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YhiQ is RsmJ, the methyltransferase responsible for methylation of G1516 in 16S rRNA of *E. coli*

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Abstract

Ten methyltransferases and one pseudouridine synthase are required for complete modification of the small ribosomal subunit in *E. coli*. Nine methyltransferases, as well as the pseudouridine synthase, are already known. Here we identify RsmJ, the last unknown methyltransferase required for methylation of m²G1516 in 16S rRNA, as the protein encoded by *yhiQ*. Reverse transcription primer extension analysis reveals that rRNA extracted from a *yhiQ* deletion strain is not methylated at G1516. Moreover, methylation is restored upon gene complementation. Also, purified recombinant YhiQ specifically methylates 30S subunits extracted from the deletion strain. The absence of the *yhiQ* gene leads to a cold sensitive phenotype. Based on these data we propose that the *yhiQ* gene be renamed *rsmJ*.

Keywords

E. coli; methyltransferase; RNA modification; small ribosomal subunit

One of the most functionally complex molecules in living organisms is RNA, which consists of a long chain of canonical nucleotides of adenine, cytosine, guanine and uracil. However, the set of four does not account for the complexity of RNAs, which is increased by the presence of modified nucleotides. RNA modification involves chemical alterations of bases and/or ribose moieties. Currently, more than 120 different types of base modifications are known, most of which are present in stable cellular RNAs such as tRNAs, rRNAs and small nuclear and nucleolar RNAs (snRNAs, and snoRNAs).^{1,2} Of the stable RNA molecules, ribosomal RNA, the most abundant in any cell, contains three main types of nucleotide modification: pseudouridines (Ψ), 2'-hydroxyl ribose methylation (Nm), and base methylation (mN).³

In bacteria, the number of base methylated nucleotides generally exceeds those of ribose methylations and pseudouridines.^{4,5,6,7} Regardless of their type and number, modified

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nucleotides are distributed in important functional, highly conserved regions of the rRNA in all organisms, such as the peptidyl-transferase center, inter-subunit bridges, tRNA binding sites, the decoding region, and the mRNA binding area.^{3,8,9,10}

Information regarding the chemical identities and sequence location of modified nucleotides in prokaryotes is limited. Most of the data come from studies of *E. coli*,^{2,11} *H. marismortui*,¹² *D. radiodurans*,^{2,12} and *T. thermophilus*^{6,7}. From the available data, it is clear that bacterial rRNA contains fewer modified nucleotides than are present in eukaryotes, and most methyl groups are attached to bases, rather than to ribose. Also, the modification process itself is performed by site-specific enzymes,¹¹ and there is no evidence of guide RNA involvement, as is the case in eukaryotes.

E. coli ribosomal RNA contains 36 modified nucleotides that include 11 pseudouridines, 24 methylated residues, one dihydrouridine² and one recently identified hydroxycytidine¹¹ (Table 1.). Twenty-eight modifying enzymes, responsible for modification at 31 residues have been identified so far (Table 1): 7 pseudouridine synthases and 21 methyltransferases. Each enzyme is site specific, with three exceptions: two pseudouridine synthases, RluC and RluD, which modify three different sites in rRNA, and RsmA(KsgA), a methyltransferase, which double-methylates two adjacent m⁶2A residues. Also, each residue is modified by just one enzyme, except for the double- methylated C1402 in 16S rRNA and the methylated pseudouridine at position 1915 in 23S rRNA.

In this study, we show that the protein encoded by *rsmJ*, previously named *yhiQ*, is responsible for methylation at G1516 in 16S rRNA. The gene was identified by analysis of putative methyltransferase deletion strains and was confirmed by gene complementation, cloning and overexpression, and *in vitro* characterization of the purified recombinant protein. By identifying *yhiQ* as the gene coding for RsmJ, we have identified the last unknown enzyme responsible for 16S rRNA modification in *E. coli*. Only two methyltransferases, one dihydrouridine synthase and one hydroxylase responsible for modification of A2030, G2069, U2449 and C2501, all in 23S rRNA, still remain to be found (Table 1).

