AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Identification and Characterization of Genes Required for 5-Hydroxyuridine Synthesis in *Bacillus subtilis* and *Escherichia coli* tRNA

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ABSTRACT In bacteria, tRNAs that decode 4-fold degenerate family codons and have uridine at position 34 of the anticodon are typically modified with either 5-methoxyuridine (mo^{5}U) or 5-methoxycarbonylmethoxyuridine (mcmo^{5}U). These modifications are critical for extended recognition of some codons at the wobble position. Whereas the alkylation steps of these modifications have been described, genes required for the hydroxylation of U34 to give 5-hydroxyuridine (ho^{5}U) remain unknown. Here, a number of genes in *Escherichia coli* and *Bacillus subtilis* are identified that are required for wild-type (wt) levels of ho^{5}U. The *yrrMNO* operon is identified in *B. subtilis* as important for the biosynthesis of ho^{5}U. Both *yrrN* and *yrrO* are homologs to peptidase U32 family genes, which includes the *rlhA* gene required for ho^{5}C synthesis in *E. coli*. Deletion of either *yrrN* or *yrrO*, or both, gives a 50% reduction in mo^{5}U tRNA levels. In *E. coli*, *yegQ* was found to be the only one of four peptidase U32 genes involved in ho^{5}U synthesis. Interestingly, this mutant shows the same 50% reduction in (m)cmo^{5}U as that observed for mo^{5}U in the *B. subtilis* mutants. By analyzing the genomic context of *yegQ* homologs, the ferredoxin YfhL is shown to be required for ho^{5}U synthesis in *E. coli* to the same extent as *yegQ*. Additional genes required for Fe-S biosynthesis and biosynthesis of prephenate give the same 50% reduction in modification. Together, these data suggest that ho^{5}U biosynthesis in bacteria is similar to that of ho^{5}C, but additional genes and substrates are required for complete modification.

IMPORTANCE Modified nucleotides in tRNA serve to optimize both its structure and function for accurate translation of the genetic code. The biosynthesis of these modifications has been fertile ground for uncovering unique biochemistry and metabolism in cells. In this work, genes that are required for a novel anaerobic hydroxylation of uridine at the wobble position of some tRNAs are identified in both *Bacillus subtilis* and *Escherichia coli*. These genes code for Fe-S cluster proteins, and their deletion reduces the levels of the hydroxyuridine by 50% in both organisms. Additional genes required for Fe-S cluster and prephenate biosynthesis and a previously described ferredoxin gene all display a similar reduction in hydroxyuridine levels, suggesting that still other genes are required for the modification.

KEYWORDS Fe-S cluster protein, tRNA, tRNA modification

Organisms from all kingdoms of life take advantage of redundancy patterns in the genetic code to reduce the number of unique tRNAs required to translate the 61 sense codons (1). To do this, tRNAs utilize wobble pairing (2) with a G or U at position 34, the first position of the anticodon, to recognize U or G, respectively, at position 3 of the codon. In addition, modified bases are heavily employed at position 34 to either extend or restrict wobble capacity beyond standard wobble rules (3–6). For example, eukaryotes invariably use inosine in $I_{34}N_{35}N_{36}$ anticodons to extend pairing to A and C,

Citation Lauhon CT. 2019. Identification and characterization of genes required for 5-hydroxyuridine synthesis in *Bacillus subtilis* and *Escherichia coli* tRNA. J Bacteriol 201:e00433-19. https://doi.org/10.1128/JB.00433-19.

Editor Tina M. Henkin, Ohio State University

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Received 27 June 2019
Accepted 19 July 2019
Accepted manuscript posted online 29 July 2019
Published 20 September 2019

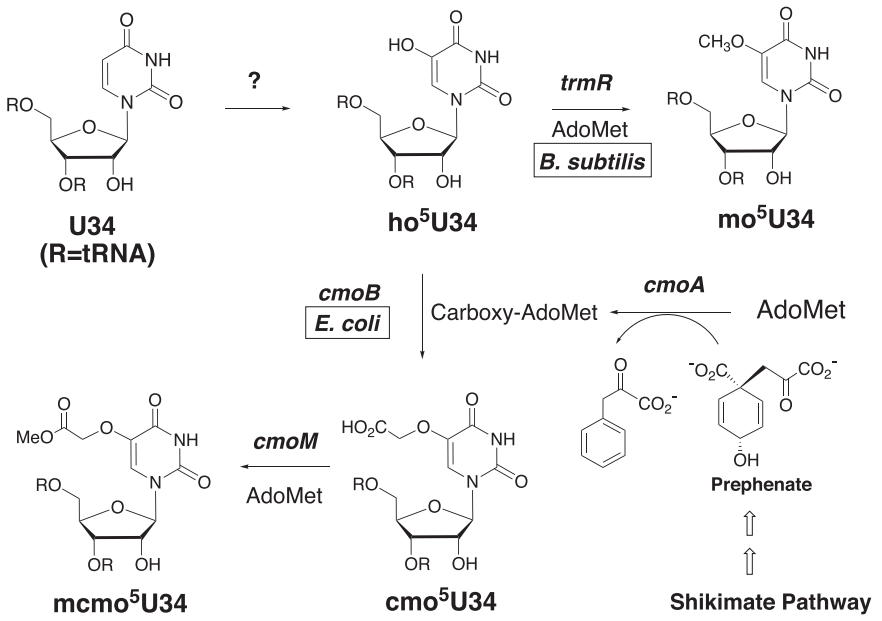

FIG 1 Biosynthesis of ho^{5}U derivatives in bacterial tRNA. In *E. coli* and other gammaproteobacteria, CmoA and CmoB convert AdoMet to carboxy-AdoMet and transfer the carboxymethyl group to ho^{5}U to give cmo^{5}U. The latter is then methylated by CmoM to give the terminal modification mcmo^{5}U. In *B. subtilis* and most other bacteria, ho^{5}U is methylated directly by the recently identified TrmR (YrrM) methyltransferase (22). The genes required for ho^{5}U synthesis have yet to be described and are the subject of this work.

while bacteria rely mostly on modified uridines in the 4-fold degenerate (four box) amino acid codon families to extend pairing to U and C (7, 8). Such modifications allow organisms to further reduce the number of required tRNAs. A recent analysis of bacterial tRNA usage and evolution suggests that a highly dynamic population of mandatory and "auxiliary" tRNAs exists in bacterial species and that these populations fluctuate via both mutation and horizontal gene transfer (9).

