

Functional characterization of the YmcB and YqeV tRNA methylthiotransferases of *Bacillus subtilis*

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ABSTRACT

Methylthiotransferases (MTTases) are a closely related family of proteins that perform both radical-S-adenosylmethionine (SAM) mediated sulfur insertion and SAM-dependent methylation to modify nucleic acid or protein targets with a methyl thioether group (–SCH₃). Members of two of the four known subgroups of MTTases have been characterized, typified by MiaB, which modifies *N*⁶-isopentenyladenosine (i⁶A) to 2-methylthio-*N*⁶-isopentenyladenosine (ms²i⁶A) in tRNA, and RimO, which modifies a specific aspartate residue in ribosomal protein S12. In this work, we have characterized the two MTTases encoded by *Bacillus subtilis* 168 and find that, consistent with bioinformatic predictions, *ymcB* is required for ms²i⁶A formation (MiaB activity), and *yqeV* is required for modification of *N*⁶-threonylcarbamoyl-adenosine (t⁶A) to 2-methylthio-*N*⁶-threonylcarbamoyl-adenosine (ms²t⁶A) in tRNA. The enzyme responsible for the latter activity belongs to a third MTTase subgroup, no member of which has previously been characterized. We performed domain-swapping experiments between YmcB and YqeV to narrow down the protein domain(s) responsible for distinguishing i⁶A from t⁶A and found that the C-terminal TRAM domain, putatively involved with RNA binding, is likely not involved with this discrimination. Finally, we performed a computational analysis to identify candidate residues outside the TRAM domain that may be involved with substrate recognition. These residues represent interesting targets for further analysis.

INTRODUCTION

Transfer RNA molecules from all three domains of life undergo numerous, and often complex, post-transcriptional modifications in the course of maturation. Residue 37, which is 3'-adjacent to the anticodon and almost invariably purine, is a frequent target of modification, the specific type of which appears to vary with the identity of residue 36 (the third residue of the anticodon) (1). Among the modified residues found exclusively at this position are *N*⁶-isopentenyladenosine (i⁶A) and *N*⁶-threonylcarbamoyl-adenosine (t⁶A), and their methylthiolated derivatives 2-methylthio-*N*⁶-isopentenyl-adenosine (ms²i⁶A) and 2-methylthio-*N*⁶-threonylcarbamoyl-adenosine (ms²t⁶A) (Figure 1). In *Escherichia coli* and *Bacillus subtilis*, i⁶A or ms²i⁶A occur in most tRNAs with A36 (reading UNN codons), while t⁶A or ms²t⁶A occur in most tRNAs with U36 (reading ANN codons) (1,2). Experimental evidence supports the hypothesis that these hydrophobic modifications stabilize the relatively weak A:U and U:A base pairs formed by the third base of the anticodons of these tRNAs with the first base of their complementary codons, thereby improving translational fidelity (1,3,4).

To the extent that the pathways are known, these modifications are constructed in a stepwise manner, and some of the enzymes involved have been discovered and characterized. Modification of adenosine to i⁶A is carried out by the gene products of *miaA* in *E. coli* and *MOD5* in yeast, which transfer the Δ²-isopentenyl group from dimethylallyl diphosphate, a mevalonic acid derivative, to A37 *N*⁶ (5–8). Mutations in *miaA* (originally called *trpX*) result in the accumulation of tRNA with strictly unmodified A37, indicating the formation of i⁶A is a prerequisite for any further modification (9). Formation of t⁶A from adenosine is less well characterized but is known to be an ATP-dependent reaction requiring threonine and

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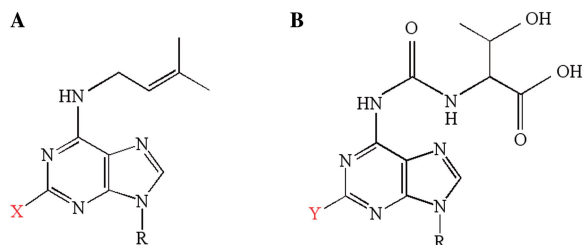


Figure 1. Schematic structures of the methylthiolated nucleic acid residues from *B. subtilis*. (A) *i*⁶A when X = H and *ms*²*i*⁶A when X = SCH₃. (B) *t*⁶A when Y = H and *ms*²*t*⁶A when Y = SCH₃.

carbonate (10). Recent genetic evidence shows that *E. coli yrdC* and yeast *SUA5* are required for this modification, but the involvement of other proteins in the reaction cannot yet be ruled out (11). Although not yet demonstrated, it seems reasonable to expect that *t*⁶A formation is a requisite first step for further modification based on analogy to the *i*⁶A pathway.

Methylthiolation (–SCH₃ addition) at C² of the adenosine ring forms *ms*²*i*⁶A and *ms*²*t*⁶A from *i*⁶A and *t*⁶A, respectively (Figure 1). The *miaB* gene product has been shown to catalyze the formation of *ms*²*i*⁶A in both *E. coli* and *Thermotoga maritima* (12–14). This reaction was originally hypothesized to occur by sequential steps, possibly catalyzed by separate enzymes: sulfur insertion by a *miaB* activity, followed by *S*-adenosylmethionine (SAM)-dependent methylation of a thiolated intermediate by a *miaC* activity (15,16). This was seemingly borne out by experiments using an *E. coli rel met cys* mutant (auxotrophic for methionine and cysteine yet capable of RNA synthesis in their absence). Under conditions of methionine starvation, such cells accumulated an uncharacterized *i*⁶A derivative that was capable of subsequent labeling using ¹⁴C-SAM, presumably by methylation (15). It was speculated that this uncharacterized nucleoside was the intermediate *s*²*i*⁶A, but this has yet to be confirmed, and this intermediate has not been observed elsewhere. Furthermore, recent results with the closely related enzyme RimO contradict this, suggesting instead that methylation of the sulfur atom occurs on an enzyme-bound FeS cluster prior to insertion (17). In any case, once discovered, purified MiaB was shown to be a methylthiotransferase (MTTase), responsible for both thiolation and methylation of *i*⁶A (18).

Phylogenetic analysis of the MTTase family shows that it consists of four clades, members of two of which have been characterized (19,20). One clade includes MiaB and its homologs, and is found exclusively in bacteria and eukaryotic organelles. The second characterized clade, also exclusively bacterial, includes RimO, a MTTase that modifies D88 of ribosomal protein S12 in *E. coli* (19). A third bacterial clade, typified by *B. subtilis* YqeV, and a fourth clade, found exclusively in archaea and eukaryotes and typified by *Methanocaldococcus jannaschii* Mj0867, remain uncharacterized. Given that the nucleoside *ms*²*t*⁶A has been observed both in bacteria [including *B. subtilis* (21) but not *E. coli*] and in archaea (22), it seems reasonable to expect that members of both of these clades are responsible for methylthiolating *t*⁶A to

*ms*²*t*⁶A, with the phylogenetic distinction reflecting the ancient split of the bacterial domain from archaea and eukaryotes rather than a functional differentiation within the protein family.

