YibK is the 2'-O-methyltransferase TrmL that modifies the wobble nucleotide in *Escherichia coli* tRNA^{Leu} isoacceptors

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ABSTRACT

Transfer RNAs are the most densely modified nucleic acid molecules in living cells. In *Escherichia coli*, more than 30 nucleoside modifications have been characterized, ranging from methylations and pseudouridylations to more complex additions that require multiple enzymatic steps. Most of the modifying enzymes have been identified, although a few notable exceptions include the 2'-O-methyltransferase(s) that methylate the ribose at the nucleotide 34 wobble position in the two leucyl isoacceptors tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA}. Here, we have used a comparative genomics approach to uncover candidate *E. coli* genes for the missing enzyme(s). Transfer RNAs from null mutants for candidate genes were analyzed by mass spectrometry and revealed that inactivation of *yibK* leads to loss of 2'-O-methylation at position 34 in both tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA}. Loss of YibK methylation reduces the efficiency of codon–wobble base interaction, as demonstrated in an amber suppressor *supP* system. Inactivation of *yibK* had no detectable effect on steady-state growth rate, although a distinct disadvantage was noted in multiple-round, mixed-population growth experiments, suggesting that the ability to recover from the stationary phase was impaired. Methylation is restored in vivo by complementing with a recombinant copy of *yibK*. Despite being one of the smallest characterized α/β knot proteins, YibK independently catalyzes the methyl transfer from S-adenosyl-L-methionine to the 2'-OH of the wobble nucleotide; YibK recognition of this target requires a pyridine at position 34 and N⁶-(isopentenyl)-2-methylthioadenosine at position 37. YibK is one of the last remaining *E. coli* tRNA modification enzymes to be identified and is now renamed TrmL.

Keywords: tRNA modification; comparative genomics; wobble base; MALDI-MS; SPOUT methyltransferases; *yibK/trmL*

INTRODUCTION

The stable RNAs (tRNAs and rRNAs) of all organisms are post-transcriptionally modified to improve their functions in protein synthesis (Grosjean 2005). The tRNAs exhibit the densest concentration of modifications with generally ~10% of their nucleotides being modified. In *Escherichia coli* tRNAs, 31 distinct types of modified nucleotide have been characterized (Björk and Hagervall 2005) requiring the investment of ~1% of the genome in the tRNA modification

process in addition to the array of enzymes required for biosynthesis of donor groups such as tetrahydrofolate or S-adenosylmethionine (SAM).

Modified nucleotides cluster in two main regions of tRNAs: in the L-shaped core and in the anticodon loop (Grosjean 2009). Most modifications in the structural core are generated by relatively simple biosynthesis reactions involving methylation, pseudouridylation, or dihydrouridine formation, and they serve to stabilize the tRNA tertiary structure (Helm 2006). Modifications within the anticodon loop include methylations and pseudouridylations together with more complex additions, which collectively enhance the accuracy of codon recognition, maintain the translational reading frame (Björk and Hagervall 2005), and facilitate the engagement of the ribosomal decoding site in these processes (Agris 2008). Loss of anticodon modifications, particularly at

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the 34 wobble position, disrupts gene expression and affects a range of phenotypic traits including virulence, pathogenicity, and cellular response to stress (Karita et al. 1997; Forsyth et al. 2002; Gong et al. 2004; Sha et al. 2004; Shin et al. 2009). Formation of the more complex nucleotide modifications involves a series of steps by different enzymes, and the pathways for the majority of these have been characterized (e.g., Hagervall et al. 1987; Björk and Hagervall 2005; Ikeuchi et al. 2006; Lundgren and Björk 2006; El Yacoubi et al. 2009; Moukadiri et al. 2009).

Modification of nucleotide 34 in the two *E. coli* isoacceptors tRNA^{Leu}_{cmnm5UmAA} and tRNA^{Leu}_{CmAA} is one of the few pathways that still await complete characterization. Formation of the 5-carboxymethylaminomethyl modification (cmnm) on the base of uridine-34 in tRNA^{Leu}_{cmnm5UmAA} by the enzymes MnmE and MnmG (formerly GidA) has recently been described in detail (Moukadiri et al. 2009); however, identification of the 2'-O-methyltransferase(s) that modifies nucleotide 34 in this and the tRNA^{Leu}_{CmAA} isoacceptor has remained elusive (Purta et al. 2006). In this study, we have applied a comparative genomics approach to prioritize *E. coli* gene candidates that could encode the undiscovered 2'-O-methyltransferase(s). Particular attention was paid to SPOUT enzymes, a class of SAM-dependent methyltransferases that exhibit an unusual fold and members of which have been associated with 2'-O-methyl additions (Schubert et al. 2003; Tkaczuk et al. 2007). Analysis by MALDI-MS of the tRNAs from null mutants conclusively revealed that a single SPOUT-class enzyme, YibK, introduces the 2'-O-methyl groups into both tRNA^{Leu} isoacceptors. The motifs in tRNA^{Leu} required for YibK recognition and catalysis were investigated in vitro and include the N⁶-(isopentenyl)-2-methylthioadenosine (ms²i⁶A) at position 37. The in vitro methylation assay also established that YibK catalyzes 2'-O-methylation without the aid of other proteins, and thus functions independently despite being one of the smallest α/β -knot proteins presently characterized.

RESULTS AND DISCUSSION

Selection of candidate genes

Candidates for previously uncharacterized tRNA-modifying enzymes were sought using comparative-genomics approaches (Gabaldon and Huynen 2004; Gabaldon 2008). We made use of phylogenetic profiles (Pellegrini et al. 1999) showing correlated evolution between genes. This was combined with other approaches such as gene chromosomal neighborhood (Overbeek et al. 1999; Zheng et al. 2002), and gene fusion (Snel et al. 2000; Yanai et al. 2001) to predict more significant evolutionary relationships. The phylogenetic profiles of all the genes encoding the currently known *E. coli* tRNA modification enzymes (Table 1) were analyzed in the context of 300 genomes (Kersey et al. 2005), and the gene clustering and gene fusion criteria were

analyzed using the STRING server (von Mering et al. 2007). The 15 top-scoring (STRING score ≥ 0.6), and previously incompletely characterized, *E. coli* open reading frames are shown in Table 2, and their domain architectures are summarized in Figure S1. All the ORFs share a genomic context with known tRNA modification enzymes and/or with components of the ribosome or other proteins involved in the translation process (Fig. 1). These findings support a tight coevolution between tRNA modification pathways and components of the translation machinery and suggest that, in addition to candidates for tRNA modification, uncharacterized proteins participating in other aspects of the translational process have also been unearthed in this search.