In *Escherichia coli*, tRNAs that decode the four degenerate codon family amino acids Ala, Pro, Thr, Val, Ser, and Leu have a hypermodified U34 substituted at the 5 position with a 5-methoxycarbonylmethoxy (5-oxyacetic acid methyl ester) (mcmo^5) group (Fig. 1) (8, 9). The ester moiety is unstable to alkali and even at pH 7.5 results in degradation with a half-life of 20 h (10). Both the fully modified 5-methoxycarbonylmethoxyuridine (mcmo^{5}U) (10) and its biosynthetic precursor 5-carbonylmethoxyuridine (cmo^{5}U) (11) are found *in vivo*, with mcmo^{5}U predominating in $tRNA_1^{Ala}$, $tRNA_3^{Pro}$, $tRNA_1^{Ser}$, and $tRNA_4^{Thr}$, while cmo^{5}U predominates in $tRNA_3^{Leu}$ and $tRNA_1^{Val}$ (12). The amount of methyl ester modification increases significantly with growth phase in $tRNA_3^{Pro}$ in *E. coli* (12). Both biochemical (13) and structural work (14) have indicated that tRNAs with the (m)cmo^{5}U modification are able to not only decode A3 and G3 at the third codon position but also bind to U3 and C3 containing codons (3). This expanded role was first verified *in vivo* by the observation that a single $tRNA^{Pro}_{cmo5UGG}$ is sufficient for recognizing all four codons in a *Salmonella enterica* serovar Typhimurium mutant lacking the other two proline tRNA genes (15). Nasvall et al. also found in later work that (m)cmo^{5}U-enhanced codon recognition *in vivo* is highly context dependent, such that in *S.* Typhimurium, U3- and C3-containing codons can be decoded by (m)cmo^{5}U-modified $tRNA_1^{Ala}$, $tRNA_3^{Pro}$, and $tRNA_1^{Val}$, while for the Leu, Ser, and Thr codon families, they cannot (16). The most surprising result from this work is that cmo^{5}U was found to be required for efficient classic wobble decoding of G3 as well. Structurally, the carboxymethyl group of cmo^{5}U is required for maximum decoding efficiency *in vivo*, as mutants which contain only 5-hydroxyuridine (ho5U) at position 34 display a significant growth defect in many cases where the (m)cmo^{5}U tRNA is the sole isoacceptor (16). Crystal structures of cmo^{5}U-modified tRNAVal anticodon stem loop RNA (ACSL^{Val})

bound to different codons on the ribosome (14) show that the modification promotes several stabilizing interactions that preorganize the ACSL for interaction with codons. These include a C3′-*endo* conformation of the ribose and hydrogen bonding from the ether oxygen of cmo^{5}U to the ribose 3′-OH of highly conserved U33. Earlier studies showed that the alkyl group of mo^{5}U shifts the tautomeric equilibrium toward the enol (lactim) form at C4 of the uridine ring (17). Together these effects promote Watson Crick, not wobble, pairing geometry to G3 ending codons via the enol form of cmo^{5}U, as well as conformational stabilization of the anticodon loop necessary to entropically compensate for weak hydrogen bonding with U3 and C3 (14).

While (m)cmo^{5}U is found mainly in gammaproteobacteria (12), *Bacillus subtilis* and possibly most other bacteria utilize the structurally simpler 5-methoxyuridine (mo^{5}U) at the same position in the respective tRNAs (18) (Fig. 1). As shown in Fig. 1, the biosynthesis of both modifications is believed to begin with a novel anaerobic hydroxylation at C5 (4). Seminal work by Björk and coworkers showed that the biosynthesis of cmo^{5}U in *S.* Typhimurium is dependent on two methyltransferase-like genes, *cmoA* and *cmoB*, both of which were required for cmo^{5}U production (15), although the exact mechanism of constructing the carboxymethyl group was not clear. An interesting clue was provided by their demonstration of an additional requirement for chorismate or a downstream metabolite. This metabolite was required for both carboxymethyl formation and for full hydroxylation activity (19). Structural studies of CmoA later showed that the chorismate-derived metabolite is prephenic acid, which is decarboxylated to provide CO_2 in the CmoA active site (20). The nascent CO_2 then adds to an unprecedented ylide form of *S*-adenosylmethionine (AdoMet) to give the novel product carboxymethyl-AdoMet. CmoB then transfers the carboxymethyl group of carboxy-AdoMet directly to ho^{5}U to give cmo^{5}U. The *cmoM* gene was recently identified by Sakai et al. as coding for the methyltransferase required for methylation of the carboxylic acid in most cmo^{5}U-containing tRNAs (12).

While genes have been identified for the biosynthesis of the alkylation steps in the ho^{5}U-derived modification (xo^{5}U) pathways, those involved in the hydroxylation have remained unknown. Kimura et al. recently reported that the hydroxylation step in the synthesis of structurally similar 5-hydroxycytidine (ho^{5}C) in rRNA is dependent on the *rlhA* gene (formerly *ydcP*) (21). RlhA is a member of a group of proteins found in bacteria annotated as U32 peptidases (NCBI conserved domain accession number COG0826) but with no experimentally known function. These authors also showed that the ho^{5}C modification requires Fe-S cluster biosynthetic machinery and that prephenic acid is also the likely source of the oxygen of the hydroxyl. In the work reported here, the genes required for the biosynthesis of ho^{5}U in tRNA in both *E. coli* and *B. subtilis* have been identified and the corresponding mutants characterized. The findings are consistent with results for ho^{5}C biosynthesis, with the interesting exception that (as Björk and coworkers demonstrated) for ho^{5}U other genes and possibly other substrates are required to give full ho^{5}U modification levels in tRNA. In addition, a novel low potential ferredoxin, YfhL, of previously unknown function, has been identified as a requirement for ho^{5}U synthesis in *E. coli*. Differences in the genetic organization, as well as the mechanistic role of the orthologous proteins in *E. coli* versus *B. subtilis* are presented and discussed.