MTTases are members of the so-called ‘radical-SAM’ superfamily of proteins, which use a reducing equivalent from a prosthetic [4Fe–4S]¹⁺ cluster to cleave SAM, generating methionine and a reactive 5′-dA• radical (23). In the case of MiaB and RimO, this radical facilitates the difficult C–H to C–S bond conversion in the nucleoside or amino acid substrate, respectively, by abstracting the hydrogen atom and creating a reactive substrate radical that is amenable to sulfur insertion. All MTTases share a common tripartite domain structure, with the central domain responsible for this radical-SAM chemistry. The N-terminal domain has been shown in MiaB and RimO to harbor a second FeS cluster (17,24), a feature common to other radical-SAM proteins that catalyze sulfur insertion reactions such as BioB and LipA (25); this cluster is speculated to serve as the immediate sulfur donor in the thiolation reaction. The C-terminal TRAM domain has been shown to bind RNA in the context of other proteins (26), suggesting a similar function in tRNA-modifying MTTases. Its presence in the protein-modifying RimO is less obvious, but a recently published structure suggests that in this particular protein it has adapted to bind the protein substrate rather than RNA (27).

In this work, we identify and characterize the two MTTases encoded in the genome of *B. subtilis* str. 168, products of the *ymcB* and *yqeV* genes. We confirm the earlier prediction that YqeV performs the novel *t*⁶A methylthiolation function, and verify that YmcB, a MiaB ortholog, methylthiolates *i*⁶A. We then went on to construct several chimeric proteins derived from these two closely-related enzymes and show that, RNA-binding function of the TRAM domain notwithstanding, the ability of these two enzymes to discriminate between *i*⁶A and *t*⁶A resides in the N-terminal and/or radical-SAM domains, and not in the TRAM domain. Finally, we were unable to complement the loss of *yqeV* in *B. subtilis* with either the *mj0867* gene or a mesophilic ortholog *in trans*. We suggest that *yqeV* be renamed *tmtB*, for the second step in tRNA-methylthiolation, reserving *tmtA* for the *t*⁶A modifying activity.

MATERIALS AND METHODS

Enzymes

All restriction enzymes and T4 DNA ligase were from New England Biolabs (Ipswich, MA, USA). All PCR reactions were performed using Phusion DNA polymerase (New England Biolabs) in the HF buffer, unless otherwise noted.

Bacterial strains and media

Table 1 describes the bacterial strains and plasmids used in this study. *Bacillus subtilis* strains BSF2608 and YQEVD were derived from strain 168 by single-crossover (Campbell type) insertion of the plasmid pMUTIN (28). The insertion in BSF2608 is after *ymcB* nt 533, and that in

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	(52)
BSF2608	168, <i>ymcB</i> ::pMUTIN	(29)
YQEVd	168, <i>yqeV</i> ::pMUTIN	(29)
B(124)	BSF2608 × pDM124c7	This work
V(124)	YQEVd × pDM124c7	This work
B(B)	BSF2608 × pDMymcB	This work
B(MM)	BSF2608 × pDMmmar	This work
V(V)	YQEVd × pDMyqeV	This work
V(MJ)	YQEVd × pDMmj0867	This work
V(MM)	YQEVd × pDMmmar	This work
B(B1V)	BSF2608 × pDM-B1V	This work
B(B2V)	BSF2608 × pDM-B2V	This work
B(V1B)	BSF2608 × pDM-V1B	This work
B(V2B)	BSF2608 × pDM-V2B	This work
V(B1V)	YQEVd × pDM-B1V	This work
V(B2V)	YQEVd × pDM-B2V	This work
V(V1B)	YQEVd × pDM-V1B	This work
V(V2B)	YQEVd × pDM-V2B	This work
Plasmids		
pDM124c7	<i>E. coli/B. subtilis</i> shuttle plasmid with the promoter region from the <i>B. amyloliquefaciens</i> α -amylase gene followed by a multiple cloning site	J. Benner and D. Martin (unpublished)
pDMymcB	pDM124c7 with <i>ymcB</i> cloned between NdeI and XhoI	This work
pDMyqeV	pDM124c7 with <i>yqeV</i> cloned between NdeI and XhoI	This work
pDMmj0867	pDM124c7 with <i>mj0867</i> cloned between NdeI and XhoI	This work
pDMmmar	pDM124c7 with <i>mmar</i> cloned between NdeI and XhoI	This work
pDM-B1V	pDM124c7 with <i>ymcB/yqeV</i> chimera B1V between NdeI and XhoI	This work
pDM-B2V	pDM124c7 with <i>ymcB/yqeV</i> chimera B2V between NdeI and XhoI	This work
pDM-V1B	pDM124c7 with <i>ymcB/yqeV</i> chimera V1B between NdeI and XhoI	This work
pDM-V2B	pDM124c7 with <i>ymcB/yqeV</i> chimera V2B between NdeI and XhoI	This work

YQEVd is after *yqeV* nt 280 (29). Insertions were confirmed by PCR amplification (data not shown).

To make *B. subtilis* competent cells, 5 ml Rich medium supplemented with 3 mM MgSO₄ was inoculated with a single colony and grown at 37°C with vigorous aeration to OD₆₀₀ ~1.0. Then, 0.5 ml of this culture was used to inoculate 10 ml minimal medium (0.9× PC buffer, 2% glucose, 3 mM MgSO₄, 2.5 mg/ml potassium aspartate, 11 µg/ml ferric ammonium citrate, 50 µg/ml phenylalanine, 50 µg/ml tryptophan), which was then grown 4 h at 37°C with vigorous aeration. Three milliliters of 50% glycerol was then added to the culture, which was frozen at -80°C in 1 ml aliquots until ready for use. The composition of 1× PC buffer was 0.1 M potassium phosphate, 3 mM trisodium citrate, pH adjusted to 7.5 with KOH.

Transformation of *B. subtilis* was accomplished by mixing 1.5 µg plasmid DNA with 200 µl competent cells, growing 37°C 1 h with vigorous aeration, and plating on Rich medium supplemented with 12.5 µg/ml chloramphenicol as required. Plasmids used for *B. subtilis* transformation were purified from *E. coli* NEBTurbo (New England Biolabs), a *recA*⁺ strain.