Among the candidate proteins, YifF and YibK were particularly interesting by reason of their SPOUT domain, which is indicative of enzymes catalyzing 2'-O-ribose methylation (Tkaczuk et al. 2007), and were thus selected for further investigation.

Mass spectrometric analyses of tRNAs

We analyzed bulk tRNA from *yifF* and *yibK* mutants using Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). This technique offers precise mass measurements (>99.98% accuracy) for RNA oligonucleotides in the trimer to 20-mer range (Douthwaite and Kirpekar 2007). Intact tRNAs are thus too large for direct analysis, but, fortuitously, the anticodon regions of the two isoacceptors tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA} yield unique 15-mer fragments after digestion with RNase T1 (Fig. 3C). In their fully modified state (Björk and Hagervall 2005), these fragments have *m/z* values of 4933.1 and 4974.1, respectively; under the analytical conditions applied here, these values correspond to the fragment masses in daltons plus a single proton. MALDI-MS can be expected to measure fragments in this mass range to within 0.5 Da, and thus loss of a single methyl group is readily detectable. Theoretical calculations of all the RNase T1 fragments obtained from bulk *E. coli* tRNAs (Dunin-Horkawicz et al. 2006) show that the masses of these and many other large oligonucleotides are unique and, furthermore, that they retain a distinctive mass even after the loss of a methyl group (Table 3).

The RNase T1 digestion products from bulk wild-type *E. coli* tRNAs were run over reverse phase columns to separate the smaller fragments (up to and including hexamers) from the larger ones. MALDI-MS analysis of the larger fragments (Fig. 2; Table 3) detected distinctive masses corresponding to the anticodon regions of tRNA^{Ser}_{CGA} (*m/z* 2403.8), tRNA^{Ser}_{UGA} (*m/z* 2403.8), tRNA^{Tyr}_{GUA} (*m/z* 2687.7), tRNA^{Phe}_{GAA} (*m/z* 3319.9), tRNA^{Trp}_{CCA} (*m/z* 3944.9), and tRNA^{Thr}_{CGU} (*m/z* 4100.5), as well as tRNA^{Leu}_{CmAA} (*m/z* 4933.1) and tRNA^{Leu}_{cmnm5UmAA} (*m/z* 4974.1). The last two peaks were relatively small, possibly reflecting that their parent molecules are minor components within the *E. coli* tRNA population (Horie et al. 1999). The corresponding

TABLE 1. tRNA-modifying enzymes and their nucleotide modifications

Nucleotide position	Modification	Enzyme	Former gene name(s)	Description of function in <i>E. coli</i>	Reference	SWISS-PROT Identifier
8	s ⁴ U	ThiI	<i>yajK</i>	Thiamine biosynthesis protein ThiI	Mueller et al. 1998	P77718
13	Ψ	TruD	<i>ygbO</i>	tRNA pseudouridine synthase D	Kaya and Ofengand 2003	Q57261
16, 17, 20, 20a	D	DusA	<i>yjbN</i>	tRNA-dihydrouridine synthase A	Bishop et al. 2002	P32695
16, 17, 20, 20a	D	DusB	<i>yhdG</i>	tRNA-dihydrouridine synthase B	Bishop et al. 2002	P0ABT5
16, 17, 20, 20a	D	DusC	<i>yohl</i>	tRNA-dihydrouridine synthase C	Bishop et al. 2002	P33371
18	Gm	TrmH	<i>spoU</i>	tRNA guanosine-2'-O-methyltransferase	Persson et al. 1997	P0AGJ2
32	s ² C	TtcA	<i>ydaO</i>	tRNA 2-thiocytidine biosynthesis protein	Jager et al. 2004	P76055
32	Cm/Um	TrmJ	<i>yfhQ</i>	tRNA (cytidine/uridine-2'-O-)-methyltransferase	Purta et al. 2006	P0AE01
32	Ψ	RluA	<i>yabO</i>	Ribosomal large subunit pseudouridine synthase A (dual rRNA/tRNA function)	Wrzesinski et al. 1995	P0AA37
34	s ² U	MnmA	<i>ycfB</i> , <i>trmU</i>	tRNA-specific 2-thiouridylase	Kambampati and Lauhon 2003	P25745
34	cmnm ⁵ U	MnmE	<i>thdF</i> , <i>trmE</i>	tRNA modification GTPase	Elseviers et al. 1984	P25522
34	cmnm ⁵ U	MnmG	<i>gidA</i>	tRNA uridine 5-carboxymethylaminomethyl modification enzyme	Bregeon et al. 2001; Yim et al. 2006	P0A6U3
34	mn ⁵ U	MnmC	<i>yfcK</i>	tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein	Hagervall and Bjork 1984; Bujnicki et al. 2004	P77182
34	Se ² U	SelU	<i>ybbB</i>	tRNA 2-selenouridine synthase	Wolfe et al. 2004	P33667
34	Se ² U	SelD	<i>fdhB</i>	Selenide, water dikinase	Leinfelder et al. 1990	P16456
34	Q	Tgt	<i>vacC</i>	Queuine tRNA-ribosyltransferase	Okada et al. 1979	P0A847
34	Q	QueA	<i>tsaA</i>	S-Adenosylmethionine: tRNA ribosyltransferase-isomerase	Slany et al. 1993	P0A7F9
34	Q	QueE	<i>ygcF</i>	7-Cyano-7-deazaguanosine (PreQ0) biosynthesis protein	Reader et al. 2004	P64554
34	Q	QueC	<i>yvaX</i>	Queuosine biosynthesis protein	Gaur and Varshney 2005	P77756
34	Q	QueF	<i>yqcD</i>	NADPH-dependent 7-cyano-7-deazaguanine reductase	Van Lanen et al. 2005	Q46920
34	k ² C	TilS	<i>yaeN</i>	tRNA ^{Ile} -lysine synthase	Soma et al. 2003	P52097
34	I	TadA	<i>yfhC</i>	tRNA-specific adenosine deaminase	Wolf et al. 2002	P68398
34	mo ⁵ U	CmoB	<i>yecP</i>	tRNA (mo5U34)-methyltransferase	Nasvall et al. 2004	P76291
34	mcmo ⁵ U	CmoA	<i>yecO</i>	tRNA (cmo5U34)-methyltransferase	Nasvall et al. 2004	P76290
34	ac ⁴ C	TmcA	<i>ypfI</i>	tRNA N4-acetylcytidine synthase	Ikeuchi et al. 2008	P76562
34	Cm/Um	TrmL	yibK	tRNA (cytidine/uridine-2'-O-)-methyltransferase	This study	P0AGJ7
37	i ⁶ A	MiaA	<i>trpX</i>	tRNA delta(2)-isopentenylpyrophosphate transferase	Caillet and Droogmans 1988	P16384
37	ms ² i ⁶ A	MiaB	<i>yleA</i>	(Dimethylallyl)adenosine tRNA methylthiotransferase	Esberg et al. 1999	P0AEI1
37	ms ² i ⁶ A	IscA	<i>yfhF</i>	Iron-sulfur cluster assembly protein	Leipuvienė et al. 2004	P0AAC8
37	m ¹ G	TrmD	<i>trmD</i>	tRNA (guanine-N(1)-)-methyltransferase	Bystrom and Bjork 1982	P0A873
37	t ⁶ A	RimN	<i>yrnC</i>	tRNA threonylcarbamoyladenosine synthase	El Yacoubi et al. 2009	P45748
37	m ⁶ A	TrmN6	<i>yfiC</i>	tRNA (adenine-N(6)-)-methyltransferase	Golovina et al. 2009	P31825
38, 39, 40	Ψ	TruA	<i>asuC</i> , <i>hisT</i>	tRNA pseudouridine synthase A	Kammen et al. 1988	P07649
46	m ⁷ G	TrmB	<i>yggH</i>	tRNA (guanine-N(7)-)-methyltransferase	De Bie et al. 2003	P0A815
54	m ⁵ U	TrmA	<i>rumT</i>	tRNA (uracil-5-)-methyltransferase	Ny and Bjork 1980	P23003
55	Ψ	TruB	<i>yhbA</i>	tRNA pseudouridine synthase B	Gutgsell et al. 2000	P60340
65	Ψ	TruC	<i>yqcB</i>	tRNA pseudouridine synthase C	Del Campo et al. 2001	P0AA41