RESULTS

The *yrrMNO* operon contains genes required for wild-type levels of ho^{5}U biosynthesis in *B. subtilis*. A bioinformatics approach was initially used with *E. coli cmoB* as query to search for genes required for ho^{5}U synthesis. This approach led to the identification of the *yrrMNO* operon in *B. subtilis* via the likely terminal methyltransferase YrrM. High-pressure liquid chromatography (HPLC) analysis of enzymatic digests of bulk tRNA extracted from a *yrrM* deletion strain (Bacillus Genetic Stock Center) showed a complete absence of mo^{5}U (Fig. 3A) and the appearance of ho^{5}U in the HPLC trace indicating *yrrM* coded for the methyltransferase. While this work was in progress, Ryu et al. published this result along with the crystal structure of the protein and named

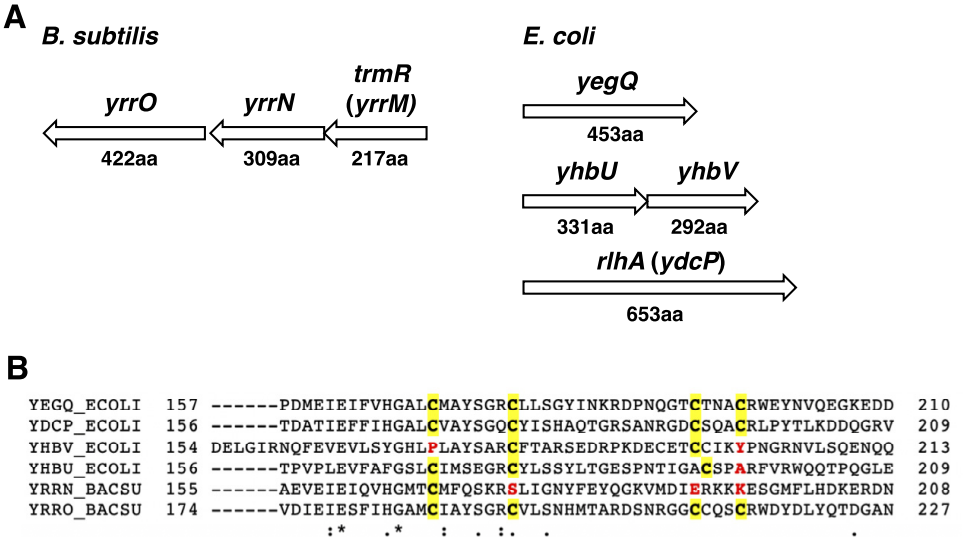

FIG 2 (A) Organization of the two peptidase U32 genes in *B. subtilis* and four genes in *E. coli*. Numbers indicate corresponding protein amino acid length. (B) Clustal-omega amino acid alignment of peptidase U32 homologs from *E. coli* and *B. subtilis*. Putative Fe-S cluster cysteines are highlighted in yellow although there are other cysteines in the region. Amino acids that differ from cysteine in the conserved positions are shown in red (YhbU is the exception in which an adjacent cysteine has been included as a potential Fe-S ligand).

the gene *trmR* (22) (Fig. 1). As shown in Fig. 2A, located downstream of the *yrrM* gene are two additional genes, *yrrN* and *yrrO*, that are adjacent and predicted to be included in the operon. Both YrrN and YrrO are annotated as U32 peptidases or collagenases (23). However, iterative PSI-BLAST (24) searches showed that the proteins had multiple conserved cysteines, as shown in Fig. 2B, and also weak homology to Fe-S cluster-containing proteins in both bacteria and archaea. In addition, Kimura et al. (21) published that the peptidase U32 gene *rlhA* was required for ho^{5}C synthesis in *E. coli* rRNA. Thus, it was likely that one or both of these genes were required for ho^{5}U synthesis in tRNA in *B. subtilis*.

Surprisingly, analysis of tRNA from both the *yrrN* and *yrrO* single mutants still showed the presence of mo^{5}U; however, as shown in Fig. 3, careful quantitation of the peak areas of mo^{5}U relative to pseudouridine (Ψ) showed that the mo^{5}U levels were reduced by ~50% in both mutant strains relative to the wild-type (wt) strain (Fig. 3B). Complementation of both the *yrrN* (not shown) and *yrrO* (Fig. 3B) mutants using the pLIKErep expression plasmid (25) with bacitracin induced expression of *yrrN* and *yrrO*, respectively, and showed full complementation of mo^{5}U to wt levels. The possibility that *yrrN* and *yrrO* are paralogs and each is required for 50% of ho^{5}U biosynthesis was quickly proven false by the successful preparation of a *yrrNO* double mutant. The method of Koo et al. (26) was used to replace the adjacent *yrrNO* genes with either a kanamycin or erythromycin resistance gene via double crossover. These mutants were viable with no observable growth defects in rich medium (LB) and, as shown in Fig. 3A and quantitated in Fig. 3B, showed no further reduction in mo^{5}U levels when compared to either the *yrrN* or *yrrO* single mutants. Complementation studies showed that both *yrrN* and *yrrO* were alone not able to complement the double mutant. This was intriguing because it indicates that the YrrN and YrrO proteins may be required together for the same step in mo^{5}U synthesis and that this step is not sufficient to account for all of the mo^{5}U synthesis.

The *yegQ* gene is the only peptidase U32 gene that is involved in the synthesis of cmo^{5}U in *E. coli*. Because deletion of *yrrO* or *yrrN* or both resulted in the same 50% reduction in mo^{5}U levels in *B. subtilis*, attention was turned toward the analogous modification in *E. coli*. In this organism, both mcmo^{5}U and cmo^{5}U are present and functional, depending on the tRNA species (12). The ester of mcmo^{5}U is significantly hydrolyzed to cmo^{5}U during usual enzymatic digestion for HPLC analysis (12) and can