Plasmid construction

The shuttle plasmid used for complementation, pDM124c7 (J. Benner and D. Martin, unpublished data), contains a ColE1 origin of replication and ampicillin-resistance marker for propagation in *E. coli* and a chloramphenicol-resistance marker for selection in

B. subtilis. Genes were cloned at the NdeI-XhoI sites, with expression in *B. subtilis* driven constitutively by the *B. amyloliquefaciens* α -amylase promoter and expressed proteins targeted to the cytoplasm.

Genes encoding MTTases were cloned by PCR amplification, with *ymcB* and *yqeV* from *B. subtilis* 168 genomic DNA, gene *mj0867* from *M. jannaschii* genomic DNA, and gene *mmar* from *Methanococcus maripaludis* genomic DNA, using primers described in Table 2. All four genes were digested at the NdeI and XhoI sites (underlined in Table 2) and inserted at the same sites in pDM124c7. Cloning steps were performed in *E. coli*, and constructs verified by DNA sequencing.

An internal NdeI site in *mmar* necessitated a second step in the cloning of that gene, as follows. The cloning steps above yielded a construct containing a truncated gene, with the 450 bp at the 5' end of the gene missing. The missing fragment was amplified using primers *mmar_F* and *mmar_I* (Table 2), digested with NdeI and inserted at the NdeI site of the truncated construct, thus reconstructing the intact gene. Our strain of *M. maripaludis* is distinct from the four strains whose genome sequences have been published to date, so we use the generic name *mmar* to refer to the *mj0867* ortholog from our strain. The *mmar* gene sequence and a protein sequence alignment of Mmar with the four published *M. maripaludis* orthologs are shown in Supplementary Figure S1.

Plasmids containing *ymcB/yqeV* chimeric genes were constructed using the USER Friendly Cloning Kit (New

Table 2. Oligonucleotide primers used in this study

Primer	Sequence	Target site ^a
ymcB_F	ATAAAACATATGAATGAAAAACAAAATTAGAGAG	<i>ymcB</i> nt 1–26, forward
ymcB_R	ATAAAA <u>CTCGAG</u> TCATTTACCTCGATTGCTTCTCTTACC	<i>ymcB</i> nt 1530–1503, reverse
yqeV_F	TATTTTCATATGGCAACTGTTGCTTCCATACGCTTG	<i>yqeV</i> nt 1–28, forward
yqeV_R	TACCCCTCGAGTTAAGAAGACAAAACGCATGTGTTTCAG	<i>yqeV</i> nt 1356–1331, reverse
mj0867_F	ATGGCGCATATGTGGTTATATTATTTACAAGTGGTG	<i>mj0867</i> nt 1–27, forward
mj0867_R	ATGCCGCTCGAGTTAAAGGATAAGCTCCCCTTTCAATCC	<i>mj0867</i> nt 1284–1258, reverse
mmar_F	ATGGCGCATATGAAAATTTACATTGAAGGATACGG	<i>mmar</i> nt 1–26, forward
mmar_R	ATGCCGCTCGAGTTAATTTATCAGTTTACCCGAAAGTCC	<i>mmar</i> nt 1278–1252, reverse
mmar_I	CCTTCA <u>CATATGGG</u> TAGTGC GGTAATTAATCC	<i>mmar</i> nt 446–421, reverse
ymcBch_F1	AACACCAGCUGCTAAGATGAAAGATAATG	<i>ymcB</i> nt 1224–1243, forward
ymcBch_R1	AGCTGGTGTUCCTTCACGCGGAGAGTAAATG	<i>ymcB</i> nt 1214–1194, reverse
ymcBch_F2	AGAATACGCUAAGGAATACGAAGGCAAGG	<i>ymcB</i> nt 1318–1336, forward
ymcBch_R2	AGCGTATTCUGCAGAAATTCATTCACCAG	<i>ymcB</i> nt 1307–1288, reverse
yqeVch_F1	AACACCAGCUGCAGCAATGGAAGACCAAG	<i>yqeV</i> nt 1011–1039, forward
yqeVch_R1	AGCTGGTGTUCCTGTACGCTTACTGTAAGG	<i>yqeV</i> nt 1020–991, reverse
yqeVch_F2	AGAATACGCUTCTCAGTATGAAAATGAAG	<i>yqeV</i> nt 1104–1132, forward
yqeVch_R2	AGCGTATTCUTTTGCAAGCTGGTCAGAAAAG	<i>yqeV</i> nt 1113–1084, reverse
bla_F	AGTTACATGAUCCCCCATGTTGTGCAAAAAAG	<i>bla</i> nt 474–443, reverse
bla_R	ATCATGTAACUCGCCTTGATCGTTGGGAAC	<i>bla</i> nt 464–493, forward

^aRegions of hybridization are: for forward cloning primers, from the ATG within the NdeI site (underlined); for reverse cloning primers, 3' of the XhoI site (underlined); for the internal *mmar* cloning primer (*mmar_I*), from the NdeI site (underlined) to the 3' end; for *ymcBch* USER primers, 3' of the uracil used for nicking (underlined); for *yqeVch* and *bla* USER primers, the entire primer. *bla* is oriented counter-clockwise in pDM124c7, so *bla_F* is forward/clockwise with respect to the plasmid but reverse/bottom-strand with respect to the gene sequence, and vice versa for *bla_R*.

England Biolabs). Two fragments (designated 'N' and 'C'), which together encompassed the entirety of the plasmid template, were amplified from each of pDMymcB and pDMyqeV using PfuCx Hotstart polymerase (Stratagene, Cedar Creek, TX, USA), with primers described in Table 2. Junctions between the two fragments were (i) within the *ymcB* or *yqeV* gene, as described in 'Results' section, and (ii) within the ampicillin-resistance marker. Following treatment with the USER enzyme, fragments were annealed such that fragment N derived from one plasmid was paired with fragment C derived from the other. Constructs were verified by DNA sequencing.

tRNA purification

tRNA was purified from 500 ml *B. subtilis* cultures grown at 37°C in Rich medium and harvested at OD₆₀₀ between 0.8 and 1.0. Cell pellets were washed with 4 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), then incubated 37°C 3 h in 4 ml TE with 40 mg/ml lysozyme. Samples were then vigorously mixed with 16 ml TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) and left 10 min at room temperature. Chloroform (3.2 ml) was added, and samples were left for 5 min at room temperature before centrifuging at 8000 r.p.m. for 10 min. Total RNA was precipitated from the aqueous phase with 0.3 M sodium acetate and 70% ethanol and collected by centrifugation at 12 000 r.p.m. for 20 min. tRNA was solubilized by vortexing pellets in 3.2 ml TE with 2 M LiCl and recentrifuging at 8000 r.p.m. for 10 min, then precipitated from the supernatant with 70% ethanol and resuspended in 450 µl TE.

