

Identification of an archaean 2′-O-methyltransferase acting at position 32 of tRNA

2′-O-methylation of the nucleoside at position 32, the first position of the anticodon loop, occurs in tRNAs from organisms belonging to all domains of life. While the bacterial and eukaryal enzymes catalyzing this modification (respectively, TrmJ and Trm7) have been identified several years ago (Pintard et al. 2002; Purta et al. 2006), their archaean counterpart remained elusive. Therefore, Blastp analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed on the S. acidocaldarius proteome using TrmJ of E. coli (EcTrmJ) and Trm7 of Saccharomyces cerevisiae as the query. Only one homolog of EcTrmJ (named Saci_0621) was found (E value = 5 × 10^{-22}, identity = 34%, and coverage of 73%), which was annotated as a SpoU tRNA MTase. In order to experimentally determine the function of this protein, we recombinantly expressed and purified the protein in E. coli. Unfortunately the purified protein easily aggregated, likely due to the formation of inappropriate disulfide bonds. To increase the solubility, we substituted the sole cysteine (Cys38) into alanine. Subsequent activity assays showed that this mutant was equally active as the wild-type protein, and it was therefore used in all further studies (see Supplemental Data; Supplemental Fig. S1). As shown in Figure 1A, the Saci_0621 encoded protein catalyzes the SAM-dependent formation of 2′-O-methylcytidine (Cm) in [α^{32}P]CTP-labeled tRNA\textsubscript{Met} of S. acidocaldarius (see Materials and Methods). This modification could have occurred on two positions in the tRNA: nucleotides 32 and 56 (Kuchino et al. 1982). However, since the enzyme catalyzing the formation of Cm56 is known (Saci_0653 encoded protein) (Renalier et al. 2005) and the replacement of C32 by U in [α^{32}P]CTP-labeled C32U tRNA\textsubscript{Met} abolishes the Cm formation by Saci_0621 (Fig. 1B), we conclude that the Saci_0621 encoded protein is responsible for 2′-O-methylation at position 32 of S. acidocaldarius tRNA. Therefore, in analogy with its bacterial homolog EcTrmJ, we propose to rename Saci_0621 to SaTrmJ.

FIGURE 1. Purified Saci_0621 catalyzes the formation of Cm at position 32 of tRNA in vitro. Autoradiograms of two-dimensional chromatograms of P1 hydrolysates of [α^{32}P]CTP-labeled transcripts of wild-type tRNA\textsubscript{Met} of S. acidocaldarius (A) and the C32U mutant (B) incubated 30 min at 60°C in presence or in absence of purified Saci_0621. Circles in dotted lines show the migration of the pA, pG, and pU nucleotides used as UV markers. Solvent B was used for the second dimension of the chromatography. The arrows indicate the direction of migration, while the numbers indicate the order of migrations. (C) Secondary structure of tRNA\textsubscript{Met} of S. acidocaldarius. The C32U mutation is indicated.
SaTrmJ shows a narrower specificity for the type of nucleoside at position 32 than EcTrmJ.

EcTrmJ can catalyse the 2′-O-methylation of both cytidine and uridine residues at position 32 (Purta et al. 2006). The lack of specificity of this enzyme for its target nucleoside prompted us to question whether the EcTrmJ protein is at all sensitive for the identity of the nucleoside at position 32. We therefore generated E. coli tRNASer mutants containing either A, G, or U at position 32 and tested the ability of EcTrmJ to methylate these targets. Remarkably, EcTrmJ efficiently methylated all these tRNA mutants (Fig. 2A,B), indicating that EcTrmJ shows no selectivity toward the identity of its target nucleoside. Although a cytidine or a uridine is generally found at position 32, two E. coli tRNAs (tRNAPro(GGG) and tRNAAla(GGC)) have an adenosine at this position (Juhling et al. 2009). To find out if the 2′-O-methylation of purine nucleosides is of any significance in vivo, these two tRNAs were tested as substrates of EcTrmJ. As shown in Figure 2C, the adenosine at position 32 of tRNAPro(GGC) is not methylated. This is in accordance with tRNA sequencing data (Juhling et al. 2009). In contrast, the tRNAPro(GGG), for which no sequencing data exist, is a substrate of EcTrmJ. This result suggests that ribose methylation of pyrimidine nucleosides, as well as adenosine, could be catalyzed by EcTrmJ in vivo.

A similar experiment was performed to determine the specificity of SaTrmJ for the type of nucleoside at position 32. Here, the reaction temperature was increased to 60°C to allow for maximum activity of the enzyme in vitro (see Supplemental Data; Supplemental Fig. S2). Surprisingly, SaTrmJ only methylates the tRNA with a cytidine residue at position 32 (even when higher concentrations and/or a

![Figure 2](https://example.com/figure2.png)
longer incubation time are used) (Fig. 2A). This result was confirmed using a homologous system, in which two S. acidocaldarius tRNAs were tested as substrates of $s_a$TrmJ: [α-32P]UTP-labeled tRNA$^{Pro}_E$ (which has a uridine at position 32) and the mutant tRNA$^{Met}_M$ C32U. Neither of these two tRNAs was methylated (see Supplemental Data; Supplemental Fig. S3), whereas the wild-type tRNA$^{Met}_E$ (which has C32) was efficiently modified by $s_a$TrmJ (Fig. 1B). Taken together, these results clearly demonstrate that the enzyme of S. acidocaldarius has a narrower specificity for the identity of the nucleoside at position 32 (i.e., only methylating C32) than its homolog of E. coli (methylating all nucleosides). This specificity of $s_a$TrmJ toward C is in agreement with the dominance of C at position 32 of S. acidocaldarius tRNAs (only two of these tRNAs possess a U at position 32) (Juhling et al. 2009).

Elements of the tRNA molecule important for $E_c$TrmJ and $s_a$TrmJ activity

In vivo, not all E. coli tRNAs are 2′-O-methylated at position 32 (Juhling et al. 2009). Hence, $E_c$TrmJ must present a specificity toward certain tRNAs. A difference in specificity exists between $E_c$TrmJ and $s_a$TrmJ since unfractionated tRNA extracted from wild-type E. coli cells can be methylated in vitro by $s_a$TrmJ but not by $E_c$TrmJ, as shown in Figure 3. This indicates that tRNAs not methylated by $E_c$TrmJ in vivo are substrates of $s_a$TrmJ (and not $E_c$TrmJ) in vitro.