analysis of tRNAs from the $\Delta yfiF$ strain produced the same range of masses as the wild type. However, the bulk tRNA from the $\Delta yibK$ exhibited a different MS spectrum at the 4900–5000 m/z interval where the anticodon regions of the tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA} isoacceptors were

shifted 14 Da downstream, respectively, to m/z 4919 and m/z 4960 (Fig. 3A), corresponding to the loss of a methyl group in the $\Delta yibK$ strain.

As the tRNA^{Leu} isoacceptors are modified at other sites in addition to the nucleotide 34 ribose (Table 3), it was

TABLE 2. Candidate genes potentially involved in tRNA modification

Identifier	Molecular function electronically inferred (database)	SWISS-PROT Identifier
YQCC_ECOLI	None	Q46919
YCFC_ECOLI	None	P25746
YCHF_ECOLI	GTP-dependent nucleic acid-binding protein engD (SWISS-PROT, Pfam)	P0ABU2
YAJC_ECOLI	Preprotein translocase subunit (Pfam)	P0ADZ7
HFLX_ECOLI	GTPase of unknown function (SWISS-PROT, Pfam)	P25519
YBEY_ECOLI	Putative metalloprotease (SWISS-PROT)	P0A898
YBEZ_ECOLI	PhoH-like predicted ATPase that is induced by phosphate starvation (SWISS-PROT, Pfam)	P0A9K3
YHBZ_ECOLI	GTP binding protein belong to OBG family (SWISS-PROT, Pfam)	P42641
YGGL_ECOLI	None	P38521
YHBC_ECOLI	None	P0A8A8
YRAL_ECOLI	Possible methyltransferase (Pfam)	P67087
ERA_ECOLI	GTPase of unknown function (SWISS-PROT)	P06616
RSGA_ECOLI	(ENG) GTPase that catalyzes rapid hydrolysis of GTP with a slow catalytic turnover (SWISS-PROT)	P39286
YfiF_ECOLI	Uncharacterized tRNA/rRNA methyltransferase yfiF	P0AGJ5
YibK_ECOLI	Uncharacterized tRNA/rRNA methyltransferase yibK	P0AGJ7

conceivable that the 14 Da had been lost from elsewhere in the fragment. To test whether this was the case, the bulk tRNAs were digested with RNase A to cleave after pyrimidines. In the wild-type strain, cleavage of tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA} with RNase A yields distinctive hexamer fragments of *m/z* 2074 and *m/z* 2162, respectively (Fig. 4A). These fragments still contain the position 34 pyrimidine because the 2'-O-methyl group on this nucleotide prevents RNase A cleavage (Burrell 1993); the same spectral pattern was observed for the $\Delta yfiF$ strain (data not shown). In the tRNA digestion products from the $\Delta yibK$ strain, however, the *m/z* 2074 and *m/z* 2162 fragments were missing and a more intensive top was observed at the mono-isotopic *m/z* of 1755 (Fig. 4B). These observations fit the pattern expected after loss of the 2'-O-methyl at position 34 followed by the removal of this nucleotide with RNase A to produce the smaller pentamer AAm^{2:6}iAAUp (Table 3).

Restoring 2'-O-methylation at U34 and C34

The RNase T1 digestion procedure described for tRNAs from wild-type and $\Delta yibK$ strains (Fig. 3A) was used to test the ability of a recombinant YibK protein to complement the *yibK*-null mutant

in vivo. The sequence encoding the full-length YibK protein with an N-terminal histidine tag was cloned into plasmid pET15b and was used to transform the *yibK*-null mutant. Expression of the recombinant 6His-YibK protein restored the mass of the T1 fragments from the anticodon region of tRNA^{Leu}_{cmnm5UmAA} and tRNA^{Leu}_{CmAA} to that of wild-type strains (Fig. 3B). Thus, it could be concluded that YibK promotes the 2'-O-methylation of U34 and C34. It is noted that this reaction proceeded efficiently in vivo even at very low expression levels where the amounts of recombinant YibK were too small to be detected by Western blotting with an anti-His antibody (data not shown).

Determinants for enzyme-tRNA^{Leu} recognition and catalysis of methyl addition

An in vitro assay was developed to determine the components that are required for specific recognition and 2'-O-methylation at U34 and C34 in the tRNA^{Leu} isoacceptors. The His-YibK recombinant was shown

FIGURE 1. (A) The global network of shared genomic context for tRNA-modification proteins. Genes are represented as spheres, which are colored according to their functional role. Lines linking the spheres represent instances of shared genomic context between the linked genes, including shared gene clustering, co-occurrence in genomes, and gene-fusion events. Strong and weak interactions are marked as red or blue links, respectively. (Orange spheres) Genes coding for tRNA modification enzymes used as baits; (white spheres) the chosen candidate genes. As can be observed, genes coding for tRNA modification enzymes and proteins involved in other translation processes form a densely connected network (i.e., they tend to share the same genomic contexts). (B) Details of YibK and YfiF subnetworks. Networks were projected graphically using Biolayout Express 3D (Freeman et al. 2007).