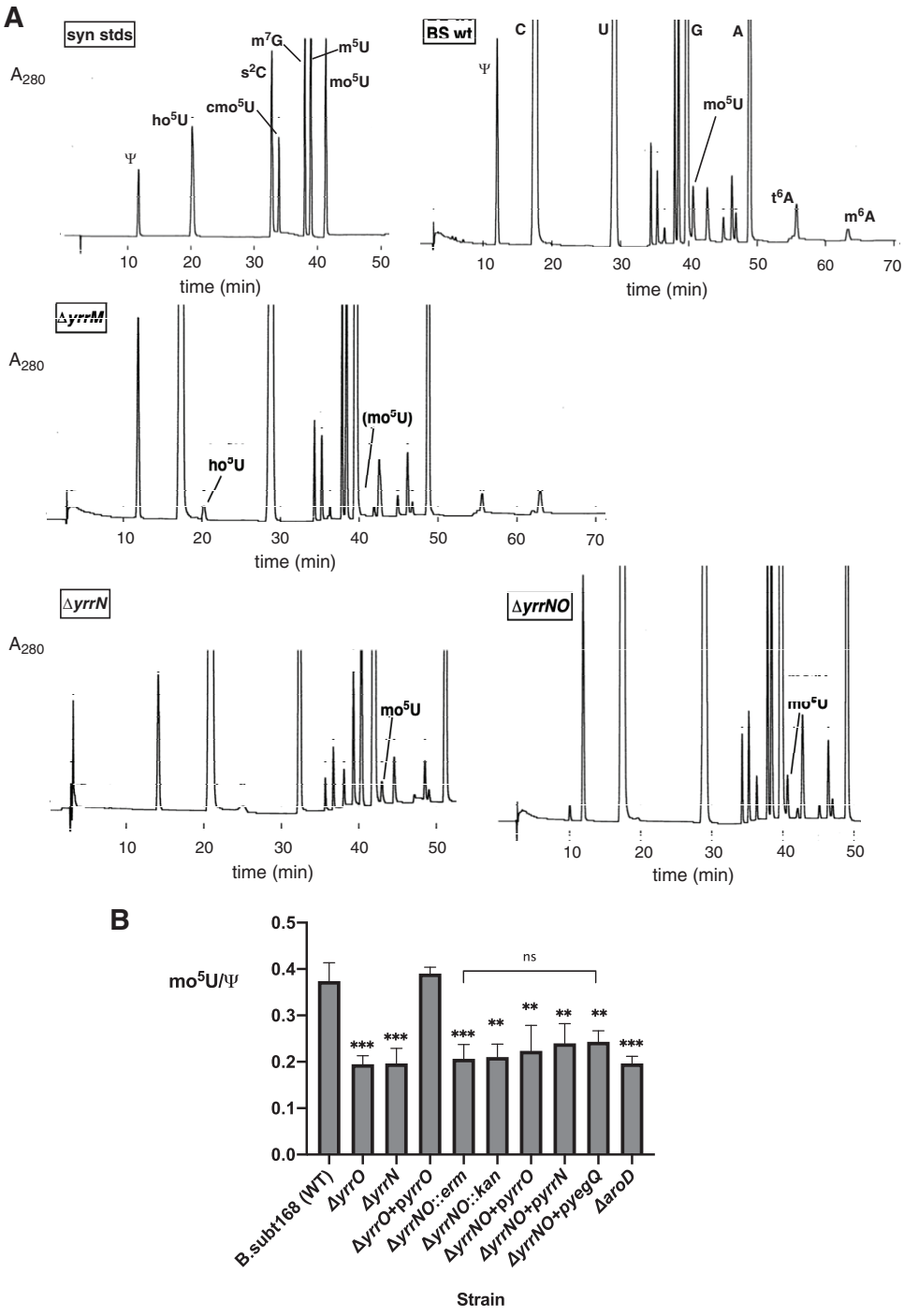

FIG 3 (A) Representative HPLC analysis of mo^{5}U levels of *B subtilis* wt and mutant strains. The first trace shows synthetic standards of modified nucleosides relevant to this work. Total tRNA isolated from each strain (12.5 μg) is enzymatically digested to nucleosides and analyzed by HPLC with detection by absorbance at 280 nm. (B) Quantitative effect of selected *B. subtilis* peptidase U32 gene deletions on levels of mo^{5}U in purified total tRNA based on the ratio of peak integration relative to pseudouridine (Ψ). ***, $P < 0.001$ (unpaired *t* test); **, $P < 0.01$ compared to wt strain; ns = no statistically significant difference between levels in these strains.

be completely hydrolyzed in 4 h at pH 8.3, which simplifies analysis. For the Keio collection reference *E. coli* strain BW5113 (27), the level of cmo^{5}U in bulk tRNA was found to be approximately 0.17 mol/mol tRNA, 70% lower than the mo^{5}U level in *B. subtilis* 168 (0.24 mol/mol tRNA). This lower value for *E. coli* is expected based on lower copy numbers of mcmo^{5}U-modified tRNA genes in the *E. coli* genome (12 copies in *E.*