In general, tRNA MTases can be divided in two groups based on their sensitivity to structural perturbations in the tRNA molecule (Grosjean et al. 1996). The first group can efficiently modify nucleosides within a local (truncated) tRNA fragment, while the second group requires correctly folded, full-length tRNA molecules as substrates. To determine to which group the two TrmJ homologs belong, [α-32P]UTP-labeled wild type and a series of truncated tRNA$^{Ser}_E$ from E. coli (Fig. 4) were tested as substrates. Here, $E_c$TrmJ was only able to methylate full-length tRNA$^{Ser}_E$, whereas $s_a$TrmJ could methylate tRNA fragments lacking the D- and T-stem/loop (Fig. 4). A minimal anticodon stem/loop fragment (Fig. 4) was not methylated by $s_a$TrmJ. However this minimal tRNA construct (having a stem of 8 bp) might not be correctly folded at the reaction temperature used (60°C). We therefore included two tRNA constructs: one having a stem of 10 bp and another with a stem of 12 bp. This latter is formed by the anticodon stem/loop directly fused to the acceptor stem. Only the construction having a stem of 12 bp was efficiently methylated by $s_a$TrmJ (Fig. 4), demonstrating that the only requirement for $s_a$TrmJ substrates is a correctly folded anticodon stem/loop fused to the acceptor stem. In summary, the here-studied TrmJ homologs belong to different groups of MTases: The bacterial $E_c$TrmJ requires full-length tRNAs as a substrate (Group 2), whereas the archaeal $s_a$TrmJ needs only the anticodon stem/loop together with the acceptor stem (Group 1). In this context, it is worth noting that the $E_c$TrmD enzyme, which is also a SPOUT tRNA MTase acting on the anticodon loop (G37), is not restricted to full-length tRNA, similarly to $s_a$TrmJ (Christian and Hou 2007).

The difference in specificity reported above suggests that the enzymes recognize different elements on the tRNA molecule. To identify some of these elements, hybrids between substrate tRNA$^{Ser}_E$ of E. coli and nonsubstrate tRNA$^{Met}_M$ of E. coli (both containing a cytidine at position 32) were constructed, and [α-32P]CTP-labeled transcripts of these hybrid tRNAs were incubated with either of the TrmJ homologs. Initially, each separate stem/loop of substrate tRNA$^{Ser}_E$ was replaced by the equivalent of the nonsubstrate tRNA$^{Met}_M$ (Fig. 5A). Interestingly, $E_c$TrmJ efficiently methylated all of these hybrid tRNAs except for the hybrid in which the D-stem/loop was replaced (Fig. 5A). In addition, $E_c$TrmJ gained the ability to methylate nonsubstrate tRNA$^{Met}_M$ when its D-stem/loop was replaced by the one from tRNA$^{Ser}_E$ (Fig. 5B). Hence, $E_c$TrmJ relies on identity elements within the D-stem/loop for recognition of its tRNA substrate. To further pinpoint the exact identity elements of $E_c$TrmJ within the D-stem/loop, additional hybrid tRNAs were made with either the D-loop or -stem of tRNA$^{Ser}_E$ exchanged by the corresponding parts of tRNA$^{Met}_M$. None of these hybrid tRNAs were modified by $E_c$TrmJ (Fig. 5C), indicating that both the D-stem and -loop are important for substrate recognition in $E_c$TrmJ. In contrast, the activity of $s_a$TrmJ solely depends on the identity of the anticodon stem/loop: tRNA$^{Ser}_M$ in which the anticodon stem/loop was replaced by the one of tRNA$^{Met}_M$ was no longer methylated by $s_a$TrmJ, while tRNA$^{Met}_M$ could be made a substrate of $s_a$TrmJ by exchanging its anticodon stem/loop with the one from tRNA$^{Ser}_E$ (Fig. 5A,B). In analogy to $E_c$TrmJ, we next tested hybrids in which the respective anticodon loop or stem of tRNA$^{Ser}_E$ was replaced by the corresponding loop or stem from tRNA$^{Met}_M$. Here, only the hybrids with the anticodon stem of tRNA$^{Ser}_E$ remained substrate for methylation by $s_a$TrmJ (Fig. 5D). Thus, substrate recognition by $s_a$TrmJ requires the presence of correct identity elements within the anticodon stem. In conclusion, we demonstrated that the two TrmJ homologs recognize tRNA differently: $E_c$TrmJ recognizes identity elements within the
**FIGURE 4.** $\text{SaTrmJ}$ can modify truncated tRNAs, whereas $\text{EcTrmJ}$ modifies only full-length tRNA. Secondary structures of wild-type and truncated tRNA$^{\text{Ser}}$ of *E. coli* are shown above the autoradiograms of two-dimensional chromatograms of T2 hydrolysates of [α$^{32}$P]UTP-labeled transcripts incubated in presence of purified $\text{SaTrmJ}$ or $\text{EcTrmJ}$. Solvent B was used for the second dimension of the chromatography. The arrows indicate the direction of migration, while the numbers indicate the order of migrations. For the meaning of abbreviations, see Supplemental Table S2 in the Supplemental Data.
FIGURE 5. Elements in the D-stem/loop are important for the tRNA recognition by \( \varepsilon \) EcTrmJ, whereas elements in the anticodon stem are important for the recognition by \( \varepsilon \) SaTrmJ. Secondary structures of the substrate \( E. coli \) tRNA\(^{Ser} \) (in black), the nonsubstrate tRNA\(^{Met} \) (in red), and hybrid tRNAs (in black and red) are shown at the left or above the autoradiograms of two-dimensional chromatograms of P1 hydrolysates of [\( ^{32} \)P]CTP-labeled tRNA transcripts incubated in presence of purified \( \varepsilon \) EcTrmJ or \( \varepsilon \) SaTrmJ. (A) tRNA\(^{Ser} \) and tRNA\(^{Met} \) and hybrids of tRNA\(^{Ser} \) with each separate stem/loop of tRNA\(^{Met} \). (B) tRNA\(^{Met} \) with D- or anticodon stem/loop of tRNA\(^{Ser} \). (C) tRNA\(^{Ser} \) with D-stem or -loop of tRNA\(^{Met} \). (D) tRNA\(^{Ser} \) with anticodon stem or loop of tRNA\(^{Met} \). Circles in dotted lines show the migration of the pA, pG, and pU nucleotides used as UV markers. Solvent B was used for the second dimension of the chromatography. The arrows indicate the direction of migration, while the numbers indicate the order of migrations. For the meaning of abbreviations, see Supplemental Table S2 in the Supplemental Data.
D-stem/loop, whereas the identity elements for _SaTrmJ_ are localized in the anticodon stem.