TABLE 3. RNase T1 and the relevant RNase A fragments from the *E. coli* bulk tRNA digestion

tRNA	Nucleotide positions	Sequence 5'–3'	Theoretical <i>m/z</i>	Empirical <i>m/z</i>
RNase T1 fragments				
Ser _{CGA/UGA}	36–42	A[ms ² i ⁶ A]AACCGp	2403.8	2404.1
Tyr _{GUA}	35–42	UA[ms ² i ⁶ A]AΨCUGp	2687.7	2687.3
Phe _{GAA}	35–44	AA[ms ² i ⁶ A]ΨCCCCGp	3319.9	3320.2
Trp _{CCA}	31–42	U[Cm]UCCA[ms ² i ⁶ A]AACCGp	3944.9	3945.3
Thr _{CGU}	58–70	ACUCCUAUUUAUCGP	4100.5	4100.3
Leu _{CAA}	31–45	AΨU[Cm]AA[ms ² i ⁶ A]AΨCAACCGp	4933.1	4933.5
Leu _{UAA}	31–45	AΨU[cmnm ⁵ Um]AA[ms ² i ⁶ A]AΨCCCUCGP	4974.1	4974.5
RNase A fragments				
Trp _{CCA}	36–40	A[ms ² i ⁶ A]AACp	1753.8	1754.2
Leu _{CAA}	34–39	[Cm]AA[ms ² i ⁶ A]AΨp	2073.7 / <u>1754.8</u>	2074.2 / <u>1755.2</u>
Ser _{CGA/UGA}	35–40	GA[ms ² i ⁶ A]AACp	2098.8	2099.2
Phe _{GAA}	34–39	GAA[ms ² i ⁶ A]AΨp	2099.8	2100.2
Leu _{UAA}	34–39	[cmnm ⁵ Um]AA[ms ² i ⁶ A]AΨp	2161.8 / <u>1754.8</u>	2162.1 / <u>1755.2</u>

to function in vivo, and its purification in vitro yielded a correctly folded protein in its native dimeric form (see Materials and Methods) that was shown by Surface Plasmon Resonance to bind its SAM cofactor with a K_d of 2.1 μ M. The substrate for the reactions was a chimera version of tRNA^{Leu}_{CAA} that essentially contains the complete tRNA^{Leu}_{CAA} structure fused at its 3'- and 5'-ends to the truncated anticodon stem of human cytosolic tRNA^{Lys}, producing an RNA of ~170 nt (Fig. 5A). Other fused constructs have been shown to be recognized by structure-specific enzymes inside *E. coli* (Ponchon and Dardel 2007), so it was reasonable to assume that the tRNA^{Leu}_{CAA} moiety in this chimera would contain the same modifications as the native tRNA^{Leu}_{CmAA}, except in the cases in which the enzymes for these had been inactivated.

The tRNA^{Leu}_{CAA} chimera was overexpressed and isolated from the $\Delta yibK$ strain for testing in the in vitro modification assay with recombinant YibK. Chimeric RNA substrates were then digested with nuclease P1 and alkaline phosphatase prior to nucleoside analysis by HPLC. This assay demonstrated the formation of Cm by purified recombinant YibK and showed that the reaction was dependent on the presence of SAM cofactor (Fig. 5B). To test whether the modification was located at the C34 wobble nucleotide of the tRNA^{Leu}_{CAA} chimera, we constructed a C34A mutant (tRNA^{Leu}_{AAA} chimera). No Cm was incorporated into the tRNA^{Leu}_{AAA} chimera (Fig. 6), indicating that the YibK-dependent formation of Cm in the parental chimera indeed occurs at position 34. Thus, despite being one of the smallest knotted proteins (~18 kDa) belonging to the SPOUT class of SAM-dependent methyltransferases (Lim et al. 2003; Watanabe et al. 2006; Tkaczuk et al. 2007), YibK modifies its wobble ribose target without the help of auxiliary proteins or other factors. We do note, however, that YibK has strict requirements con-

cerning the RNA sequence and the presence of other modifications on its tRNA substrate.

In this context, it should be mentioned that when we substituted the in vivo transcribed tRNA^{Leu}_{CAA} chimera in our assay system for a fully synthetic in vitro transcript of tRNA^{Leu}_{CAA}, absolutely no Cm was formed by YibK. This observation agrees with a previous study of YibK that failed to elicit methylation activity under similar conditions (Purta et al. 2006). Obviously, an in vitro transcript of tRNA^{Leu}_{CAA} would lack all the natural modifications present in in vivo transcripts, and one or more of these modifications could be essential for substrate recognition and modification by YibK. The key modification that guides YibK activity was revealed after isolating the tRNA^{Leu}_{CAA} chimera from a *miaA/yibK* double mutant strain of *E. coli*. MiaA is involved in formation of the ms²i⁶ modification at nucleotide A37 (Fig. 3C), where it catalyzes the addition of dimethylallyl diphosphate to the N6-exocyclic amino group forming i⁶A37 in a subset of tRNAs that includes tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm⁵UmAA} before formation of ms²i⁶ is completed

FIGURE 2. MALDI-MS spectra of RNase T1 oligonucleotides from bulk *E. coli* tRNA. The theoretical *m/z* values of fragments are shown in Table 3 and match well with the empirical values shown above the peaks.

FIGURE 3. (A) Expanded region of the RNase T1 MALDI-MS spectra. Fragments from tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmm5UAAA} with *m/z* values of 4933.5 and 4974.5 are seen in the wild-type and $\Delta yfiF$ samples, and the corresponding peaks are shifted to masses that are 14 Da smaller in the $\Delta yibK$ mutant. For all spectra, the 2′–3′-cyclic forms are apparent; these are 18 Da smaller and seen to the left of the linear phosphate forms, which are indicated with their *m/z* values. (B) In vivo complementation of BW25113 $\Delta yibK$ cells by recombinant 6His-YibK. (C) Secondary structures of tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmm5UAAA}. (Gray) Unique fragments resulting from T1 digestion.

by MiaB. Without ms²i⁶ at A37, YibK was rendered virtually incapable of modifying its own target nucleotide at C34 either in vitro (Fig. 6) or in vivo (Fig. S2).