tRNA was cleaned for LC/MS by three rounds of precipitation with 0.3 M ammonium acetate and 70% ethanol, and finally resuspended in 450 µl TE. Typical

yield was roughly 1 mg. Eighty micrograms of this was then purified over Nucleobond AX-R 80 ion exchange columns (Macherey-Nagel, Bethlehem, PA, USA), collecting 1.2 ml eluate. tRNA was precipitated from the eluate with 45% isopropanol and resuspended in 40 µl H₂O. Typical yield was 65 µg tRNA.

tRNA was digested to nucleosides as follows. Forty microliters (roughly 65 µg) of tRNA was denatured 3 min at 100°C, then rapidly chilled in an ice-H₂O slurry. Four microliter 0.1 M ammonium acetate pH 5.3 and 8 U Nuclease P1 (Sigma-Aldrich) were added, and samples were incubated at 45°C for 2 h. Four microliters of 0.1 M ammonium bicarbonate and 0.1 U Phosphodiesterase I (Worthington Biochemical, Lakewood, NJ, USA) were added, and samples were incubated at 37°C for 2 h. Five units Antarctic phosphatase (New England Biolabs) were added, and samples were incubated at 37°C for 1 h.

Liquid chromatography and ESI-MS analysis

Nucleosides were separated, at room temperature, on a Hitachi HPLC system (L-7100 pump) with UV detection at 260 nm (L-7400 UV detector). The column used was a Supelcosil LC-18-S (25 cm × 2.1 mm, 5 µm diameter particles, with a 2 cm × 2.1 mm guard column), which was run at a flow rate of 0.3 ml min⁻¹. The mobile phases used were (i) 5 mM ammonium acetate pH 5.3, and (ii) acetonitrile/water (40:60, v/v) with the gradient described by Pomerantz and McCloskey (30) with minor alterations. The column effluent was split, with one-third directed to a Thermo LTQ-XL (or LTQ-FT) mass spectrometer and two-thirds to the UV detector. Mass spectra were recorded in the positive ion mode from *m/z* 103 to 510. Electrospray and MS conditions were optimized using adenosine, introduced post-column. The capillary temperature used was 275°C, source voltage 3.7–5 kV, sheath gas flow 45

arbitrary units, auxiliary gas flow 25 arbitrary units and sweep gas flow 10 arbitrary units.

RESULTS

Identification of *B. subtilis* MTTase genes

A previous BLASTP search of the translated *B. subtilis* 168 genome using *E. coli* MiaB as the query revealed two putative MTTases, products of *ymcB* (BSU17010) and *yqeV* (BSU25430). Based on phylogenetic considerations, *ymcB* was predicted to be a true *miaB* ortholog, with its product responsible for the ms^2i^6A modification observed in *B. subtilis* (31), while *yqeV* belonged to a novel subfamily predicted (19) to be responsible for the observed ms^2t^6A modification (32). *ymcB* appears to be a part of bicistronic operon with the gene *ymcA*, with potential -35 and -10 elements upstream of *ymcB* and a putative rho-independent terminator downstream of *ymcA* (33). *yqeV* is the terminal gene of the heptacistronic *dnaK* operon, whose transcripts have been mapped and regulation described by Homuth and coworkers (who refer to *yqeV* as *orf50*) (34,35). It is expressed from both a heat-inducible promoter upstream of *hrcA* and from a constitutive promoter upstream of *dnaJ*. The tetracistronic constitutive transcript encodes, between *dnaJ* and *yqeV*, two other methyltransferases involved with modifying the translational machinery: *yqeT*, a probable homolog of the ribosomal protein L11 MTase *prmA*, and *yqeU*, a probable homolog of the 16S rRNA MTase *rsmE*.

Characterization of mutant phenotypes

Mutant strains with insertions in both *ymcB* and *yqeV* have been constructed (29), indicating that neither gene is essential under normal growth conditions. We obtained these mutant strains as well as the parental strain 168 from the *B. subtilis* sequencing consortium (<http://bacillus.genome.ad.jp>). To investigate the effect of these insertion mutants on tRNA modification, we purified and digested to nucleosides total tRNA from the parental and two mutant strains. These nucleoside digests were analyzed by LC/UV and LC/MS to determine the presence or absence of post-transcriptional modifications, specifically t^6A , ms^2t^6A , i^6A and ms^2i^6A . Figure 2 shows the UV chromatogram of the nucleoside digest of tRNA from wild-type strain 168, indicating the presence of peaks at retention times expected for all four modifications (highlighted with 'asterisks'). The identities of these peaks were confirmed by LC/MS, as shown in Figure 3. The characteristic collision-induced fragmentation pattern of modified nucleosides involves the cleavage of the *N*-glycosidic bond and the neutral loss of ribose (132 mass units when the 2'-OH is unmethylated, as is the case for the four modified adenosines in question). Thus, the major ions detected by MS are the protonated molecular ion (MH^+) and the protonated base ion (BH_2^+). The corresponding mass spectra in Figure 3 reveal that in each case, the MH^+ and BH_2^+ ions characteristic of the expected nucleoside are present and track to the equivalent retention time as the UV peak.

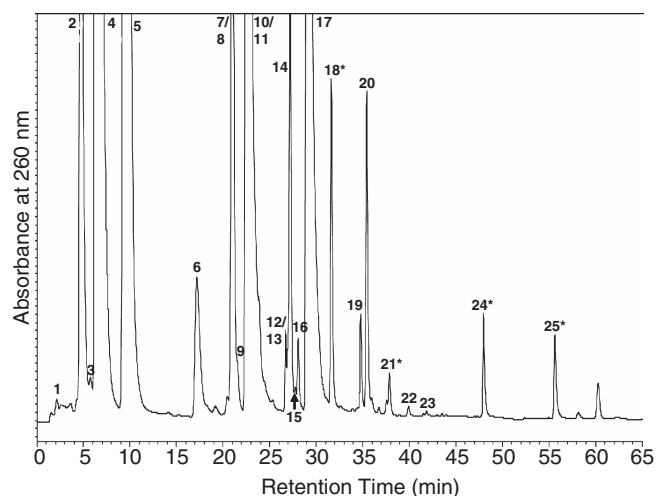


Figure 2. UV chromatogram of wild-type *B. subtilis* 168 total tRNA digested to nucleosides. Numbered peaks are as follows: (1) dihydrouridine (D), (2) pseudouridine (Ψ), (3) 5-carboxymethylaminomethyluridine (cmnm⁵U), (4) cytidine, (5) uridine, (6) 1-methyladenosine (m¹A), (7) 5-methyluridine (m⁵U), (8) 5-methoxyuridine (mo⁵U), (9) inosine (I), (10) guanosine, (11) 7-methylguanosine (m⁷G), (12) 2'-*O*-methylguanosine (Gm), (13) queuosine (Q), (14) 1-methylguanosine (m¹G), (15) lysidine (k²C), (16) *N*²-methylguanosine (m²G), (17) adenosine, (18) t^6A , (19) 2-methyladenosine (m²A), (20) N^6 -methyladenosine (m⁶A), (21) ms^2t^6A , (22) 2-methylthioadenosine (ms^2A), (23) N^6,N^6 -methyladenosine (m⁶₂A), (24) i^6A , (25) ms^2i^6A . The four peaks relevant to this work are marked with asterisks.