The recognition elements mentioned above reside on a global level of the tRNA molecule. We next questioned whether the local environment around position 32 has an influence on the MTase activity. A typical tRNA anticodon loop consists of 7 nucleotides (nt) in which the first (at position 32) and the last (at position 38) form a bifurcated hydrogen bond (Auffinger and Westhof 1999). This noncanonical interaction together with the U-turn motif (Quigley and Rich 1976), which involves stacking interactions between U33 and nucleotide 35, characterizes the structure of the anticodon loop. To evaluate the importance of these interactions on the activity of both _TrmJ_ proteins, U33C, A38C, and A38U mutants were made in _E. coli_ tRNAser. In addition, the effect of inserting or deleting 1 nt in the anticodon loop was also tested. Here, the activity of _EcTrmJ_ was not affected by single mutations (U33C, A38C, or A38U) within the anticodon loop or by the enlargement or the reduction of the loop (see Supplemental Data; Supplemental Fig. S4). Similarly, the activity of _SaTrmJ_ was not influenced by the loop length, but the substitution of U33 to C33 completely abolished the activity of _SaTrmJ_ (see Supplemental Data; Supplemental Fig. S4). Hence, _SaTrmJ_, but not _EcTrmJ_, relies on the identity of the nucleoside at position 33 of the tRNA for activity. The observation that neither _EcTrmJ_ nor _SaTrmJ_ is affected by insertion or deletion of 1 nt in the anticodon loop suggests that the _TrmJ_ enzymes bind their target by unfolding the preformed tRNA structure rather than by binding a rigid tRNA structure (Byrne et al. 2009 and references therein).

### The N-terminal domains of _EcTrmJ_ and _SaTrmJ_ adopt a SPOUT fold

To determine the molecular mechanism behind the difference in substrate specificity between the two _TrmJ_ orthologs, we set out to solve the crystal structure of both enzymes. Crystallization trials on _EcTrmJ_ yielded single plate-like crystals following a period of 2–3 mo. These crystals diffracted X-rays to a high resolution (Table 1). However, analysis revealed the presence of only the N-terminal domain of _EcTrmJ_ (residues 1–164) in the crystal. This conserved domain with SPOUT fold is shown by sequence analysis to be linked to a more variable C-terminal domain in both _EcTrmJ_ and _SaTrmJ_ (Fig. 6A). The crystal structure of this N-terminal domain of _EcTrmJ_ (EcTrmJ1-164) was solved using molecular replacement with the structure of a hypothetical MTase of _Haemophilus influenzae_ (PDB: 3ILK) as search model. In light of these results and with the crystallization attempts of _SaTrmJ_ yielding no usable protein crystals, we generated, based on

| TABLE 1. Data collection, refinement and validation statistics of the TrmJ crystal structures |
|-----------------------------------------------|----------------------|----------------------|----------------------|----------------------|
|                                  | _EcTrmJ_1-164        | _SaTrmJ_1-157        |
|                                  | APO                  | SAH bound            | APO                  | SAH bound            |
| **Data collection and processing**   |                      |                      |                       |                      |
| X-ray source                      | ESRF ID14-1          | SOLEIL PROXIMA 1     | DIAMOND I04          | DIAMOND I24          |
| Wavelength (Å)                    | 0.9334               | 0.9801               | 0.9795               | 0.9763               |
| Resolution range (Å)*             | 40–1.5 (1.6–1.5)     | 20–1.9 (2.0–1.9)     | 43–1.4 (1.5–1.4)     | 43–1.1 (1.2–1.1)     |
| Total/unique reflections          | 355,438/49,018        | 103,691/24,619       | 910,877/63,289       | 530,844/128,921      |
| _R__meas (%)                      | 5.8 (87.8)           | 5.0 (43.5)           | 14.6 (243.4)         | 5.7 (94.3)           |
| I/σ                             | 23.2 (2.0)           | 17.2 (3.4)           | 11.1 (1.2)           | 12.5 (1.8)           |
| _CC_1/2                          | 99.9 (67.7)          | 99.9 (91.9)          | 99.7 (49.7)          | 99.7 (52.5)          |
| Completeness (%)                 | 97.5 (80.3)          | 96.5 (91.9)          | 99.9 (99.3)          | 97.5 (93.3)          |
| Redundancy (%)                   | 7.2 (5.4)            | 4.2 (3.7)            | 14.4 (14.0)          | 4.1 (3.5)            |
| Spacegroup                       | P 2_1                | P 2_1                | P 2_1, 2_1           | P 2_1, 2_1           |
| Cell dimensions (Å, °)            | 42.4, 73, 53.3       | 42.4, 72.8, 55.5     | 45.1, 53.7, 130.3    | 45.8, 54.1, 130.6    |
| _α, β, γ_ (%)                    | 90, 105, 90          | 90, 108, 90          | 90, 90, 90           | 90, 90, 90           |
| **Model refinement**              |                      |                      |                       |                      |
| _R_ work/R_free (%)              | 19.85/22.51          | 18.0/22.7            | 16.6/20.06           | 16.85/19.16          |
| RMSD bond length (Å)              | 0.015                | 0.017                | 0.017                | 0.013                |
| RMSD bond angle (°)               | 1.71                 | 1.52                 | 1.56                 | 1.56                 |
| Ramachandran favored/allowed/disallowed regions (%) | 97.8/2.2/0          | 97.5/2.5/0          | 99.3/0.7/0          | 98.0/2.0/0          |
| PDB code                         | 4CND                 | 4CNE                 | 4CNF                 | 4CNF                 |

*Values for the highest-resolution shell are given in between brackets. CC1/2 values were used as a guide for selecting the highest usable resolution shell (Karplus and Diederichs 2012). For comparison: I/σ values of 2 were reached at 1.6 Å and 1.2 Å for, respectively, the _SaTrmJ_1-157 and _SaTrmJ_1-157_SAH data sets.

bAn identical subset of 5% of the reflections was used for calculating _R_ work for the _EcTrmJ_1-164 and _EcTrmJ_1-164_SAH structures and for the _SaTrmJ_1-157 and _SaTrmJ_1-157_SAH structures.