Growth rate and growth competition

A slow-growth phenotype has previously been noted in *E. coli* *mnmE* and *mnmG* mutants that lack complete modification on the base of U34 in several tRNAs including tRNA^{Leu}_{cmm5UAAA} (Yim et al. 2006). Although it was feasible that lack of the ribose methylation at the same nucleotide might cause similar growth defects, no significant difference in the steady-state growth rate between the wild-type and the $\Delta yibK$ mutant was observed in rich medium at either 37°C (Table 4) or 42°C (17.1 ± 0.3 and 18.0 ± 0.7 min, respectively). Moreover, comparison of the growth rate of the *mnmG*::Tn10 strain with a *mnmG*::Tn10/

$\Delta yibK$ double mutant failed to reveal any significant difference (Table 4).

Additional growth rate comparisons were made between *miaA* and *miaA/yibK* strains. As described above, the MiaA modification at A37 is a prerequisite for YibK modification at C34, and the absence of any significant difference in the doubling times of the single *miaA* mutant (22.8 ± 0.1 min) and the double *miaA/yibK* mutant (24.2 ± 1.2 min) is fully consistent with this observation. Taken together, these results indicate that the 2′-O-methylation mediated by YibK does not play a crucial role for exponential growth in rich medium.

Direct growth rate comparisons have previously been shown to be inconclusive in cases in which measurement errors overshadow subtle growth differences. A more precise method is to grow cells in competition with each other over many generations; growth over several cycles also gives an indication of how well cells recover from the stationary-phase stress conditions. The $\Delta yibK$, $\Delta mnmE$, and $\Delta ttcA$ mutants, each of which carries a kanamycin resistance cassette, were grown in competition with the wild-type strain (lacking the resistance cassette). Expression of the kanamycin resistance gene can have a biological cost (Purta et al. 2008), although loss of *ttcA* has no additional cost (Jager et al. 2004). Approximately equal numbers of wild-type cells were mixed with $\Delta yibK$::*kan* cells, $\Delta mnmE$::*kan* cells, or $\Delta ttcA$::*kan* cells and were incubated

during several growth cycles in rich medium (Table 5). As expected, all cells with the kanamycin resistance cassette were eventually out-competed by the wild-type strain in the absence of kanamycin. However, the *yibK* and *mnmE* mutants clearly fared worse than the *ttcA* cells, indicating that loss of YibK (and MnmE) function has an additional biological cost.

In order to verify the phenotype associated with the YibK inactivation, we transferred mutations $\Delta ttcA$::*kan* and $\Delta yibK$::*kan* to strain IC4639, which has a genetic background different from BW25113. We found that the expression of the kanamycin resistance gene had a smaller biological cost in the IC4639 background, but, importantly, the $\Delta yibK$::*kan* mutation reduced the relative ratio of viable cells by ~ 10 -fold in comparison with the $\Delta ttcA$::*kan* mutation (Table 5). Therefore, we conclude that translation of specific mRNAs, probably related to the ability for

FIGURE 4. MALDI-MS spectra of RNase A oligonucleotides from bulk *E. coli* tRNA. Empirical m/z values of fragments are indicated above the peaks, and match well with the theoretical values (Table 3). (A) The m/z 2074.2 and 2162.1 peaks correspond to fragments from tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA}. Both fragments are missing in the $\Delta yibK$ strain. (B) Enlargement of the region containing the AAms²₁⁶AAcP fragment from tRNA^{Trp} at monoisotopic m/z of 1754.2 and the AAms²₁⁶AAUp fragments at monoisotopic m/z of 1755.2 that arise from RNase A digestion of the $\Delta yibK$ strain tRNA^{Leu} isoacceptors. Although the naturally occurring $^{12}\text{C}:^{13}\text{C}$ ratio ($\sim 99:1$) in all the samples makes it impossible to distinguish unambiguously between these two fragments, the proportionally higher peak in the $\Delta yibK$ sample at m/z 1755.2 is consistent with the presence of the AAms²₁⁶AAUp fragments.

recovering from stationary phase, is impaired by the loss of YibK-mediated modification. Interestingly, it has been reported that tRNA^{Leu}_{CmAA} expression is important for survival of *E. coli* cells in stationary phase (Newman et al. 1994).

YibK methylation and codon–anticodon interaction

Methylation of the 2'-hydroxyl group favors the C3'-endo ribose conformation for all nucleosides, although the effect is more marked with pyrimidines (Kawai et al. 1992). Such conformational rigidity of the modified pyrimidine nucleosides located at the tRNA anticodon may aid recognition of the correct codon. We studied the effect of the YibK-mediated modification on the codon–anticodon interaction using a *lambda* mutant (*limm21cI⁻ int6 red3 Oam29*) that requires an amber suppressor in order to replicate (Ogawa and Tomizawa 1968). The *E. coli* strain XA106 has a mutation in the anticodon of tRNA^{Leu}_{CmAA} with a change from CAA to CUA (mutation *leuX151* also known as *supP*), which facilitates amber suppression and thus supports replication of the mutant λ phage.

We followed the replication of wild-type and mutant λ phages in the *supP* strain and compared this with their replication in an isogenic *supP*/ $\Delta yibK$ strain. Inactivation of *yibK* reduced the burst size of the mutant λ phage by $\sim 35\% \pm 1\%$ but had no effect on the development of the wild-type phage. Complementation experiments showed that the burst size of the mutant λ phage in the *supP*/ $\Delta yibK$ strain expressing active recombinant YibK from a plasmid was similar to that seen in the *supP* mutant, and this contrasted with a 25% lower mutant λ burst size in the *supP*/ $\Delta yibK$

strain with an empty plasmid. These results suggest that YibK-mediated methylation supports the functional role of the suppressor tRNA in decoding the UAG amber stop codon.

The difference in mutant λ burst size, while being statistically significant, was not as large as might be expected, and this led us to question the extent to which YibK was capable of methylating the suppressor tRNA. Reading of amber codons by the suppressor tRNA is facilitated by its A35U mutation, which is adjacent to the YibK target at C34 and could thus conceivably affect methylation. This idea was tested by introducing the A35U mutation into the in vitro test system in the form of a tRNA^{Leu}_{CUA} chimera. As a consequence, YibK methylation fell to less than one-fifth of the level seen with the wild-type chimera (Fig. 6; Fig. S2), clearly indicating that nucleotide A35 functions as an identity element for recognition and methylation by YibK.