Deletion of *yhiQ* gene is associated with lack of methylation at G1516 in 16S rRNA

Seven open reading frames *yhiQ*, *smtA*, *yafS*, *yfiF*, *yafE*, *yfmD* and *yjtD*, predicted to be S-adenosyl-L-methionine dependent methyltransferases, were considered as candidates for the enzyme catalyzing m²G methylation at nucleotide 1516 in 16S rRNA of *E. coli*. To test this prediction, total RNA was extracted from *E. coli* deletion strains lacking one of these genes, constructed as described in “Materials and Methods” each, and analyzed.¹³ However, reverse transcriptase primer extension analysis¹⁴ with a primer specific to nucleotides 1523–1542 in 16S rRNA, initially revealed no conclusive difference in rRNA modification at its 3' end between wild type and these deletion strains. Strong stops in the RT extension reaction were seen due to methylation at the RsmA specific sites¹⁵ A1518 and A1519 in all strains, including the isogenic wild type. A much weaker stop was also observed at site 1517 as a result of G1516 methylation in all strains, and was barely detectable in the *yhiQ* deletion

(data not shown). Based on this initial data, we reasoned that the weak stop at 1517 might be caused by the inability of the RT to bypass the highly methylated bases A1518 and A1519 (Fig. 1), making it difficult to determine whether the absence of a stop in the *yhiQ* deletion was real. In order to allow extension past nucleotides 1518 and 1519, and to allow better analysis of the methylation state of G1516, we reexamined RNA isolated from a strain lacking both RsmA and YhiQ using the same RT-primer extension method. As shown in Figure 2, a strong stop was now revealed at nucleotide 1517 in the absence of RsmA (lane 1), while no stop in the extension reaction was obtained in the double mutant (lane 2), indicating that rRNA in this strain lacks the methyl group at position G1516.

Methyl group at G1516 is recovered by gene complementation

To confirm that *yhiQ* is the gene responsible for methylation at G1516, we performed gene complementation. Plasmids pCA24N-*lacZ* and pCA24N-*yhiQ* encoding His-tagged proteins under the control of an IPTG-inducible promoter were transformed into the double mutant strain.¹⁶ Cells were grown to exponential phase in either the presence or absence of IPTG, and tested for G1516 methylation. Figure 2 shows that G1516 is methylated when the mutant strain is transformed with the plasmid containing *yhiQ* (lanes 4 and 5), but there is no stop at G1517 with RNA extracted from cells transformed with the control plasmid (lane 3). These data indicate that *yhiQ* is required for methylation at G1516 in 16S rRNA. Methylation at G1516 in cells harboring pCA24N-*yhiQ* was recovered even in the absence of IPTG (lane 4), and this is due to the leakiness of the inducible *lac* promoter. Apparently, only a low level of YhiQ expression is sufficient for complementation as its overexpression (lane 5) does not intensify the stop at G1517.

Purified protein encoded by *yhiQ* has m²G1516 specific methyltransferase activity

To examine the activity of the *yhiQ* gene product *in vitro*, the gene was subcloned into pET28a such that an N-terminal His-tag fusion protein could be overexpressed. Induction of protein expression by IPTG led to a protein product of ~29 kDa, as expected from the DNA sequence. The protein was purified to homogeneity by metal affinity, ion exchange and size exclusion chromatography as described in “Materials and Methods” (Fig. 3), and was assayed for methyltransferase activity. The enzyme was able to incorporate [³H]-methyl groups from [³H]-SAM into 30S particles purified from the *yhiQ* deletion strain, but not into 30S particles extracted from wild type cells (Fig. 4). No more than 0.4 pmol methyl group was incorporated per pmol of mutant 30S subunit, even at high enzyme concentrations, suggesting that the modification is site specific and that not all the substrate 30S particles may be active. Additionally, we do not exclude the possibility that some endogenous SAM might be present in the enzyme preparation, reducing the specific activity of the [³H]-SAM and the apparent level of methyl group incorporation.