UV chromatograms of nucleoside digests from the two mutant strains are shown in Figure 4. Strain BSF2608 (*ymcB*) shows specific loss of the ms^2i^6A peak (Figure 4B), and strain YQEVd (*yqeV*) shows specific loss of the ms^2t^6A peak (Figure 4C). Both genes were separately subcloned into the plasmid pDM124c7 ('Materials and methods' section) and reintroduced *in trans* into the respective mutant strains to complement the disrupted alleles. In both cases, the intact plasmid-encoded gene rescued the modification-deficient phenotype, whereas the empty plasmid vector did not, indicating that *ymcB* and *yqeV* are required for ms^2i^6A and ms^2t^6A modification, respectively, confirming previous phylogenomic predictions (19). These results are summarized in Table 3, and complete LC/UV and LC/MS data for all samples are included as Supplementary Figure S2. Although we will continue to refer to these genes and their products here as *ymcB* and *yqeV* for clarity, we suggest that *ymcB* be renamed *miaB* and suggest the name *tmtB* for *yqeV*.

The ms^2i^6A - and ms^2t^6A -deficient *B. subtilis* strains identified above are, in addition, suitable for characterizing heterologous MTTases of unknown substrate specificity. We cloned *mj0867* from *M. jannaschii* (a hyperthermophile), and the orthologous gene *mmar* from *M. maripaludis* (a mesophilic relative of *M. jannaschii*), into pDM124c7 and introduced them separately into the $ms^2t^6A^-$ strain YQEVd as above. However, despite being predicted to be ms^2t^6A MTTases, neither gene was able to rescue the modification-deficient phenotype (Table 3 and Supplementary Figure S2). In addition, *mmar* failed to

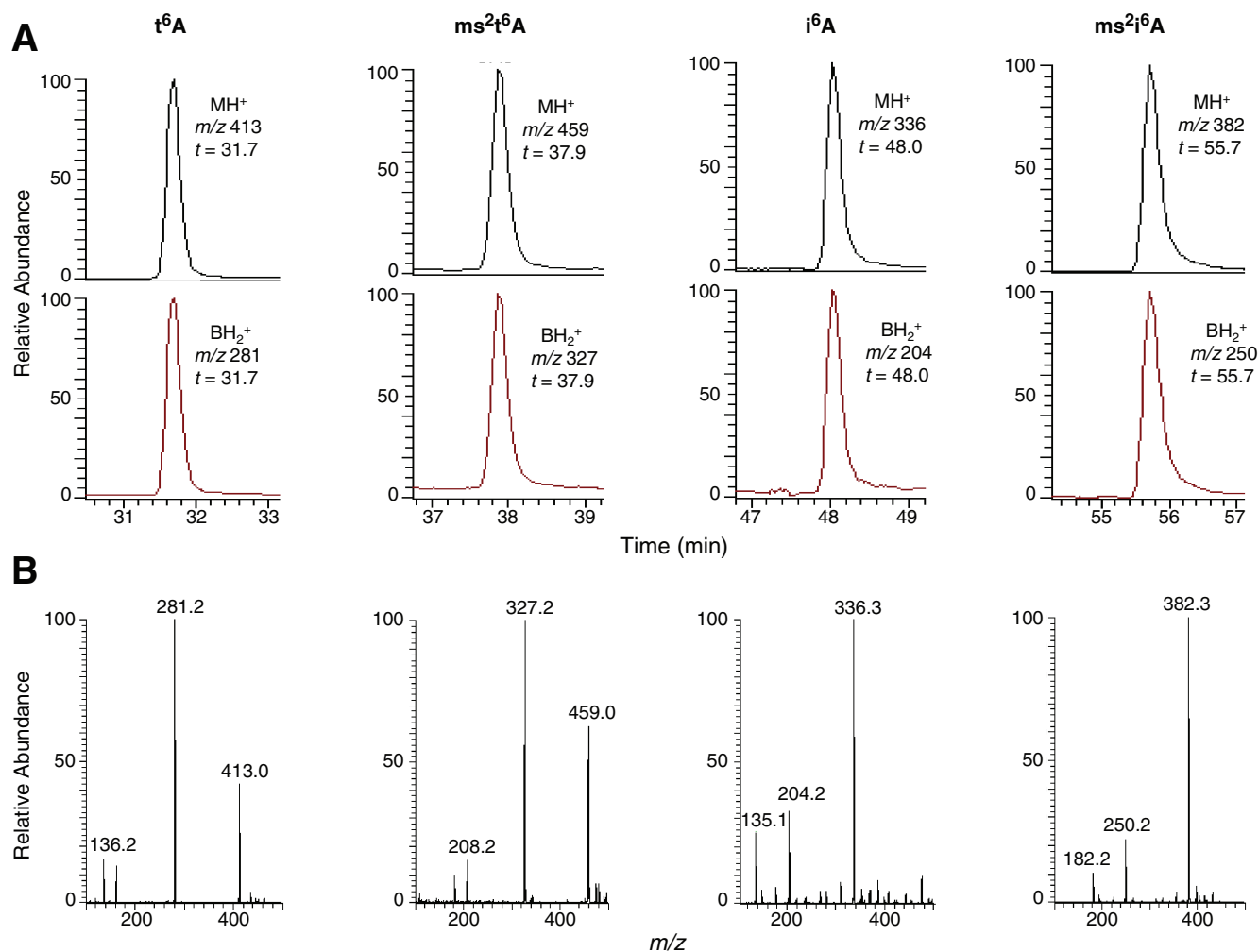


Figure 3. LC/MS spectra of the adenosine derivatives t^6A , ms^2t^6A , i^6A and ms^2i^6A from digested wild-type *B. subtilis* 168 total tRNA. (A) Selected ion chromatograms of molecular and base ions. (B) Mass spectra.

rescue the ms^2i^6A deficiency in strain BF2608. Both archaeal proteins appeared largely insoluble in the *B. subtilis* extracts (data not shown), suggesting that these proteins may be misfolded, and therefore inactive, in this heterologous context.