FIGURE 5. In vitro methylation by YibK of the tRNA^{Leu}_{CAA} chimera. (A) Expression and purification of the tRNA^{Leu}_{CAA} chimera. Bulk tRNA (first four lanes) and chimera tRNA^{Leu}_{CAA} purified from $\Delta yibK$ cells (fifth lane) were run on a 3% agarose gel. (B) HPLC analysis of the YibK activity with (left) or without (right) SAM on the chimera tRNA^{Leu}_{CAA} purified from $\Delta yibK$ cells. Absorbance was monitored at 270 nm. mAU, absorbance units $\times 10^{-3}$.

FIGURE 6. Identity determinants in tRNA^{Leu}_{CAA} for recognition by YibK. YibK activity in vitro on wild-type and mutant versions of the tRNA^{Leu}_{CAA} chimera was monitored by HPLC analysis. Chromatogram views at *top* (35–42 min) show the Cm production (percent of RNA molecules methylated by YibK) for wild-type and mutant versions of the tRNA^{Leu}_{CAA} chimera extracted from *yibK* or *miaA/yibK* strains. Chromatogram views at *bottom* (56–62 min) show the proportion (percent) of tRNA substrates modified with ms²i⁶A.

Extrapolating this result to the in vivo system, the proportion of suppressor tRNA molecules modified by YibK would be small but nonetheless sufficient to give a modest enhancement in the replication of the mutant λ phage. To sum up, the effect we observe here on phage replication is taken as an indication that 2'-O-methylation of the tRNA^{Leu} wobble nucleotide by YibK enhances cognate codon–anticodon recognition.

Concluding remarks

The bioinformatics approach used in this study pointed out *yibK* as a highly ranked gene in our search for the tRNA 2'-O-methyltransferase that modifies the wobble nucleotide in tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA}. Previous comparative genomics analyses also highlighted this gene (de Crecy-Lagard et al. 2007; Grosjean et al. 2010), although no experimental evidence was provided. YibK is a representative protein of the SPOUT family and has been widely used in biophysical and bioinformatics studies of knot formation (Mallam et al. 2008a,b; Sulkowska et al. 2009; Tuszyńska

and Bujnicki 2010). The presence of SPOUT proteins has been predicted in all proteomes (Tkaczuk et al. 2007), although only a few of these proteins have been characterized, and the functional role of YibK had previously remained elusive.

The present study demonstrates that the wobble position at nucleotide 34 in tRNA^{Leu}_{CmAA} and tRNA^{Leu}_{cmnm5UmAA} is 2'-O-methylated by YibK. YibK carries out this reaction in

TABLE 4. Growth rate of *yibK* mutants at 37°C

Strain ^a	Doubling time (min) ^b
Wild type	21.0 ± 1.0
$\Delta yibK$	21.4 ± 0.6
<i>mnmG::Tn10</i>	30.5 ± 1.5
<i>mnmG::Tn10/ΔyibK</i>	31.5 ± 2.5
<i>miaA148_{UAA}</i>	22.8 ± 0.1
<i>miaA148_{UAA}/ΔyibK</i>	24.2 ± 1.2

^aAll strains were derived from IC4639.

^bEach value is the mean ± SEM of three separate experiments.

an independent manner, without the direct participation of any other protein, and furthermore is discriminating in its choice of substrate. YibK is selective for the two tRNA^{Leu} isoacceptors and only methylates these when they present the correct anticodon loop sequence and modification pattern. Specifically, YibK requires a pyrimidine nucleoside at position 34, it has a clear preference for an adenosine at position 35, and it fails to methylate without prior addition of the ms²i⁶A modification at position 37. This latter observation further indicates that 2'-O-methylation by YibK occurs as a late step in the maturation of the tRNA^{Leu} isoacceptors. The selection against *yibK*-null mutants in competition with wild-type cells, as well as the reduction in suppressor activity upon inactivation of *yibK*, point to a role for YibK in fine-tuning the codon–anticodon recognition process. YibK is one of the few remaining *E. coli* tRNA modification enzymes that awaited identification, and a comprehensive overview of these enzymes is presented in Table 1. We propose that the YibK enzyme now be renamed as the tRNA methyltransferase L (“TrmL”).

MATERIALS AND METHODS

Comparative genomics—bioinformatics predictions

Sequence data

The list of known tRNA modification enzymes (Table 1) was compiled from the literature (Bujnicki et al. 2004; Björk and Hagervall 2005; Purta et al. 2006; Ikeuchi et al. 2008; El Yacoubi et al. 2009; Golovina et al. 2009). Proteins encoded in completely sequenced bacterial genomes were downloaded from the Integr8 database at EBI (Kersey et al. 2005).

Generation of phylogenetic profiles

Smith-Waterman searches were run using sequences from known tRNA modification enzymes as a query against the abovementioned database of completed bacterial proteomes. A particular

gene was considered to be present in a given species when it produced a hit with an *e*-value <10^{−3} aligned over 50% of the query sequence. Phylogenetic profiles were represented as matrices of 0's and 1's, indicating presences or absences, respectively. Distances between profiles were computed using the Hamming Distance, as indicated in Gabaldon (2008).

Analysis of gene fusion and chromosomal neighborhood

Analysis of gene neighborhood and search for gene fusion events in other genomes were carried out in the STRING web server (von Mering et al. 2007). The confidence score threshold was fitted to ≥0.600 in order to obtain more reliable predictions of protein interactions.

Bacterial strains

All knockout mutants of the candidate genes identified by comparative genomics, as well as the *mnmE* mutant, were obtained from the Keio collection (Baba et al. 2006). The *mnmG* mutant carrying a Tn10 insertion was kindly donated by D. Brégeon (Brégeon et al. 2001). The *miaA* mutant (containing the mutation *miaA148*_{UAA}) was donated by G.R. Björk (Landick et al. 1990). P1 transduction (Miller 1990) was used to introduce the desired null allele into strain IC4639 (Yim et al. 2006), a wild-type derivative from strain Dev16 (Elseviers et al. 1984), IC5550, an *mnmG*::Tn10 derivative of IC4639 (Yim et al. 2006), and BW25113. Correct insertion of mutations was checked by PCR using primers upstream-flanking the replaced gene and internal primers for the *kan^r* gene (Datsenko and Wanner 2000) or mini Tn10 element. The XA106 strain carrying the *supP* amber suppressor was obtained from the *E. coli* Genetic Stock Center. The *supP*/Δ*yibK* double mutant was constructed by P1 transduction of the *yibK* region from BW25113Δ*yibK* to strain XA106. Correct insertion of the *yibK* mutation into the XA106 background was checked as above.