No methyl incorporation was detected when free 16S rRNA was used as substrate, suggesting either an altered 16S rRNA structure that requires the presence of ribosomal proteins or that one or more ribosomal proteins are directly required for the methylation reaction to proceed. These findings are in agreement with the suggestion of Siibak and

Remme that modification at this site takes place during a late intermediate or late step during ribosome assembly.¹⁷

YhiQ belongs to the DUF548/UPF0341 subfamily of proteins of unknown function (pfam04445; E=2e-145), conferring a specific founder enzymatic function for this subfamily. It is also predicted to belong to a superfamily of SAM-dependent methyltransferases (cd02440; E=1e-04).¹⁸ Although there are no significant paralogs of YhiQ found using blastp, there are 13 proteins in *E. coli* K-12, including YhiQ, that match the COG0500 family profile.¹⁹ Only one other COG0500 protein is known to be an rRNA methyltransferase (RlmA), but two are tRNA methyltransferases (CmoAB). The only other enzymatically characterized COG0500 member is BioC, a small molecule (malonyl-CoA) methyltransferase, identifying COG0500 as a family of diverse methyltransferases. Crystal structures of YhiQ proteins from *E. coli* (PDB entry 2PGX), *S. flexneri* (PDB entry 2OYR), *S. typhimurium* (PDB entry 2PKW), and *N. gonorrhoeae* (PDB entry 2R6Z) have been solved^{20,21,22,23}. The structures reveal monomeric proteins except the *N. gonorrhoeae* protein that crystallized as a homodimer. The structure of YhiQ from *S. flexneri* was determined as a complex with S-adenosyl-L-homocysteine confirming its SAM binding ability.²³

Effect of *yhiQ* deletion on cell physiology

yhiQ and *prlC*(*opdA*) form a two gene, RpoH-regulated, heat-shock operon in *Salmonella typhimurium* and *E. coli*.^{24,25} The *dtbB*(*yhiP*) gene is downstream of, and convergent upon the *prlC*-*yhiQ* operon, separated by 48 bp of intergenic DNA containing a predicted bidirectional terminator ($-G = 17.7$ kcal/mole) in *E. coli* and a pair of REP sequences in *Salmonella*.

Deletion of the *yhiQ* gene leads to the absence of both the gene product and the methyl group at G1516. To determine if there is any physiological effect caused by this loss, we examined growth rates of wild type, *yhiQ*::kan mutant and *yhiQ*::kan *rsmA* double mutant strains. No significant differences in cell growth were observed when cells were grown in either LB or M9 glucose media at 22°C, 30°C or 37°C (data not shown) similarly to *Salmonella yhiQ* mutants.²⁴ We also found no competitive growth disadvantage of an *yhiQ* mutant at 30°C and 37°C in either an *rsmA*+ or an *rsmA*::kan background (data not shown). However, a markedly cold sensitivity phenotype at 16°C was noticed for *yhiQ* and *rsmA* deletion strains on plates (data not shown), in agreement with a recent high-throughput phenotyping study that found the growth rate of an *E. coli yhiQ* mutant to be significantly slower than an isogenic *yhiQ*+ strain only at 16°C, 18°C, and 20°C.²⁶

We determined the growth rate of the *yhiQ*::kan mutant at 16°C and showed that it is 88% of the growth rate of an isogenic *yhiQ*+ strain. Despite the growth defect, we observed no change in the ribosome profile of mutant cells grown at 16°C (data not shown). A normal growth phenotype was restored by gene complementation using mutant *yhiQ*::kan cells transformed with pCA24N-*yhiQ*(GFP-).

E. coli becomes resistant to kasugamycin when methyl groups at A1518 and A1519 are missing due to a mutation in *rsmA*, formerly known as *ksgA*.^{27,28} Given the proximity of G1516, we wanted to assess whether modification of this site affects kasugamycin

sensitivity or resistance. Thus, we measured growth rates of wild type, *yhiQ*, *rsmA* and double mutant *rsmA yhiQ* strains at 37°C in LB containing various concentrations of kasugamycin in 96-well microtiter plates. The MIC (minimum inhibitory concentration) of kasugamycin for wild type or *yhiQ* cells was 2 µg/ml, while *rsmA* cells and the double mutant cells were both resistant to kasugamycin up to 100 µg/ml at which point growth is slightly inhibited. These data suggest that lack of the methyl group at residue G1516 does not contribute to the antibiotic resistance phenotype conferred by *rsmA*.