Construction and characterization of YmcB/YqeV chimeric proteins

We further used the availability of the modification-deficient strains to explore the determinants of substrate recognition in YmcB and YqeV by constructing chimeric proteins. Specifically, we sought to determine whether the TRAM domain, believed to bind RNA, also conferred the recognition of the N^6 moiety that differentiates the substrates of these two proteins. YmcB and YqeV share significant sequence similarity across all three domains (Figure 5). We wished to generate a breakpoint between the radical-SAM and TRAM domains at which the TRAM domains could be swapped. However, we noted significant sequence conservation in the region between these two domains as defined by Pfam (Figure 5), and it was thus unclear where the functionally required elements

of one domain ended and the other began. Accordingly, we generated two alternative breakpoints for each construct, one close to the Pfam-defined boundary of the radical-SAM domain (breakpoint 1, Figure 5 red arrows) and the other close to the boundary of the TRAM domain (breakpoint 2, Figure 5 green arrows). Four constructs were generated in all, designated B1V, B2V, V1B and V2B, with the first letter ('B' for YmcB and 'V' for YqeV) describing the source of the N-terminal and radical-SAM domains, the second letter describing the source of the TRAM domain, and the number between them indicating the location of the breakpoint. Note that, due to the requirements of the cloning methodology, breakpoint 2 differs by three residues between B2V and V2B.

We attempted to rescue the modification defect in both BSF2608 and YQEVD with each of the four chimeric constructs as was done for *ymcB*, *yqeV* and *mj0867*. tRNA was purified and the modified nucleosides examined for each of these eight strains (four chimeras in two backgrounds), and we observed successful rescue in two of them: in strains B(B1V) and B(B2V), chimeras B1V and

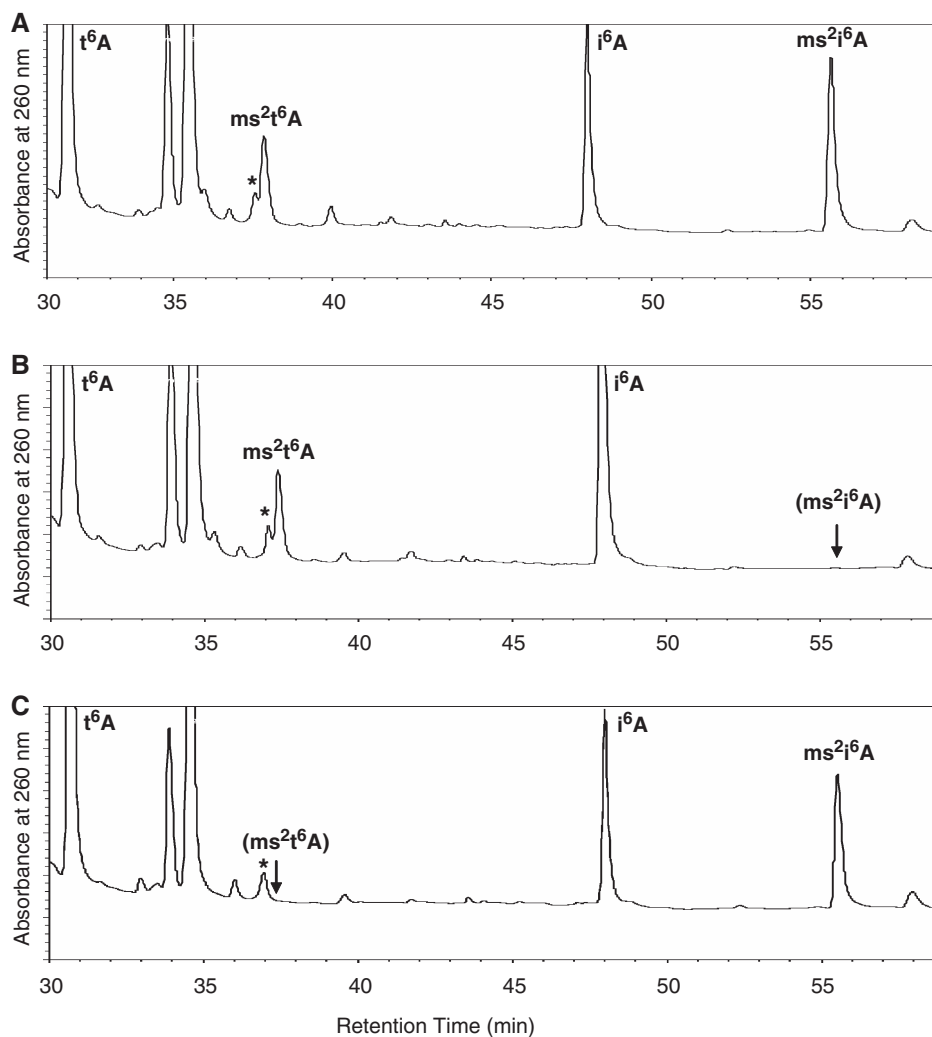


Figure 4. UV chromatograms of *B. subtilis* total tRNA nucleoside digests from the wild-type and two mutant strains, focused on later eluting species. Peaks representing t^6A , ms^2t^6A , i^6A and ms^2i^6A are labeled, with parentheses indicating loss of the expected peak. A non-nucleoside peak eluting close to ms^2t^6A is indicated by an asterisk. (A) Wild-type strain 168, (B) strain BSF2608 and (C) strain YQEVd.

B2V rescued the loss of ms^2i^6A modification (Table 3 and Supplementary Figure S2). These results are consistent with differential recognition of the i^6 or t^6 substituent residing in either the N-terminal or radical-SAM domain of these MTTases rather than in the TRAM domain. The inability of V1B and V2B to rescue the ms^2t^6A defect (or the ms^2i^6A defect) is likely due to misfolding of the proteins. Indeed, all four chimeric proteins appeared largely insoluble in the cell extracts, which was not the case with the two native proteins (data not shown). However, the B1V and B2V proteins were significantly more highly expressed than V1B and V2B, and enough protein may have folded correctly in these cases to observe enzymatic activity.

In order to pinpoint residues within the N-terminal and/or radical-SAM domains that may be responsible for substrate recognition, we performed a computational analysis that is described more fully in the Supplementary Data. Briefly, we examined a collection of sequences from the three characterized MTTase subfamilies, MiaB, YqeV and RimO, to identify residues that are conserved within each

subfamily but differ between them. Such residues are candidates for discriminating the three types of substrates specific for each subfamily. Of the 13 highest scoring residues in our analysis, shown in Supplementary Figure S3, 6 are closely proximal to the 6 invariant cysteines involved with coordinating the FeS clusters, suggesting they may be of structural importance, and 2 more are within the TRAM domain. The remaining five, all within the radical-SAM domain yet distal in the primary sequence to the FeS cluster motif, represent strong candidates for substrate interaction; these are residues 195, 231, 232, 297 and 328, using numbering from *B. subtilis* YqeV. Note that the highest scoring of these, residue 328, is technically outside of the Pfam-defined radical-SAM domain, but is just upstream of breakpoint 1 in our chimera construction, and therefore segregated with the radical-SAM domain in all of the chimeras we constructed.