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sequence alignment, an analogous construct of \( \text{SaTrmJ} \) containing only the SPOUT domain (\( \text{SaTrmJ1-157} \)). This construct yielded well-diffracting crystals from which the crystal structure could be determined by molecular replacement using the \( \text{EcTrmJ1-164} \) as a search model. In order to gain more insights in the active site, crystal structures were also solved of \( \text{SaTrmJ1-157} \) cocrystallized with the reaction-product SAH and of \( \text{EcTrmJ1-164} \) soaked with SAH. Statistics on data collection, processing, and refinement for all four data sets (\( \text{EcTrmJ1-164} \), \( \text{EcTrmJ1-164-SA} \), \( \text{SaTrmJ1-157} \), and \( \text{SaTrmJ1-157-SA} \)) are summarized in Table 1.

The \( \text{EcTrmJ1-164} \) and \( \text{SaTrmJ1-157} \) crystals both contain two protein chains in the asymmetric unit, in which each protomer displays a SPOUT fold with a central \( \beta \)-sheet (composed of six parallel \( \beta \)-strands) surrounded by seven \( \alpha \)-helices and a typical deep topological knot at the C terminus (Fig. 6B; FIGURE 6. (A) Sequence alignment of \( \text{EcTrmJ} \) and \( \text{SaTrmJ} \). The secondary structure elements, as deduced from the crystal structures, are shown below the alignment with \( \beta \)-strands shown as blue arrows and \( \alpha \)-helices as green tubes. The three predicted \( \alpha \)-helices of the C-terminal domain are colored in orange. Identical residues in the \( \text{EcTrmJ} \) and \( \text{SaTrmJ} \) sequence are highlighted in gray. (B) Crystal structures of \( \text{SaTrmJ1-164-SA} \) (left) and \( \text{SaTrmJ1-157-SA} \) (right). The biological dimers are shown in cartoon representation with one subunit colored in gray. The reaction product SAH located in the active site (in purple), as well as the catalytic tyrosine-arginine diad, is represented as sticks. In the \( \text{SaTrmJ1-157-SA} \) structure, an additional SAH molecule, shown in blue sticks, is bound to loop \( \beta 4-\alpha 5 \), which induces the formation of helix \( \alpha 5' \). (C) Electrostatic potential mapped on the solvent accessible surface of \( \text{EcTrmJ1-164-SA} \) (left) and \( \text{SaTrmJ1-157-SA} \) (right). A yellow star indicates the position of the bound SAH molecule in the active site (which is not visible in this orientation). The additional SAH molecule bound to the \( \text{SaTrmJ1-157-SA} \) structure is shown in ball-and-stick representation. Positively charged residues of \( \text{EcTrmJ1-164-SA} \) and \( \text{SaTrmJ1-157-SA} \) are indicated. These residues of \( \text{SaTrmJ1-157-SA} \) were mutated in this study.
Characterization of bacterial and archaeal TrmJ

Anantharaman et al. (2002). Similar to all currently described members of the SPOUT superfamily except for Trm10 (Shao et al. 2013), both TrmJ SPOUT domains form a homodimer with a buried dimerization surface of ~1400 Å². The dimerization surface is formed by helices a1 and a7 of one subunit interacting with the same helices from the second subunit in an almost perpendicular orientation, similar to the SpoU subfamily of SPOUT proteins (Anantharaman et al. 2002). The individual subunits of the SPOUT domains of EcTrmJ and SaTrmJ superimpose well on the archetypical SpoU protein TrmH, with a RMSD (root-mean-square deviation) between the Ca atoms of 1.6 Å. A similar RMSD value is found for the superposition of EcTrmJ1-164 and SaTrmJ1-157.

In the EcTrmJ1-164 crystal structure, clear electron density is visible for the complete SPOUT domain spanning residues 1–164 with the exception of residues 44–50 (helix a2) of the B-chain. In the B-chain of the EcTrmJ1-164_SAHe structure, this region obtains a well-defined conformation, while another region (residues 82–86 in loop β4–a5) is flexible. For SaTrmJ1-157, clear electron density is present for residues 1–82 and 85–157 in the A-chain and 1–83 and 89–156 in the B-chain. Moreover, in the SaTrmJ1-157 crystal structure a 5’ methyl-thioadenosine (MTA) molecule is observed in the active site of the A-chain, which was probably copurified with the protein. In the A-chain of saTrmJ1-157_SAHe, the region 84–88 could be traced and adopts a helical conformation. Probably, this region is stabilized by the additionally bound SAH molecule, outside the active site pocket (Fig. 6B).

Global binding of SAH causes few structural rearrangements in both TrmJ homologs. A subtle difference is found within the loop connecting β5 and a6, where a peptide bond undergoes cis-trans isomerization upon ligand binding (see Supplemental Data; Supplemental Fig. S5). In EcTrmJ1-164, the peptide bond between Glu116 and Arg117 undergoes cis-trans isomerization upon SAH binding, while in SaTrmJ1-157, the peptide flip occurs between Val115 and Gly116. This flip is, however, only visible in the B-chain of EcTrmJ1-164_SAHe and results in a minor, local effect by movement of the Arg117 side-chain. In the B-chain of SaTrmJ1-157_SAHe, the peptide flip causes the region Gly116-Thr118 to move away from the active site and loop β4–β5. The relevance of this observation is so far unclear. However loop β5–a6 is one of the three highly conserved regions within the SpoU family and was hence designated as motif II. Cis-trans isomerization of a peptide bond within this motif has already been reported in H. influenzae YibK (TrmL), where it was suggested to increase the binding affinity for the substrate SAM (Lim et al. 2003).

EcTrmJ and SaTrmJ contain a dimeric, predominantly helical C-terminal domain

So far, protein crystals of sufficient diffraction quality of full-length EcTrmJ and SaTrmJ could not be obtained. We therefore set out to investigate their C-terminal domains. First, in order to determine the secondary structure of the C-terminal domains of both TrmJ homologs, we expressed and purified these domains separately. These constructs behaved well during purification, demonstrating that the C-terminal part of both TrmJ homologs forms an autonomously folded domain. Subsequent circular dichroism experiments (see Supplemental Data; Supplemental Fig. S6A) confirmed that the C-terminal domains of both TrmJ proteins were folded and presented a predominantly helical arrangement. This is in agreement with the predicted three α-helices in these domains (Fig. 6A). Additional SAXS measurements on these constructs revealed that they exist as dimers in solution (see Supplemental Data; Supplemental Fig. S6B). Hence, both EcTrmJ and SaTrmJ are composed out of two autonomously folded, dimerizing domains in which the N-terminal domain adopts a SPOUT fold, while the C-terminal domain is predominantly helical in conformation.