Growth and competition experiments

The doubling time of exponential-phase cultures was measured by monitoring the optical density of the culture at 600 nm. Samples were taken from exponentially growing cultures after at least 10 generations of steady-state growth. Growth rate was calculated as doubling time of each strain culture at steady-state log phase by linear regression. Competition experiments were carried out as previously reported (Gutgsell et al. 2000). Briefly, wild type and mutants were grown separately to stationary phase by incubation at 37°C. Equal volumes of wild-type and individual mutant cultures were mixed, and a sample was immediately taken to count viable cells on LB plates with and without the antibiotic required to estimate the mutant cell content. Six cycles of 24-h growth at 37°C were performed by diluting mixed cultures 1/1000 on LB media; each cycle corresponding to 10 or 11 cell divisions. After the sixth cycle, the mixed culture was analyzed for its wild-type:mutant cell content as before.

TABLE 5. Effect of *yibK* mutation on growth competition

Wild type and mutant mixed 1:1 ^a	CFU/mL at mix time ^b		CFU/mL after six dilutions ^b	
	LB	LB + kan (ratio)	LB	LB + kan (ratio)
BW25113				
Δ <i>ttcA</i>	2.4 × 10 ⁸	1.1 × 10 ⁸ (0.46)	2.1 × 10 ⁸	9.4 × 10 ⁵ (0.004)
Δ <i>mnmE</i>	2.3 × 10 ⁸	1.1 × 10 ⁸ (0.48)	1.6 × 10 ⁸	3.9 × 10 ⁴ (0.0003)
Δ <i>yibK</i>	1.9 × 10 ⁸	1.0 × 10 ⁸ (0.53)	1.5 × 10 ⁸	1.8 × 10 ⁴ (0.0002)
IC4639				
Δ <i>ttcA</i>	1.1 × 10 ⁸	4.9 × 10 ⁷ (0.45)	1.3 × 10 ⁸	3.3 × 10 ⁷ (0.26)
Δ <i>yibK</i>	1.4 × 10 ⁸	6.5 × 10 ⁷ (0.46)	1.3 × 10 ⁸	4.6 × 10 ⁶ (0.03)

^aGenetic background of strains is indicated in bold.

^b(CFU) Colony forming units. Data are presented as means of three independent replicates. The ratio of CFU per milliliter recovered on LB + kan (kanamycin) versus LB is indicated in parentheses.

Phage burst size determination

A standard procedure for determination of phage burst size (number of phage progeny produced per infected bacterial cell) was used for wild-type λ and for the mutant λ imm21cI int6 red3 Oam29. In brief, bacteria were grown in LB media to $\sim 2 \times 10^8$ cells/mL, harvested by centrifugation and resuspended in 10 mM MgSO₄ to one-third of the initial volume. Cells were infected at a multiplicity of 0.05 phage/cell and incubated for 20 min to allow adsorption of the phage. After separating an aliquot for determination of infected cells (IC), samples were diluted 1/50 in pre-warmed LB and incubated with vigorous shaking. Aliquots were taken at 10, 30, 40, and 60 (F) min; chloroform was added, and, after dilution, free phages were plated on the indicator strain (XA106). Infected cells were determined immediately after the aliquot was withdrawn by plating appropriated dilutions on the indicator strain. All experiments were carried out at 37°C. The burst size was calculated as $b = F \times 50/IC$. The number of free phages was similar at 40 min and 60 min, indicating that a plateau had been reached.

Isolation of bulk tRNA and analysis of modification status by HPLC

Bacterial strains were grown overnight in LB media and then were diluted 100-fold in 100 mL of LB media and grown to 0.7 to 0.8 units at OD₆₀₀. Cells were harvested by centrifugation and resuspended in 0.4 mL of buffer A (25 mM Tris at pH 7.4, 60 mM KCl, 10 mM MgCl₂). Lysozyme (2 mg; Sigma) was added, and the suspension was incubated during 15 to 20 min at 37°C. The cell suspension was lysed by three freeze-thaw cycles using liquid nitrogen; then 0.6 mL of buffer B (buffer A supplied with 0.6% Brij35, 0.2% Na-deoxycholate, 0.02% SDS) and 0.1 mL of phenol (equilibrated to pH 4.3 with citrate) were added and mixed. The suspension was incubated for 15 min on ice, and the aqueous phase was extracted twice with 1 vol of phenol. RNA was precipitated with 2.5 vol of cold ethanol containing 1% (w/v) potassium acetate. The pellet was washed with 70% ethanol and was dissolved in 2 mL of buffer R200 (100 mM Tris-H₃PO₄ at pH 6.3, 15% ethanol, 200 mM KCl) prior to running over a Nucleobond AX500 column (Macherey-Nagel), pre-equilibrated with 10 mL of the same buffer. The column was washed once with 6 mL of R200 and once with 2 mL of R650 (R200 with 650 mM KCl). tRNA was eluted with 6 mL of R650 buffer and was then precipitated with 0.7 vol of isopropanol, washed with 70% ethanol, and redissolved in water.

For HPLC separation, 50 μ g of the tRNA mixture was hydrolyzed with nuclease P1 (Sigma) by overnight incubation in water with 1 mM ZnSO₄ followed by treatment with *E. coli* alkaline phosphatase (Sigma) at pH 8.3 for 2 h. The hydrolysate was analyzed by HPLC using a Develosil 5 μ RP-AQUEOUS C-30 column (Phenomenex) with gradient elution to obtain optimal separations of nucleosides. Buffer A contained 2.5% methanol and 10 mM NH₄H₂PO₄ (pH 5.1), while buffer B contained 25% methanol and 10 mM NH₄H₂PO₄ (pH 5.3). The time for gradient elution was extended during 100 min. All the HPLC-nucleoside mutant profiles were compared with those derived from wild type. Approximately 16 predominant and well-known (by UV spectra according to Gehrke and Kuo 1989) tRNA modifications seen in the wild-type strain were evaluated to be absent in mutants of candidate genes at 254-nm wavelength.

RNA mass spectrometry

Bulk tRNA from wild-type, $\Delta yifF$, and $\Delta yibK$ was isolated as above, and 1600 pmol was incubated overnight with 80 mM 3-hydroxypicolinic acid and 0.5 units of RNase T1 (USB) or 3 μ g of RNase A at 37°C. Digested tRNA was mixed 2:1 with 1 M triethylammonium acetate (TEAA) and loaded onto a microcolumn for reverse-phase-type chromatography on a GELoadertip containing Poros R3 matrix (Applied Biosystems) and pre-equilibrated with 10 mM TEAA. After washing twice with 10 mM TEAA and once with 10% acetonitrile, 10 mM TEAA solution, larger fragments were eluted with a 25% acetonitrile, 10 mM TEAA solution. Samples were dried and dissolved in 4 μ L of H₂O prior to analysis on a MicroMass MALDI-Q-TOF Ultima Mass Spectrometer or 4700 Proteomics Analyzer (Applied Biosystems) recording in positive ion mode (Kirpekar et al. 2000; Douthwaite and Kirpekar 2007).