CONCLUSIONS

Taken together, the data presented in this study demonstrate that *yhiQ*, a member of the DUF548/UPF0341 subfamily of proteins of unknown function, encodes the methyltransferase specific for m²G1516 in 16S rRNA, thus identifying a founder enzymatic function for this subfamily. We propose that the m²G1516 16S rRNA methyltransferase be renamed RsmJ, as previously suggested.^{11,36} A strain lacking RsmJ due to deletion of the *rsmJ(yhiQ)* gene is missing the methyl group at G1516 and exhibits a cold sensitive phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Secondary structure of the small ribosomal subunit. Enlarged insert diagram depicts the structure of the 3' minor domain (helices 44 and 45) and their methylated sites. Residues modified by RsmA, as well as nucleotide G1516 are indicated by arrows. The primer used for reverse transcription reaction spans nucleotides 1523–1542.

Figure 2.

Reverse transcriptase primer extension analysis of RNA. Total RNA was annealed to a [³²P]-labeled primer complementary to residues 1523–1542 in 16S rRNA prior to extension by AMV-RT according to the manufacturer's protocol. Primer extension products were precipitated with ethanol, dissolved in DNA loading buffer containing 89% formamide, 4% TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA), 0.12% bromophenol blue and 0.17% xylene cyanol, separated on 8% polyacrylamide/7M urea sequencing gels and visualized by exposure to a PhosphorImager screen (Molecular Dynamics). The arrow indicates position G1517 at which the reverse transcriptase stops when G1516 is methylated.

Figure 3.

SDS-PAGE gel analysis of recombinant methyltransferase. Protein was overexpressed in BL21(DE3) cells transformed with pET28a-yhiQ containing the *yhiQ* gene insert fused with an N-terminal His-tag coding region. Purification was performed as described in “Supplemental material”. The gel was overloaded with 9 μ g of purified His-YhiQ (Lane 2) next to molecular weight standards (Lane 1).

Figure 4.

In vitro methylation of wild type and mutant 30S particles. Reaction mixtures (100 μ l) contained 20 mM HEPES (pH 7), 100 mM NH_4Cl , 5 mM DTT, 200 μ M SAM (S-adenosine-l-methionine, Sigma), 0.5 μ Ci [^3H]-SAM (Amersham Pharmacia Biotech), 2 mM $\text{Mg}(\text{OAc})_2$, 100 pmole 30S subunits or 16S rRNA, and 2.7 μ g His-YhiQ. Reaction mixtures were incubated for 20 min at 37°C and the reaction terminated by vortexing in an equal volume of phenol. Seventy μ l of the aqueous layer was removed after centrifugation and precipitated at room temperature using pellet paint (EMD chemicals), as indicated by the manufacturer. RNA pellets were resuspended in water, and radioactivity was determined by scintillation counting.

Table 1Modified nucleotides of *E. coli* 16S rRNA and their modifying enzymes.

Nucleotide	Modification	Gene	Synonym	Reference
516	Ψ	RsuA	YejD	29
527	m ⁷ G	RsmG	GidB	30
966	m ² G	RsmD	YhhF	31
967	m ⁵ C	RsmB	Fmu, YhdB	32,33
1207	m ² G	RsmC	YjjT	34
1402	m ⁴ Cm	RsmH, RsmI	YabC, YraL	38
1407	m ⁵ C	RsmF	YebU	36
1498	m ³ U	RsmE	YggJ	14
1516	m ² G	RsmJ	YhiQ	This study
1518	m ⁶ ₂ A	RsmA	KsgA	15,28
1519	m ⁶ ₂ A	RsmA	KsgA	15,28