SAH adopts a different conformation in EcTrmJ and SaTrmJ

The SAM/SAH binding pocket is largely conserved between the two TrmJ enzymes and is formed by residues from the beginning of loop β4–a5, from loop β5–a6 (motif II), and from loop β6–a7 (motif III). In both TrmJ_SAHe structures, the adenosine moiety of the ligand binds in a hydrophobic cavity near the topological knot of the SPOUT domain and forms H-bonds with the backbone of the conserved residues Thr79, Gly114, Ile134, Ser141, and Leu143 in EcTrmJ1-164_SAHe and the corresponding residues Thr77, Gly111, Ile131, Pro138, and Leu140 in SaTrmJ1-157_SAHe. Remarkably, the conformation of the homocysteine moiety of SAH with respect to the adenosine moiety is different in the two enzymes (Fig. 7). In both TrmJ homologs, the homocysteine moiety of the SAH molecules adopts a δ4'–C4'–C5'–Sδ dihedral angle of 90°, similar to the conformations seen in other SPOUT-SAM/SAH complexes and sometimes referred to as “bended conformation” (Elkins et al. 2003; Kurowski et al. 2003; Lim et al. 2003; Schubert et al. 2003; Nureki et al. 2004; Liu et al. 2013). However, while in SaTrmJ1-157_SAHe and in the A-chain of EcTrmJ1-164_SAHe, the S6-Cy-Cβ-Cα dihedral angle is 162° (similar to most SPOUT-SAM/SAH complexes); in the B-chain of EcTrmJ1-164_SAHe, the homocysteine moiety of SAH adopts a S6-Cy-Cβ-Cα dihedral angle of ~69°. In this conformation, the homocysteine moiety is nearly completely folded back in a parallel orientation toward the adenosine ring of SAH. In analogy with the bended conformation, one could call this a “super-bended conformation.” This super-bended conformation might be correlated with the observed cis-trans isomerization of the peptide bond between Glu116 and Arg117 upon SAH binding, which also only occurs in the B-chain of EcTrmJ1-164_SAHe.

The super-bended conformation of SAH in the B-chain of EcTrmJ1-164_SAHe could originate from the observed H-bond between the homocysteine moiety of SAH and the
side-chain hydroxyl of Ser142 (Fig. 7). A similar interaction is impossible in SaTrmJ1-157_SAH, where the homolog of Ser142 is a valine residue (Val139). Apart from this substitution, the position of the β5-α6 loop (motif II) might also contribute to the difference in ligand conformation. This loop, which is located on the bottom of the SAM/SAH binding pocket, is located closer to the SAM binding pocket in the EcTrmJ1-164_SAH structure compared with the SaTrmJ1-157_SAH structure (Fig. 7). However, some care should be taken in the interpretation of these results, since the SAH super-bended conformation is stabilized via a hydrogen bond with Ser142 and possibly also by the different conformation of loop β5-α6 (motif II) in EcTrmJ1-164_SAH. An H-bond between Ser114 and Glu11 stabilizes this loop in a more “open” conformation in SaTrmJ1-157_SAH. The position of the catalytic tyrosine-arginine diad is also shown (note that the Arg residue is provided by the A-chain).

FIGURE 7. Differences in SAH conformation between EcTrmJ1-164_SAH and SaTrmJ1-157_SAH. A superposition of active site residues of the B-chain of EcTrmJ1-164_SAH (green) and SaTrmJ1-157_SAH (yellow) is shown. For clarity, the bound reaction product SAH is shown with paler coloring. While SAH adopts a common “bended conformation” in SaTrmJ1-157_SAH, it adopts a “super-bended conformation” in EcTrmJ1-164_SAH. This super-bended conformation of SAH in EcTrmJ1-164_SAH is stabilized via a hydrogen bond with Ser142 and possibly also by the different conformation of loop β5-α6 (motif II) in EcTrmJ1-164_SAH. An H-bond between Ser114 and Glu11 stabilizes this loop in a more “open” conformation in SaTrmJ1-157_SAH. The position of the catalytic tyrosine-arginine diad is also shown (note that the Arg residue is provided by the A-chain).

Surprisingly, the Ser150 residue of the proposed Ser150-Arg41 diad in TrmH is not conserved in SaTrmJ1-157 (the serine residue is replaced by Val139), while in EcTrmJ1-164_SAH, the corresponding Ser142 of the B-chain is not within H-bond distance to Arg23 but rather forms an H-bond with the SAH ligand (Fig. 7). However, in both TrmJ crystal structures, the catalytic arginine is within H-bonding distance (3.3 Å) to the side-chain hydroxyl group of a tyrosine, conserved in both EcTrmJ and SaTrmJ (Tyr140 and Tyr137, respectively) but not present in TrmH. To determine whether this Tyr-Arg diad is involved in SaTrmJ catalysis, we tested the activity of EcTrmJ and SaTrmJ variants in which this arginine was mutated to alanines. In agreement with the results obtained for TrmH, mutating the conserved arginine of both TrmJ homolog resulted in an inactive protein (Fig. 8).

Catalysis by TrmJ involves a Tyr-Arg diad

Several highly conserved residues, which are proposed to be catalytically important in the SpoU family (Watanabe et al. 2005), are maintained in both TrmJ homologs. In T. thermophilus TrmH, an arginine residue in motif I (Arg41) was suggested to function as catalytic base (Nureki et al. 2004). In the TrmJ crystal structures, Arg23 of EcTrmJ1-164 and Arg21 of SaTrmJ1-157 superimpose on this proposed catalytic arginine. For TrmH, it has been proposed that the backbone phosphate of the substrate tRNA together with a nearby serine residue (Ser150) first deprotonate the catalytic Arg41. Subsequently, Arg41 functions as a general base by subtracting a proton from the 2′'-O-ribose such that the latter group can conduct a nucleophilic attack on the methyl group of SAM (Nureki et al. 2004). To confirm that this arginine is indeed crucial in the reaction mechanism of TrmJ, we tested the activity of EcTrmJ and SaTrmJ variants in which these arginines were mutated to alanines. In agreement with the results obtained for TrmH, mutating the conserved arginine of both TrmJ homolog resulted in an inactive protein (Fig. 8).