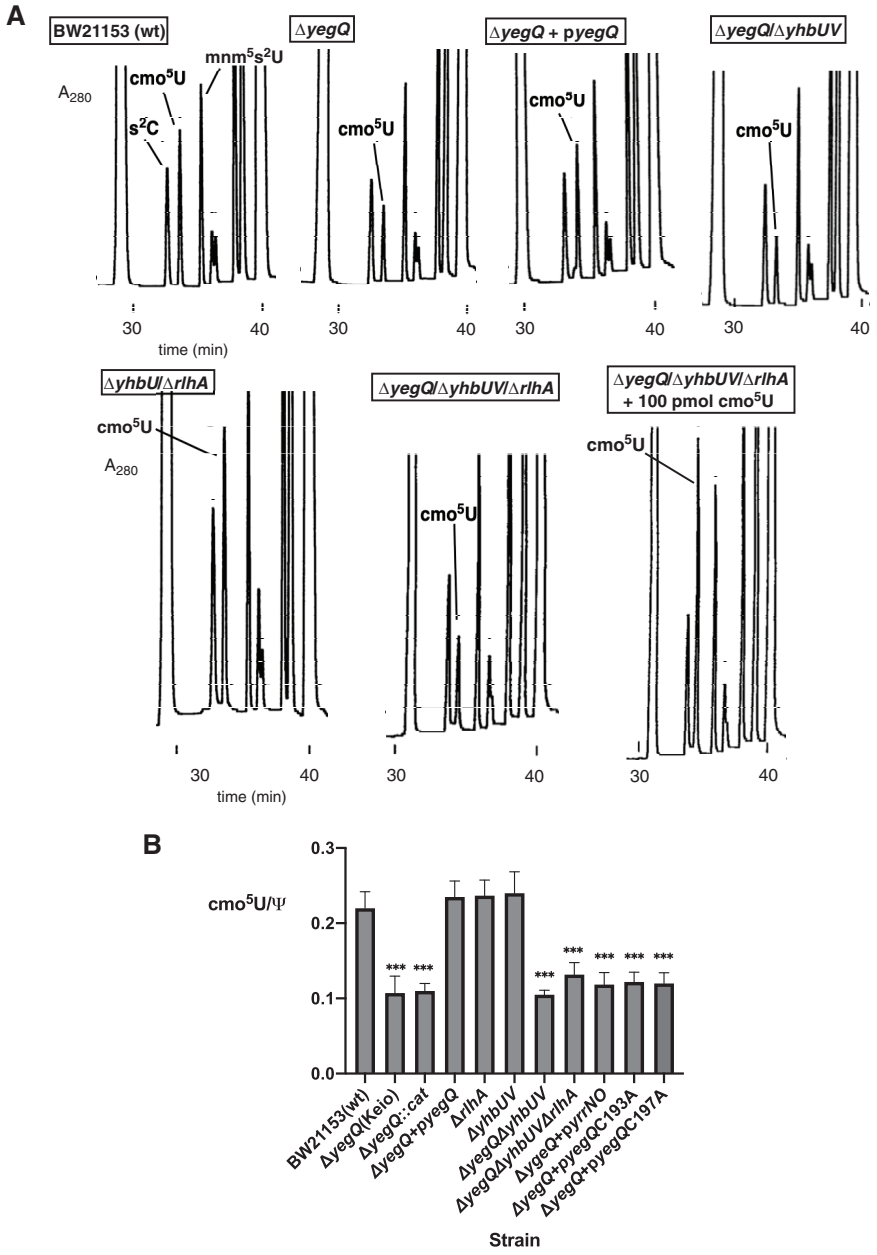

FIG 4 (A) HPLC analysis of cmo^{5}U levels of various *E. coli* peptidase U32 gene deletion mutants analyzed as described for Fig. 3A. The last two panels show the Δ*yegQ* Δ*yhbUV* Δ*rlhA* quadruple mutant without and with coinjection of cmo^{5}U standard. The cmo^{5}U peak was also isolated from this mutant and verified for identity by high-resolution mass spectrometry. (B) Quantitative analysis of selected gene deletions and complementation of *E. coli* peptidase U32 gene deletions based on HPLC analyzed levels of cmo^{5}U in purified total tRNA. ***, $P < 0.001$ (unpaired *t* test) compared to wt strain.

coli versus 18 copies in *B. subtilis*) (1). As recently described by Kimura et al. (21) and shown in Fig. 2, *E. coli* contains four peptidase U32 genes as follows: *rlhA* (*ydcP*), *yegQ*, and a putative operon containing the two adjacent genes *yhbU* and *yhbV*. The *rlhA*, *yegQ*, and *yhbU* mutants were obtained via the Keio collection (via *E. coli* Genetic Stock Center), while the *yhbV* deletion strain was prepared via the method of Datsenko and Wanner (28) by replacement of *yhbV* with the *cat* gene from plasmid pKD3 (Table 2). As shown in Fig. 4, analysis of the four single gene deletion strains for cmo^{5}U levels shows that only *yegQ* has a reduced level of cmo^{5}U compared to that of the wt. Surprisingly, the level of cmo^{5}U in this strain is reduced by the same 50%, within experimental error, as that of the *yrrN* and *yrrO* mutants in *B. subtilis*. To confirm this result, we recon-

structed the *yegQ* gene deletion mutant from the isogenic wt strain by gene replacement with the *cat* gene and also removed the antibiotic markers and observed the same levels of reduced cmo^{5}U in both of these strains. Complementation with wt *yegQ* cloned in the arabinose-inducible plasmid pBAD33 resulted in L-arabinose-dependent rescue of the reduced cmo^{5}U levels (Fig. 4). Extended growth of the *yegQ* mutant strain overnight did not result in increased levels of cmo^{5}U (data not shown). Mutation of any of the four YegQ cysteines (C170, C177, C193, and C197) that are conserved with YrrO and RlhA (Fig. 2B) eliminates restoration of cmo^{5}U to wt levels. These data show that wt levels of cmo^{5}U are dependent on expression of functional *yegQ*. To determine if the other homologous genes are able to synthesize the remaining cmo^{5}U in the absence of *yegQ*, systematic double and triple mutants were constructed as well as the quadruple mutant lacking all four peptidase U32 genes. PCR analysis confirms that all four genes were deleted in this strain (see Fig. S1 in the supplemental material). Only combinations of gene deletions that include *yegQ* were found to have reduced cmo^{5}U levels, and these levels were all reduced from wt by essentially the same amount within experimental error. The quadruple mutant still retained the same cmo^{5}U levels as those of the *yegQ* mutant. To confirm that this peak is indeed cmo^{5}U, a coinjection was conducted with pure synthetic cmo^{5}U (Fig. 4A), and the material that eluted in that peak was analyzed by high-resolution mass spectrometry to have an exact mass that identifies it as cmo^{5}U (calculated, 317.077206; found, 319.077560). All mutant combinations showed no significant growth defects in rich medium. Since the *rlhA* (*ydcP*) mutant was found to be absolutely required for ho^{5}C synthesis in rRNA, these data suggest that other genes are required for the biosynthesis of ho^{5}U at wt levels.

***E. coli iscS* mutant synthesizes cmo^{5}U but at a reduced level.** An important finding from Kimura et al. (21) was that ho^{5}C synthesis in rRNA in *E. coli* is significantly dependent on Fe-S cluster biogenesis, specifically for genes in the *isc* operon. This is consistent with the earlier mentioned PSI-BLAST results. For example, they observed the level of ho^{5}C is ~1% in *iscU* mutants at log phase. Similar to effects previously reported in *isc* mutants for the Fe-S cluster enzyme-dependent thionucleosides s^{2}C and ms^{2}i^{6}A (29), the levels of ho^{5}C increase significantly in stationary phase, which suggests auxiliary Fe-S synthesis (e.g., via genes in the *suf* operon) is able to provide Fe-S clusters to *rlhA*, albeit inefficiently. The effect is greatest for *iscS* mutants. To test if cmo^{5}U levels behave similarly, the *iscS* deletion strain from the Keio collection was analyzed for cmo^{5}U levels in tRNA. Figure 5A shows the HPLC analysis of tRNA modified nucleosides from this mutant. While the region between C and G shows the expected absence of s^{2}C and mn^{5}s^{2}U at early log phase, cmo^{5}U is clearly present, although at reduced levels. As shown in Fig. 5B, quantitative analysis shows that cmo^{5}U levels are similar on average to those for the *yegQ* mutant and also increase slightly from log phase to saturation ($P = 0.06$). Consistent with the *iscS* mutant phenotype in *E. coli*, the thionucleoside s^{2}C, which elutes just before cmo^{5}U, is undetectable at log phase but increases to near wt levels after overnight incubation. These data suggest that like ho^{5}C, ho^{5}U synthesis is dependent at least in part on *iscS* catalyzed Fe-S cluster biosynthesis. However, unlike ho^{5}C, there is either a greater intrinsic ability to synthesize cmo^{5}U via the redundant Fe-S pathway, or other genes are required that can synthesize ho^{5}U without significant Fe-S cluster protein activity.