Relative modification levels

A semi-quantitative investigation of the LC/UV data revealed apparent differences in the relative amounts of

Table 3. Modified nucleosides observed in *B. subtilis* strains

Strain	Genotype ^a		Phenotype ^b			
	<i>ymcB</i>	<i>yqeV</i>	i ⁶ A	ms ² i ⁶ A	t ⁶ A	ms ² t ⁶ A
168	+	+	+	+	+	+
BSF2608	-	+	+	- ^c	+	+
B(124)	-	+	+	- ^c	+	+
B(B)	+ ^P	+	+	+	+	+
B(MM)	(?) ^P	+	+	-	+	+
B(B1V)	(?) ^P	+	+	+	+	+
B(B2V)	(?) ^P	+	+	+	+	+
B(V1B)	(?) ^P	+	+	-	+	+
B(V2B)	(?) ^P	+	+	-	+	+
YQEVd	+	-	+	+	+	-
V(124)	+	-	+	+	+	-
V(V)	+ ^P	+	+	+	+	+
V(MJ)	+	(?) ^P	+	+	+	-
V(MM)	+	(?) ^P	+	+	+	-
V(B1V)	+	(?) ^P	+	+	+	-
V(B2V)	+	(?) ^P	+	+	+	-
V(V1B)	+	(?) ^P	+	+	+	-
V(V2B)	+	(?) ^P	+	+	+	-

^aIntact genes indicated by 'plus'; insertion mutants indicated by 'minus'; question marks indicate the presence of a heterologous gene that may or may not complement the gene in question; superscript 'P' indicates plasmid-encoded copy.

^bPhenotypes indicate the presence (plus) or absence (minus) of an LC/UV peak indicative of this modified nucleoside. MS results were consistent with the UV results except where indicated (footnote c).

^cWeak MS signal detected in these samples (Supplementary Figure S2).

the modifications noted in Table 3 for the 16 strains listed there. We first calculated the peak area ratios of t⁶A, ms²t⁶A, i⁶A and ms²i⁶A to m⁵U, a modified nucleoside found at position U54 of all sequenced *B. subtilis* tRNA species (<http://modomics.genesilico.pl>), and whose modification is independent of those studied in this work; these ratios can therefore be interpreted as the fractions of all tRNA molecules bearing t⁶A, ms²t⁶A, i⁶A or ms²i⁶A, respectively. We then calculated the ratios of ms²i⁶A and ms²t⁶A to (ms²i⁶A + i⁶A) and (ms²t⁶A + t⁶A), respectively, that is, the fractions of i⁶A- and t⁶A-containing residues that are methylthiolated. This information is summarized in Supplementary Table S2, and the original peak areas from which the ratios were calculated can be found in Supplementary Table S1.

The fraction of t⁶A residues that are methylthiolated is roughly similar to the wild-type across ms²t⁶A⁺ strains at ~10%, regardless of whether YqeV is expressed from the chromosome or the plasmid (Supplementary Table S2). The wild-type strain 168 exhibits roughly equal amounts of i⁶A and ms²i⁶A (50% methylthiolation), but in other ms²i⁶A⁺ strains this fraction ranges as high as 70% in strain B(B), where wild-type YmcB is expressed from the plasmid, to as low as 8% for strain B(B1V) (Supplementary Table S2). These more extreme values are likely caused by promiscuous modification of non-cognate tRNA by overexpressed YmcB in the case of B(B), and misfolding of a substantial fraction of chimeric protein in the case of B(B1V), as described earlier.

A final observation from the LC/UV and LC/MS data indicates possible crosstalk between the YqeV and YmcB

modification pathways. In three of the four strains where no ms²i⁶A peak could be detected by LC/UV, trace amounts of the diagnostic MH⁺ and BH₂⁺ ions could still be detected by LC/MS (Table 3 and Supplementary Figure S2). One of these three was the original insertion mutant strain BSF2608, suggesting that this trace modification could be due either to incomplete inactivation of *ymcB* by the insertion, or to trace promiscuous activity by the fully active YqeV in these strains. Levels of this nucleotide are low enough that stochastic variation between samples could explain the absence of LC/MS-detectable amounts in the fourth strain.

Other tRNA modifications present

In addition to enabling identification of t⁶A, ms²t⁶A, i⁶A and ms²i⁶A, Figure 2 represents one of the most comprehensive censuses of tRNA modification in *B. subtilis* to date, with a total of 23 modified nucleosides identified as present in physiologically relevant amounts. The spectrum of modified nucleosides in Figure 2 is largely consistent with previous studies, including those of Vold and coworkers (36,37), and the collection of sequenced tRNA species in the MODOMICS database (2). All nucleosides positively identified in at least one of those studies can be identified in Figure 2 with the exception of s⁴U and cmnm⁵s²U. In addition, we identified k²C, which was previously identified only in a more specialized analysis (38). We detected three nucleosides (m²G, m²A and m²°A) that are typical of rRNA and therefore may be indicative of trace rRNA contamination in our tRNA preparation. One other nucleoside, ms²A, is present in trace amounts in all samples and is assumed to be a breakdown product created during tRNA purification. We could identify no modified nucleosides besides those observed in previous studies.

DISCUSSION

Regulation of MTTase activity

Early studies of ms²i⁶A in bacteria revealed that the methylthiol group, in modulating codon-anticodon interactions, acts as an environmental sensor, mediating cellular responses to changing growth conditions. In *E. coli*, most i⁶A residues are methylthiolated to ms²i⁶A at all stages of growth under normal conditions (31,39). However, i⁶A is undermodified under conditions of iron limitation in *E. coli* and other Proteobacteria (40-42). An effect of the loss of ms² from tRNA^{Trp} and tRNA^{Phe} is to specifically reduce the efficiency at which these tRNAs read the regulatory leader sequences preceding the tryptophan and phenylalanine biosynthetic operons, respectively. The slower rate at which these sequences are read by ribosomes disfavors the formation of attenuator structures that prematurely terminate transcription, with the end result being specific up-regulation of the *trp* and *phe* operons in response to iron depletion.