FIGURE 8. Effect of the substitution R23A, Y140F, and R21A, Y137F on the activity of EcTrmJ and SaTrmJ, respectively. The activity measure (in cpm) corresponds to the amount of 14C transferred to tRNA using [methyl-14C] SAM as methyl donor. (A) Thirty micrograms of wild-type EcTrmJ or of the R23A, Y140F variant was incubated with 80 µg of unfractionated tRNA (from a strain of E. coli in which the gene coding for TrmJ was deleted) for increasing time intervals at 37°C. (B) Ten micrograms of wild-type SaTrmJ or of the R21A or Y137F variant was incubated with 140 µg of unfractionated tRNA from E. coli for increasing time intervals at 60°C.
Regions in TrmJ important for tRNA binding

To gain more insight in the tRNA binding surface of both TrmJ homologs, the electrostatic potential was mapped on their solvent accessible surface. As seen in Figure 6C, the deep cleft between both protomers in the EcTrmJ1-164 homodimer displays a large positively charged surface patch. This patch follows the complete length of the cleft, connecting both active sites of the dimer. In SaTrmJ1-157, a comparable positively charged surface exists, but here it is interrupted by an aromatic cluster containing Tyr14 and Phe46 near helices α2-α3. To confirm the involvement of this positively charged region in tRNA binding, we mutated the basic residues of SaTrmJ in this region to glutamate residues. Since, despite many attempts, no tRNA–enzyme complex could be observed using electrophoretic mobility shift assay (EMSA), the MTase activity assay was used to test the effect of the SaTrmJ variants on unfractionated E. coli tRNA. Given that the selected mutations are outside the SAH/SAM binding pocket, we assume that their effect will be mainly due to defective tRNA binding. In general, all these mutations either completely abolished or severely reduced the MTase activity (Table 2), confirming the involvement of this region in tRNA binding.

The electropositive surface of SaTrmJ is composed of essentially three regions: loop α2-α3, loop α4-α5, and motif II. Mutation of residue Lys49 in loop α2-α3 and of Arg119 at the end of motif II to glutamate completely abolished the MTase activity, indicating that these regions are involved in tRNA binding. A K84E mutation in the loop connecting α4 to α5 has a small effect on the MTase activity, while the R89E mutation in the same loop completely abolished the activity. This finding is remarkable since Arg89 is pointing away from the SAM/SAH binding site. Therefore this loop probably undergoes considerable rearrangements upon tRNA binding.

In general, the accessory domains linked to SPOUT domains are suggested to be involved in RNA binding (Tkaczuk et al. 2007). We therefore investigated the importance of the C-terminal domain of both TrmJ homologs in tRNA binding and catalysis. We first tested the MTase activity of the EcTrmJ and SaTrmJ constructs in which the C-terminal domain was deleted (EcTrmJ1-166 and SaTrmJ1-157). Both these constructs completely lost their activity, confirming that the C-terminal domains of TrmJ are crucial components either for catalysis or for tRNA binding. Next, we generated chimeric proteins in which the C-terminal domains were interchanged, such that the EcTrmJ SPOUT domain was linked to the SaTrmJ C-terminal domain and vice versa. Both chimeric proteins were inactive (result not shown), pointing toward a model in which the C-terminal domain functions in close collaboration with the corresponding SPOUT domain.

Structural basis for the difference in substrate specificity between EcTrmJ and SaTrmJ

Our results show that SaTrmJ and EcTrmJ have a different specificity toward the identity of the nucleoside at position 32 of tRNA. In order to identify the amino acid residues involved in substrate specificity, residues in and around the active site that differ between SaTrmJ and EcTrmJ were mutated in SaTrmJ to their respective EcTrmJ counterparts and tested for expanded substrate specificity (Table 3). In this experiment, the SaTrmJ mutants were tested specifically for their ability to methylate both C32 and U32 (similar to EcTrmJ).

Mutations E11S, Y14S, K49G, S79A, I80R, P138S, and V139S in SaTrmJ had no effect on the activity and specificity of SaTrmJ (Table 3). Four mutants nearly completely lost their activity: F46A, S47L, S114R, and R119N. The reduced activity of S47L might be caused by a reduced stability of the protein since we observed that the expression of the SaTrmJ S47L variant in E. coli was severely impaired (data not shown).

One striking difference between the SaTrmJ1-157_SAH and EcTrmJ1-164_SAH crystal structures concerns the conformation of the bound SAH ligand itself. In the B-chain of EcTrmJ1-164_SAH, SAH adopts a super-bended conformation likely due to an H-bond between the homocysteine moiety of SAH and Ser142 (Fig. 7). Additionally, the β6-o6 loop (motif II) is located closer to the SAM/SAH binding pocket in EcTrmJ1-164_SAH than in SaTrmJ1-157_SAH and might also force the SAH ligand to adopt a super-bended conformation. To test the involvement of these residues and/or the associated cofactor conformation on the specificity, a SaTrmJ V139S/E11A double mutant was made. Mutating Val139 to serine should allow H-bonding with the homocysteine moiety of SAH in a similar fashion to what Ser142 does in EcTrmJ1-164_SAH. Additionally, we destroyed the H-bond between Glu11 and Ser142 (which opens up the β6-o6 loop in SaTrmJ) by mutating Glu11 to alanine. However, also this SaTrmJ double mutant did not show expanded substrate specificity toward U32. Nevertheless, the super-bended conformation of the SAH ligand might still be implicated in the different substrate specificity, since recreating the super-bended SAH conformation in SaTrmJ1-157_SAH might not occur efficient in the SaTrmJ V139S/E11A mutant. We therefore adopted the inverse strategy and created the EcTrmJ

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**TABLE 2. Effect of SaTrmJ variants on the tRNA MTase activity in comparison to wild-type SaTrmJ**

| Mutant of TrmJ | Cm32 formation |
|---------------|----------------|
| SaTrmJ        | +++            |
| SaTrmJ K45E   | +              |
| SaTrmJ K49E   | 0              |
| SaTrmJ K84E   | +              |
| SaTrmJ R89E   | 0              |
| SaTrmJ R119E  | 0              |

The following codes were used: fully active (+++), reduced activity (+), no activity (0).
TABLE 3. Effects of S₅TrmJ variants on the activity and substrate specificity toward uridine and cytidine at position 32 of tRNA

| Mutant in TrmJ | Cm32 formation | Um32 formation |
|----------------|----------------|---------------|
| EcTrmJ         | +++            | +++           |
| S₅TrmJ         | +++            | 0             |
| S₅TrmJ E11S    | +++            | 0             |
| S₅TrmJ Y14S    | +++            | 0             |
| S₅TrmJ F46A    | 0              | 0             |
| S₅TrmJ S47L    | +              | 0             |
| S₅TrmJ K49G    | +++            | 0             |
| S₅TrmJ S79A    | +++            | 0             |
| S₅TrmJ I80R    | +++            | 0             |
| S₅TrmJ S114R   | +              | 0             |
| S₅TrmJ R119N   | +              | 0             |
| S₅TrmJ P138S   | +++            | 0             |
| S₅TrmJ V139S   | +++            | 0             |
| S₅TrmJ E11AV139S | +++          | 0             |

The following codes were used: fully active (+++), reduced activity (+), no activity (0).