Effect of *aro* mutants on the levels of cmo^{5}U in *E. coli* and mo^{5}U in *B. subtilis*. One of the most intriguing aspects of the cmo^{5}U modification in tRNA was the observation that chorismic acid or a downstream metabolite not only was required for the synthesis of the methylene carbon of cmo^{5}U but also was necessary for wt levels of ho^{5}U (19). Kimura et al. (21) have recently provided strong evidence that prephenic acid is the metabolite required for ho^{5}C synthesis in rRNA; thus, it is reasonable to suspect it is also utilized by YegQ in the synthesis of ho^{5}U. HPLC analysis of an *aroD E. coli* mutant from the Keio collection showed the expected complete absence of cmo^{5}U and the presence of ho^{5}U at reduced levels (Fig. 5A), confirming the original results from Hagervall et al. in *S.* Typhimurium (19). When *trmR* (*yrrM*) from *B. subtilis* is expressed in the *E. coli aroD* mutant, complete conversion of the ho^{5}U to mo^{5}U is

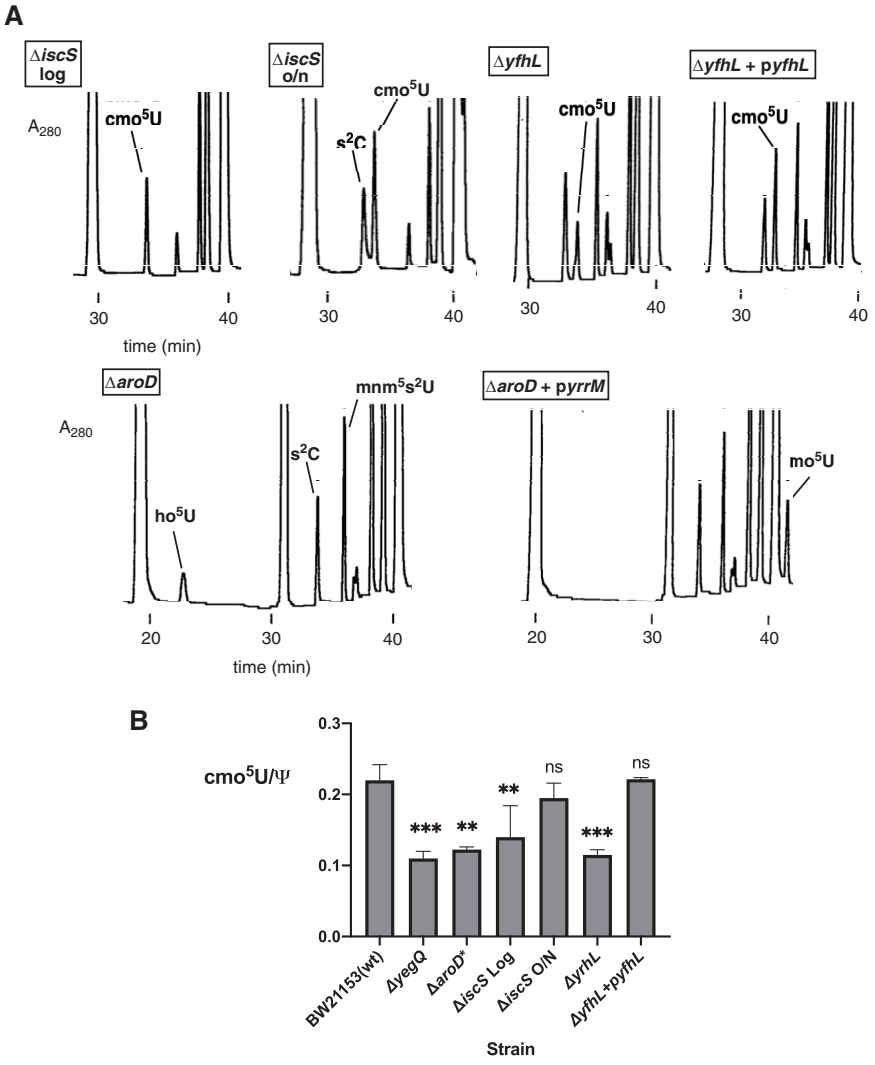

FIG 5 Effects of other genes on xo^{5}U levels. (A) HPLC analysis of *iscS*, *yfhL*, and *aroD* in *E. coli*. The *iscS* mutant lacks thionucleosides s^{2}C and mnm^{5}s^{2}U and cmnm^{5}s^{2}U in this region but still retains cmo^{5}U, which is decreased to a similar level as the *yegQ* mutant (shown in panel B). The overnight *iscS* mutant culture shows significant increases in s^{2}C and some increase in cmo^{5}U, similar to increases in other Fe-S-dependent modifications. The effect on cmo^{5}U is not statistically significant due to variability in the log-phase data. An *E. coli* strain lacking the ferredoxin *yfhL* shows a similar HPLC spectrum as that of the *yegQ* mutant and a similar level of reduction in cmo^{5}U (shown in panel B). In *E. coli*, shikimate pathway genes, such as *aroD*, are required for conversion of ho^{5}U to cmo^{5}U and for wt levels of ho^{5}U, as previously reported (15, 19). Quantifying the level of ho^{5}U shows the same reduction in ho^{5}U as for cmo^{5}U in the *yegQ* and *yfhL* mutants. (B) Quantitative analysis of HPLC data for *E. coli aro*, *iscS*, and *yfhL* mutants relative to those of the wt strain. *, the *aroD* strain lacks the ability to synthesize cmo^{5}U from ho^{5}U and thus the ratio reported here is for ho^{5}U/Ψ. ***, $P < 0.001$ compared to wt (unpaired *t* test); **, $P < 0.01$ compared to wt; *ns* = no statistical significance among these strains or with the wt strain.

observed. The level of ho^{5}U observed here is identical within experimental error to the level of cmo^{5}U in the *yegQ* mutant, and all combinatorial mutants of peptidase U32 genes in which *yegQ* is deleted. Furthermore, an *aroD yegQ* double mutant also shows the same reduced level of ho^{5}U (not shown). *E. coli* mutants in *aroA*, *aroB*, and *aroC* also show similar reductions in ho^{5}U, which is the sole xo^{5}U modification present. These data suggest that like *rlhA* in ho^{5}C synthesis, *yegQ* is part of a pathway that likely utilizes prephenate for ho^{5}U synthesis. Yet, unlike ho^{5}C, the ho^{5}U tRNA modification is not solely dependent on *yegQ* and prephenate, and other genes and substrates are likely involved.