In vivo complementation

The *yibK* open reading frame from *E. coli* was amplified using the following oligonucleotides: 5'-CGCCCCATGGGTTCATCATCACCA TCACCATATGCTAAACATCGTACTTTACGAACCAGAAATTCCG and 3'-GCCGGATCCCTAATCTCTCAATACCGCTCCCGG encoding NcoI and BamHI restriction sites, respectively (bold) and the N-terminal histidine tag (*italics*). The PCR product was digested and inserted into an NcoI/BamHI-linearized pET15b plasmid by incubation with T4 ligase overnight at 16°C. The pET15b-His-*yibK* construct was used to transform the BW25113 $\Delta yibK$ strain; empty pET15b plasmids were used to transform BW25113 wild-type and BW25113 $\Delta yibK$ cells as controls. Bulk tRNA isolation from these plasmid-bearing strains and mass spectrometry analysis were carried out as above. To study the effect of a recombinant YibK protein on replication of wild-type λ phage and λ imm21cI⁻ int6 red3 Oam29, strain *supP*/ $\Delta yibK$ was independently transformed by pET15b and pET15b-his-*yibK*, whereas its parental strain XA106 (*supP*) was transformed by pET15b. Phage burst size was determined as above.

Determining YibK activity in vitro and in vivo

For in vitro transcription, the *E. coli* gene encoding tRNA^{Leu}_{CAA} was PCR-amplified from genomic DNA using primers 5'-GATA GAATTCaattaatcagactactatagGCCGAAGTGGCGAAATCG (EcoRI site in bold and T7 promoter sequence in lowercase) and 5'-GATAGGATCCTGGTGCCGAAGGCCGACTC (BamHI site in bold) and cloned into pUC19 EcoRI/BamHI-linearized plasmid. The resulting plasmid was named pIC1581. Unmodified tRNA^{Leu}_{CAA} was prepared by in vitro transcription of BamHI-digested pIC1581 using the Riboprobe T7-transcription kit (PROMEGA) as previously described (Moukadiri et al. 2009). Recombinant His-YibK protein was purified by affinity chromatography followed by a gel filtration purification step (Superdex 75; GE Healthcare Life Sciences), where YibK eluted as a dimer of 36 kDa. To assay the YibK methyltransferase activity in vitro, the reaction mix contained 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2.5–5.0 mM KCl, 2.5–5.0 mM MgCl₂, 0.1–0.6 mM SAM, 4 μ g of in vitro-transcribed tRNA^{Leu}_{CAA}, and 5–10 μ M His-YibK. After 2 h at 37°C, tRNA was hydrolyzed, and nucleoside separation was achieved by HPLC. The possible synthesis of the nucleoside Cm

in vitro by YibK was monitored by HPLC using commercial 2'-O-methylcytidine (Sigma) as a standard.

For in vivo transcription of chimeric tRNA, the gene for tRNA^{Leu}_{CAA} was cloned in the pBSKrn plasmid (Ponchon et al. 2009) using primers 5'-GATAGATATCGCCGAAGTGGCGAAATCG and 5'-GATAGATATCTGGTGCCGAAGGCCGGACTC (EcoRV restriction sites in bold). The PCR product was digested and inserted in an EcoRV-linearized pBSKrn plasmid by incubation with T4 ligase overnight at 16°C. Chimera tRNA^{Leu}_{CAA} derivatives (tRNA^{Leu}_{AAA} and tRNA^{Leu}_{CUA}) were constructed by site-directed mutagenesis using appropriate primers. The pBSKrn constructs were used independently to transform the wild-type, $\Delta yibK$, and $miaA/\Delta yibK$ strains and chimera tRNAs were overproduced in these cells as previously described (Ponchon et al. 2009). Bulk tRNA was isolated as described above. The chimera tRNA^{Leu}_{CAA} was purified by the Chaplet Column Chromatography method (Suzuki and Suzuki 2007) with the DNA probe ^{biotin}5'-TGGCGCCGAACAGGGACTTGAACCC, complementary to the scaffold human cytosolic tRNA^{Lys} moiety of the chimera tRNA^{Leu}_{CAA}, and immobilized in a HiTrap Strep-tavidin HP column (GE Healthcare). The in vitro modification reaction contained 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5.0 mM KCl, 5.0 mM MgCl₂, 1.0 mM SAM, 5 μ M purified His-YibK, and 7 μ g of specific tRNA chimera. After 2 h at 37°C with gently shaking, the reaction was stopped with 1 vol of phenol (pH 4.3) followed by centrifugation at 16,000g during 10 min. tRNA was recovered from the aqueous phase by ethanol precipitation, followed by hydrolysis and nucleoside separation by HPLC as described above. The Cm nucleoside was monitored using commercial 2'-O-methylcytidine (Sigma) as a standard. The nucleoside area was compared and measured at maximum absorption wavelength for cytidine-derived nucleosides, 270 nm, with EZchrom Elite software. The area of the m⁷G nucleoside (monitored using commercial 7-methylguanine; Sigma) present in the scaffold human cytosolic tRNA^{Lys} (Ponchon and Dardel 2007), but absent in tRNA^{Leu}_{CmAA} (Horie et al. 1999), was used as a reference to normalize the relative accumulation of Cm and ms²i⁶A nucleosides. The in vivo modification status of purified chimera tRNA^{Leu}_{CAA} and mutant derivatives obtained from wild-type, $yibK$, and $miaA$ strains was analyzed by HPLC, as above, using 15 μ g of tRNA for each digestion reaction with nuclease P1.

S-Adenosyl-L-methionine (SAM) binding assay

Recombinant His-YibK protein was purified by affinity chromatography. Binding of SAM was determined through Surface Plasmon Resonance (Biacore T100; GE Healthcare) by linking monoclonal anti-His immunoglobulins to CM5 chip using the Amine Coupling Kit (Biacore; GE Healthcare). TBS buffer (50 mM Tris-HCl, 200 mM NaCl at pH 7.5) was used as the mobile phase. Approximately 10 μ g of protein was immobilized for 60 sec with flux at 10 μ L/min. Concentrations ranging from 100 nM to 10 μ M of SAM (Sigma) were tested to obtain the affinity of His-YibK for SAM. The contact time for SAM was 40 sec at the same flux as before; SAM affinity was calculated using the Biacore T100 Evaluation Software, V2.0 (Biacore; GE Healthcare).

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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