S142V mutant, which cannot be to form the H-bond that stabilizes the super-bended conformation. Interestingly, this mutant shows a shift to a narrower specificity: While the cytosine methylation is only slightly impaired, a strong reduced ability to methylate uridine is observed. This can be explained by two hypotheses: Either (1) Ser142 interacts directly with the substrate nucleoside, or (2) there is a link between the difference in substrate specificity and the difference in SAH/SAM conformation observed in the active sites of EcTrmJ1-166 and S₅TrmJ1-157. In the case of the latter, the difference in conformation of the methionine moiety of SAH/SAM might change the space available for the target nucleoside and, consequently, be one of the factors contributing to the specificity.

In summary, despite the high conservation between both TrmJ homologs, we could not identify a single residue responsible for the narrower substrate specificity of S₅TrmJ compared with EcTrmJ. In fact, the majority of the residues near the active site of S₅TrmJ that are not conserved in EcTrmJ are small, hydrophobic amino acids that are unable to specifically interact with the nucleoside at position 32 of the tRNA. Therefore, the origin of the different substrate specificity in the TrmJ homologs likely originates from a combinatorial effect in which the conformation of the SAM substrate might be one of the determining factors.

CONCLUSION

We have shown that despite the high sequence and structural similarity between the bacterial (E. coli) and archaeal (S. acidocaldarius) TrmJ proteins, there is a significant difference in specificity between both, not only for the nature of the nucleoside at position 32 of the substrate tRNA molecule but also for the tRNA substrates themselves. The crystal structures of the two proteins together with the biochemical analyses contributed to the partial uncovering of the molecular determinants of this difference in specificity toward the target nucleoside. Nevertheless, a crystal structure of the complex between TrmJ and its tRNA substrate will be necessary to be able to fully understand the molecular origin of this finding and to determine the interactions required for both TrmJ homologs to select their respective substrate tRNAs.

MATERIALS AND METHODS

General procedures

Ampicillin was used at a concentration of 50 µg/mL, kanamycin at 30 µg/mL, and chloramphenicol at 30 µg/mL. Restriction endonucleases and T4 DNA ligase were purchased from Thermo Scientific, and T7 RNA polymerase was purchased from Promega. [α-32P] NTPs (3000 Ci/mmol) were from PerkinElmer. Oligonucleotides (see Supplemental Data; Supplemental Table S1) were synthesized by Sigma. Nuclease P1 and RNase T2 were from Sigma. The N-terminal His-tagged EcTrmJ expression vector was a gift from J.M. Bujnicki and E. Purta (Purta et al. 2006). Genomic DNA from S. acidocaldarius was isolated according to the genomic DNA isolation method described by Roovers et al. (1997).

Cloning of the Saci_0621 gene

The S. acidocaldarius Saci_0621 gene was amplified by PCR from genomic DNA using the Saci_0621 FOR and Saci_0621 REV primers (see Supplemental Data; Supplemental Table S1) and Pfu DNA polymerase. The obtained fragment was first subcloned in the pET PCR cloning vector (Thermo Scientific). Subsequently, the insert was cloned in the pET28b expression vector (Novagen) using the restriction enzymes NdeI and XhoI, allowing the expression of an N-terminal His-tagged protein in E. coli.

Mutagenesis

All the described variants of the TrmJ proteins and all point mutations of tRNA from E. coli were prepared by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene). The constructs spanning the N-terminal domains of both TrmJ homologs (S₅TrmJ1-157 and EcTrmJ1-166) were generated by mutating the adjacent codon to a stop codon using the QuickChange site-directed mutagenesis kit (Stratagene). The constructs spanning the C-terminal domains of TrmJ (S₅TrmJ163-235 and EcTrmJ167-246) were amplified by PCR (see Supplemental Data; Supplemental Table S1) and cloned in the pET28b vector.

Expression and purification of EcTrmJ and S₅TrmJ

The His-tagged S₅TrmJ was expressed in the E. coli strain Rosetta (DE3), whereas the His-tagged EcTrmJ was expressed in the E. coli strain BL21 (DE3). Transformed cells were grown at 37°C in 2 liters of Luria broth supplemented with adequate antibiotics to an optical density at 660 nm of 0.6. At this stage, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM (for S₅TrmJ) or 1 mM (for EcTrmJ) to induce recombinant protein expression. Cells were harvested after 3 h of incubation at 37°C and
resuspended in 100 mL of buffer A (50 mM Tris-HCl at pH 8, 500 mM NaCl) for E. coli TrmJ or 100 mL of buffer B (50 mM Tris-HCl at pH 8, 1 M NaCl) for S. aureus TrmJ. Cells were lysed by 30 min of sonication at 4°C using a VibraCell 75041 sonicator. The lysates were cleared by centrifugation (12,000 rpm for 20 min) and applied to a column of chelating Sepharose fast flow (1 × 30 cm; GE Healthcare) charged with Ni2+ and equilibrated with either buffer A or B. The column was washed with the same buffers, and the bound proteins were eluted with a linear gradient (from 0 to 500 mM) of imidazole in the respective buffers. The final purification step of S. aureus TrmJ was a size-exclusion chromatography on a HiLoad superdex75 column (GE Healthcare) in buffer C (20 mM Tris at pH 8, 1 M NaCl). The E. coli TrmJ sample was desalted using a Q HiPrep 26/10 desalting column (GE Healthcare) in buffer D (50 mM Tris-HCl at pH 8, 100 mM NaCl). TrmJ mutants and truncates were purified using the same protocol as for the wild-type protein.