Unlike in *E. coli*, the *B. subtilis* shikimate pathway late stage genes (*aroK*, *aroE*, and *aroF*) that convert shikimic acid to chorismic acid are required for viability (26).

However, the *aroD* mutant for shikimate dehydrogenase has no paralog, and the deletion mutant is viable, although it has a slow growth phenotype. Analysis of total tRNA from the *aroD* deletion mutant BKK27340 shows a reduced level of mo^{5}U that is essentially the same as that in the *yrrN* and *yrrO* mutants (Fig. 3B). The synthesis of mo^{5}U by TrmR uses AdoMet and, thus, does not require prephenate; thus, the effect of the *aroD* mutation is solely expressed as a reduction in mo^{5}U. Both *pheA* and *tyrA* mutants in *B. subtilis* were observed to have wt mo^{5}U levels (data not shown). Thus, it is likely that *B. subtilis* also uses prephenate as the hydroxyl donor for partial ho^{5}U synthesis, and similar to *E. coli*, there are other genes and/or substrates involved in the biosynthesis of this modification.

Identification of the low potential ferredoxin YfhL as a requirement for ho^{5}U synthesis in *E. coli*. Because deletion of all peptidase U32 genes in both *E. coli* and *B. subtilis* did not abolish ho^{5}U synthesis in tRNA, other genes must be involved. To search for additional genes, the genomic context of peptidase U32 orthologs was analyzed in a wide range of bacteria using the BioCyc platform (30). The genomic neighborhood as well as the homology of these genes is highly variable among bacteria. In Gram-negative organisms, there is no proximity between the peptidase U32 genes and homologs of *cmoA*, *cmoB*, or *cmoM*. In Gram-positive organisms, there is some consistency with colocalization of *yrrO* orthologs with *O*-methyltransferases that are similar to *trmR* (*yrrM*), but otherwise no consistent theme emerges. A number of candidate genes located adjacent to peptidase U32 genes, which have orthologs in *E. coli* and *B. subtilis* and possibly relevant annotation, were analyzed, but the deletion strains showed no effects on cmo^{5}U or mo^{5}U levels, respectively. However, one candidate gene was intriguing due to its annotation as a small ferredoxin. A number of bacteria in the *Gammaproteobacteria* class have *yegQ* and/or *yrrO* orthologs that are adjacent to downstream ferredoxin genes. The ferredoxins may be relevant to ho^{5}U synthesis since YegQ and its homologs likely bind Fe-S clusters. In *E. coli*, the most similar homolog is YfhL, a unique low potential ferredoxin of unknown function (31). To test this hypothesis, total tRNA was isolated from the *E. coli yfhL* deletion mutant (Keio strain JW2546). HPLC analysis of the digested tRNA showed an identically reduced level of cmo^{5}U as the isogenic *yegQ* mutant (Fig. 5). The reduced level could be fully complemented by plasmid-based overexpression of *yfhL*. A similar search for homologs in *B. subtilis* did not result in a clear hit, which is not surprising given the known restricted presence of *yfhL* orthologs to gammaproteobacteria (32). These results suggest that one of the elusive physiological roles of YfhL in these organisms may be to provide electrons to an Fe-S cluster-bound YegQ to support ho^{5}U formation. Construction of a *yegQ yfhL* double knockout showed cmo^{5}U levels identical to those of each single mutant, indicating that both genes are likely required in the same pathway for cmo^{5}U synthesis. *E. coli* YfhL was overexpressed and purified aerobically. Attempts to observe formation of a YegQ-YfhL complex by native gel analysis under aerobic conditions were unsuccessful. It is possible that these proteins interact only under anaerobic conditions or that each protein has a distinct role in ho^{5}U synthesis that does not require direct interaction.

Analysis of mo^{5}U levels of individual tRNAs in the *B. subtilis yrrO* mutant. A 50% reduced level of mo^{5}U in bulk tRNA from *yrrO* and *yrrN* mutants could be the result of a complete loss of ho^{5}U in half of the substrate tRNAs or a partial loss of ho^{5}U in all tRNAs, the sum of which leads to the observed 50% reduction. To find out which is more likely to be the case, individual tRNAs were purified from *B. subtilis* wt and *yrrO* mutant strains and analyzed for mo^{5}U levels. Analysis of the individual tRNAs would also reinforce the observed decrease in mo^{5}U since the effects in bulk tRNA are not large. A previously described oligonucleotide capture method (33, 34) was used to purify individual tRNAs from purified total tRNA isolated from both the wt and *yrrO* deletion strains. In many cases, the tRNAs were homogeneous after one round of affinity purification, as judged from the modification HPLC profile and PAGE analysis. As determined by PAGE (see Fig. S2 in the supplemental material) and HPLC analysis