The same effect of iron-limitation has been observed in *B. subtilis* (43), but in addition, the degree of ms² modification in this differentiating bacterium is growth-phase dependent. During exponential growth, ms²i⁶A levels are



Figure 5. Protein sequence alignment of *B. subtilis* MTTases YqeV (NP_390421) and YmcB (NP_389583). Boundaries of the three domains as defined by Pfam are boxed in pink (UPF0004; PF00919), cyan (radical-SAM; PF04055) and brown (TRAM; PF01938). Arrows indicate the breakpoints 1 (red) and 2 (green) used to construct chimeric proteins (see text).

low relative to i^6A , whereas the situation is reversed at stationary phase and sporulation (31,43). These two conditions are not independent, since cells grown in iron-deficient media sporulate much less efficiently than in iron-replete media. However, ms^2i^6A modification also covaries with other activities affecting sporulation such as catabolite repression, NADPH oxidase and isocitrate dehydrogenase, and as a result has been hypothesized to be itself required for sporulation (43). The effect of growth conditions and growth phase on ms^2i^6A modification has been less closely studied. However, in tRNA^{Lys}, the sole ms^2i^6A -modified species identified in *B. subtilis*, the extent of ms^2 modification appears to be somewhat greater in exponentially growing cells than in stationary phase cells (32,44), in direct contrast to the pattern of ms^2i^6A methylthiolation. Thus, although it is tempting to speculate that growth phase-related changes in methylthiolation are due to fluctuations in intracellular iron concentration, most likely acting through the FeS clusters of MTTases, the inconsistency of behavior of YmcB and YqeV suggests there are additional levels of regulation at work.

Confirmation of the functions encoded by the *ymcB* and *yqeV* MTTase genes should facilitate better understanding of the precise regulatory networks that coordinate their activities. The *ymcB* gene appears to be co-transcribed with *ymcA*, a master regulator of biofilm formation that is required for pellicle formation and colony differentiation (33). This gene pair is extensively conserved within the Bacilli, but the functional relationship between the two genes remains unclear. The fact that both genes are associated with cell differentiation is nonetheless intriguing. Based on published results, these genes do not appear to be part of any well-characterized regulon, particularly those associated with sporulation (σ^E , σ^H , Spo0A) or iron utilization (Fur) (45–49). Given the condition-dependent pattern of ms^2i^6A modification described earlier, this is somewhat surprising and suggests that some regulatory element remains to be discovered. Transcription of *yqeV* appears to be dual regulated: a background level of transcript (along with other modification-related genes and *dnaJ*) expressed from a vegetative promoter, supplemented with expression

from a second, longer *dnaK* transcript governed by a CIRCE-regulated σ^A promoter. Again the functional connection between the *hrcA*-related genes and the translation-related modification genes is unclear, but this gene order is again well conserved among both Bacilli and Clostridia, so there appears to be some selective pressure to maintain this arrangement.

Substrate recognition

The substrate elements recognized by MTTases are not completely defined. However, the absence of observed ms^2A in *miaA* mutants indicates that the i^6A group is absolutely required for MiaB modification. Given the strong similarity among all MTTases, it seems reasonable to expect that YqeV should correspondingly require the t^6A group for modification; however, no t^6A^- phenotype has been created in an organism harboring an YqeV-type MTTase to test this. Our results with YmcB/YqeV chimeric proteins, while incomplete, nonetheless suggest that recognition of the N^6 substituent lies in either the N-terminal or radical-SAM domain, not in the C-terminal TRAM domain. The TRAM domain may function as a non-specific RNA clamp by which the MTTase holds its substrate, such that TRAM domains are completely interchangeable, or alternatively, TRAM may confer additional substrate specificity, perhaps at the RNA sequence level. We have shown that the B1V and B2V chimeras form ms^2i^6A , but without further characterization of individual tRNA species, it cannot be assumed that these chimeras modify the same set of tRNAs as YmcB.

Bacillus subtilis YmcB and YqeV both appear relatively restrictive in their additional substrate requirements. Of the 7 i^6A -modified tRNA species, only tRNA₁^{Phe} and tRNA₁^{Tyr} are methylthiolated (2), and of the 9 t^6A -modified species, only tRNA₁^{Lys} is methylthiolated (2,21). Pierrel and coworkers have shown that *E. coli* MiaB can modify a 17 base RNA oligonucleotide derived from the anticodon stem-loop of tRNA^{Phe} *in vitro* when the residue corresponding to A37 is isopentenylated (18) so we examined the corresponding regions from the tRNA species above for elements common to those that are methylthiolated. (Alignments

of these regions are provided as Supplementary Figure S4.) In the case of i^6 A-bearing tRNAs, the only sequence feature outside the anticodon that is unique to the two methylthiolated species is the A31: Ψ 39 bp at the end of the stem-loop. Ψ :A base pairs at the corresponding position of a synthetic stem-loop oligonucleotide have been shown to have a stabilizing effect on the structure (50). However, tRNA₁^{Trp}, which has a Ψ 31:A39 bp, is not methylthiolated, suggesting that if this base pair has any significance with respect to YmcB recognition, the important element may be Ψ 39 itself (and/or A31) rather than the stem structure it confers. In the case of t^6 -bearing tRNAs, there is only a single methylthiolated example, tRNA₁^{Lys}. It again contains an A31: Ψ 39 bp, but tRNA₁^{Thr} also contains this base pair yet is not methylthiolated. Indeed, the sequences of tRNA₁^{Lys} and tRNA₁^{Thr} are nearly identical in this region outside the anticodon, except for the U27:A39 bp, which is unique to tRNA₁^{Lys}.

Escherichia coli MiaB appears much less restrictive in its modification than *B. subtilis* YmcB: of 10 i^6 A-modified tRNA species, nine are methylthiolated. In addition, mutant tRNAs that have been altered to become MiaA substrates, such as tRNA₂^{Gly} C36A, tRNA₁^{Lys} U36A and tRNA^{fMet} A35U U36A (51), acquire ms^2i^6 A37, indicating they too are MiaB substrates when isopentenylated. It is unclear to what degree the difference in the suite of methylthiolated tRNA species between these two organisms is due to changes in the proteins and to what degree it is due to changes in tRNA, as both enzyme and substrate coevolve. In *B. subtilis*, ms^2i^6 A modification has come to be associated with sporulation, a process that has no analog in *E. coli*. We speculate that this additional role may have influenced these changes, but alternative explanations are certainly possible. In any case, proper assessment of any differences in substrate specificity between *E. coli* MiaB and *B. subtilis* YmcB will require complementation of one with the other and full analysis of the modified tRNA species. This work provides a first step towards an understanding of the process of substrate recognition by MTTases, and further progress in this area may shed light on multiple key biological processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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