Generation of 32P-labeled tRNAs by in vitro transcription

The general procedure for generating in vitro transcripts of tRNA genes is based on the method described in Reyes and Abelson (1987). tRNA genes were amplified with Pho DNA polymerase using the respective oligonucleotide sets (see Supplemental Data; Supplemental Table S1). The obtained fragments were cloned into the SmaI site of pUC18, and their sequences were checked. In the resulting plasmids, the tRNA genes were flanked by a 5′ T7 promoter and a 3′ Mval restriction site. After Mval digestion of the plasmids, tRNA transcripts were generated in the presence of T7 RNA polymerase and NTPs, of which one was [α-32P]-labeled.

tRNA MTase assays

The two types of tRNA MTase assays used in this work were described by Droogmans et al. (2003). The first method consisted in measuring the amount of 14C transferred to total tRNA MTase activities using [methyl-14C]SAM as methyl donor. The reaction mixture (400 µL) consisted of 50 mM Tris (pH 8), 5 mM MgCl2, 60 µg unfractionated merase and NTPs, of which one was [α-32P]-labeled tRNA, 25 nCi [methyl-14C]SAM (50 mCi/mmol; GE Healthcare), and enzyme (5 to 10 µg) or crude extract. The second type of tRNA MTase assay involved in vitro transcribed 32P-labeled tRNAs as substrates. The reaction mixture (400 µL) consisted of 50 mM Tris-HCl (pH 8), 5 mM MgCl2, 10°C c.p.m. of the radioactive transcript and 500 µM SAM and 1 µg of purified enzyme. In both methods, after an incubation of 30 min at 37°C for E. coli TrmJ or 60°C for S. aureus TrmJ, the reaction was stopped by phenol extraction and the tRNAs were ethanol precipitated. The recovered radioactive tRNA was then completely digested by either nuclease P1 (1 unit) or RNase T2 (0.1 unit).

Modified nucleotides were analyzed by two dimensional thin-layer chromatography (2D-TLC) on cellulose plates (Merck). The first dimension was developed with solvent A (isobutyric acid/concentrated NH4OH/water; 66/1/33; v/v/v); and the second dimension was developed with solvent B (0.1 M sodium phosphate at pH 6.8/(NH4)2SO4/n-propanol; 100/60/2; v/v/v) or with solvent C (concentrated HCl/n-propanol/water; 17.6/68/14.4; v/v/v). The radioactive spots were visualized by autoradiography. The nucleotides were identified using reference maps (Grosjean et al. 2007).

Crystallization, data collection, and structure determination

Attempts to crystallize full-length E. coli TrmJ, using sitting-drop vapor diffusion experiments at 20°C, led (after a period of 2–3 mo) to the spontaneous formation of crystals containing only the SPOUT domain (referred to as E. coli TrmJ-164). These crystals were obtained by mixing E. coli TrmJ at a concentration of 15 mg/mL in a 1/1 ratio with 0.02 M Na/K phosphate, 0.1 M MES (pH 6.5), and 20% PEG3350. For data collection, crystals were frozen in liquid nitrogen with crystallization buffer supplemented with 15% PEG200 as cryoprotectant. SAH-soaked crystals were prepared by overnight soaking of these E. coli TrmJ-164 crystals at 20°C in crystallization buffer with 1 mM SAH.

E. coli TrmJ-157 crystals were obtained by mixing protein (at a concentration of 20 mg/mL) with an equal volume of crystallization buffer in a hanging drop vapor diffusion set-up. The crystallization buffer contained 0.1 M sodium acetate (pH 5.5), 20%–25% PEG2000MME, and 0.2 M of either MgCl2, KBr, or sodium acetate. Crystals typically appeared within 1–2 d at 20°C. E. coli TrmJ-157 was co-crystallized with SAH by addition of 1 mM SAH to the crystallization solution. Single crystals were flash frozen in liquid nitrogen with crystallization buffer supplemented with 15% glycerol as cryoprotectant.

All diffraction data were collected at 100 K, and data sets of diffraction crystals were processed with the XDS suite (Kabsch 2010) using either Xscale or Scala (Evans 2006) for scaling and merging of the reflections. Initial data quality was assessed in phenix.xtriage (Zwart et al. 2008). Phase information was obtained by molecular replacement with the Phaser program (McCoy 2007; McCoy et al. 2007) in the CCP4 software package (Winn et al. 2011). The initial E. coli TrmJ-164 data set was phased using the structure of the SPOUT domain of a hypothetical MTase from H. influenzae (PDB: 3ILK) as search model. The obtained E. coli TrmJ-164 crystal structure was subsequently used as a model to solve the S. aureus TrmJ-157 apo structure. The SAH containing crystals of both proteins were phased using their respective apo structures, making sure that the same set of reflections were set aside for cross-validation.

The ARP/wARP program (Langer et al. 2008) was used for automated model building. Model building was finalized by manual building cycles in Coot (Emsley and Cowtan 2004), alternated with refinement in Reinafin (Murshudov et al. 1997). Temperature factors were anisotropically refined in all S. aureus TrmJ-157 structures, while TLS refinement with 11 groups was performed for the E. coli TrmJ-164_SAH structure. The amount of TLS groups used during refinement was determined by the TLSMD server (Painter and Merritt 2006). The temperature factors of the E. coli TrmJ-164 structure were isotropically refined. The obtained models were validated with the Molprobity server (Davis et al. 2007). All structure figures were prepared in PyMOL (http://www.pymol.org/). Data collection and processing statistics are summarized in Table 1.

Structural analysis

Sequence alignments were performed with the T-coffee server (Notredame et al. 2000). Poisson-Boltzmann electrostatics were calculated with the PARSE force field in PDBePQR (Dolinsky et al. 2007) and visualized with APBS (Baker et al. 2001) in PyMOL. Structural alignments were performed by PDBBfOLD (Kriisinel and Henrick 2004), while the area of the dimerization interface of
both Trm1 homologs was calculated using the PISA software (Krissinel and Henrick 2007), both accessible via the PDBe server.

SAXS

All SAXS measurements were performed at 15°C on the SWING beamline (SOLEIL, France). An inline size-exclusion chromatography set-up was used prior to data collection. Here, 50 μL of sTrm|163-235 or sTrm|167-246 at 8 mg/mL was injected on an Agilent Bio SEC-3 column (300 Å pore size), which ran at 0.2 mL/min in 20 mM Tris (pH 8), 150 mM NaCl buffer. Data processing was done using the ATSAS package (Potokhov and Svergun 2013). The multimerization state of these C-terminal constructs was determined through the SAXSMoW application (Fischer et al. 2010).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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