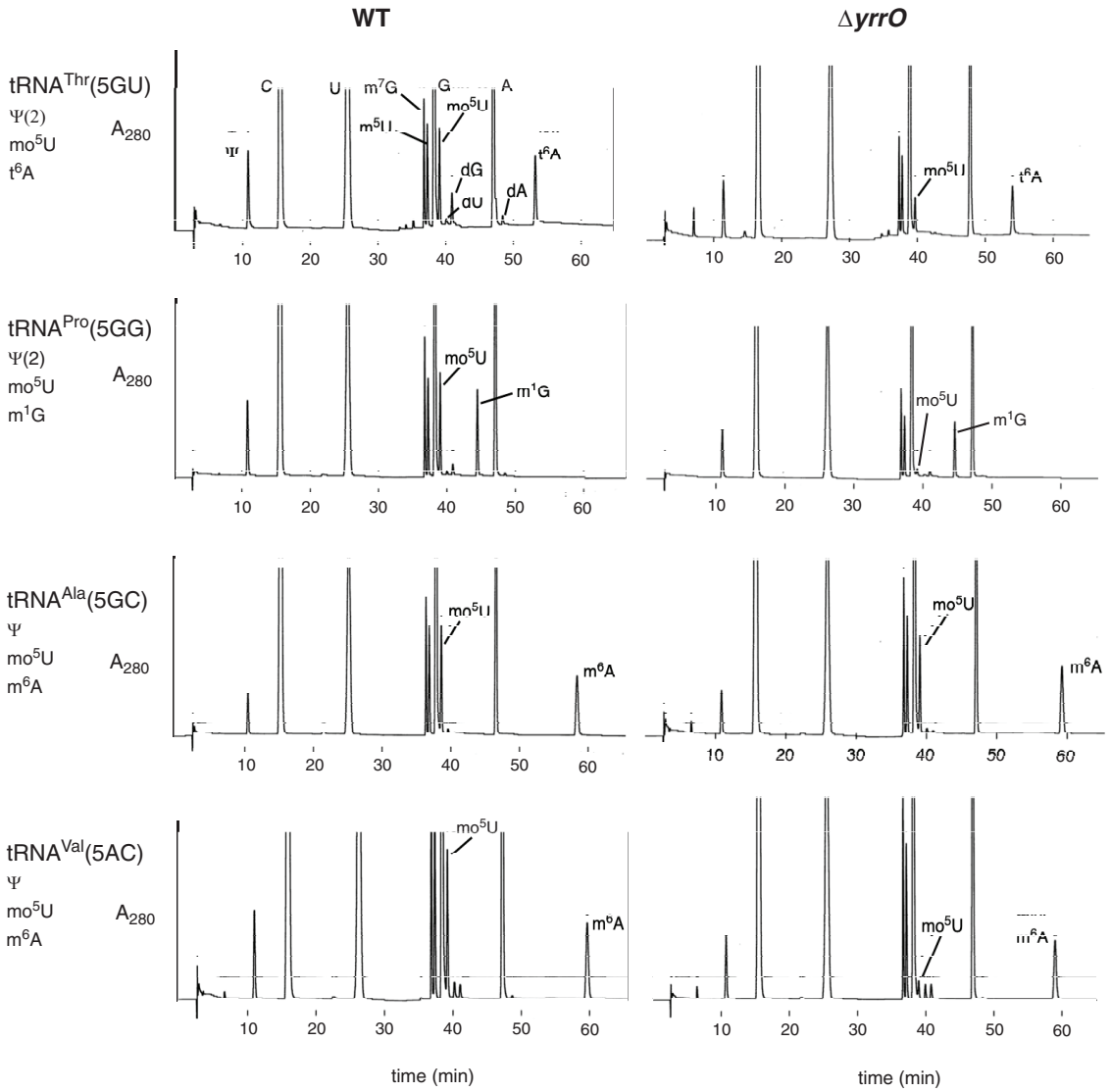

FIG 6 HPLC profiles of enzymatic digests of individual tRNAs (ca. 6 μg each) purified from *B. subtilis* wt and *yrrO* mutant strains. The expected modifications for each tRNA are listed on the left for reference. Some residual deoxynucleosides remain after DNase treatment and gel filtration. Detection is by absorbance at 280 nm.

(Fig. 6), four tRNA species were sufficiently purified from both wt and *yrrO* mutant strains as follows: $tRNA^{Ala}_{mo5UGC}$, $tRNA^{Pro}_{mo5UGG}$, $tRNA^{Thr}_{mo5UGU}$, and $tRNA^{Val}_{mo5UAC}$. Unmodified transcripts were used as standards, insomuch as the modifications do not affect the mobility greatly. The purity of $tRNA^{Thr}$(mo^{5}UGU) is lower (Fig. S2, lane 6), perhaps due in part to a degradation product; however, HPLC analysis (Fig. 6) shows a high level of purity based on the absence of modifications that are found in the other tRNAs. Purification of $tRNA^{Ser}$ and $tRNA^{Leu}$ has been unsuccessful in that HPLC traces showed multiple modifications not ascribed to those tRNAs (7, 8). Of the tRNAs that have been purified sufficiently, measurement of mo^{5}U levels (Fig. 6) show particularly significant reductions in $tRNA^{Pro}$ and $tRNA^{Val}$ ($<$20% relative to those of the wt), while $tRNA^{Thr}$ retains 50% mo^{5}U and $tRNA^{Ala}$ mo^{5}U levels are essentially unaffected. None of the other modifications in these purified tRNAs were found to vary significantly in amount between the two strains. More work will need to be done, but these initial experiments show that there is a significant degree of tRNA specificity associated with YrrO-mediated ho^{5}U biosynthesis. The overall reduced level of mo^{5}U appears to be the result of variable reduction of mo^{5}U in each tRNA. A similar analysis in the *B. subtilis yrrN*

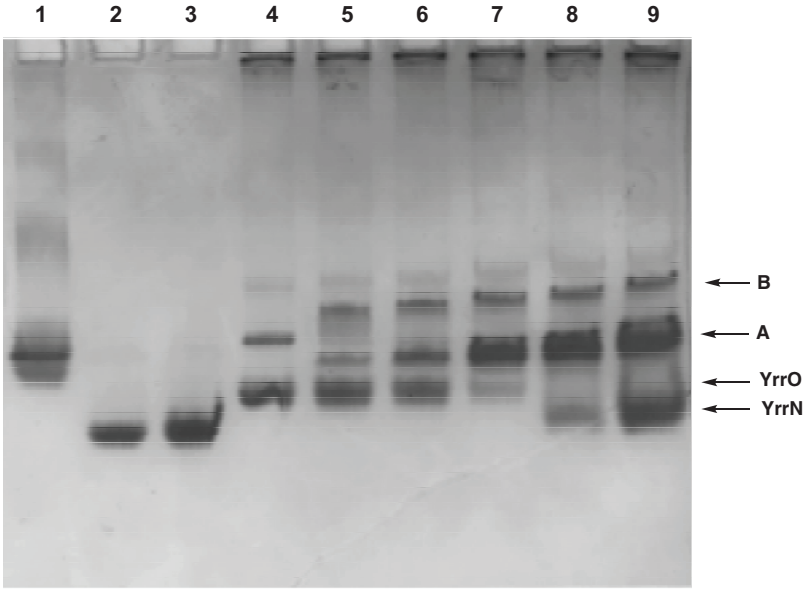

FIG 7 Native PAGE analysis shows evidence of a YrrN-YrrO complex. Increasing concentrations of His_6-YrrN are incubated with 2 μM His_6-YrrO in 30 mM Tris-glycine with 5 mM DTT (20 μl total volume) for 30 min at RT and then analyzed on a 10% nondenaturing polyacrylamide gel using 30 mM Tris-glycine, pH 7.5, as the buffer. Gel was stained with Coomassie blue. Lane 1, 2 μg bovine serum albumin; lane 2, 2 μg purified His_6-YrrN; lane 3, 4 μg YrrN; lane 4, 2 μg purified His_6-YrrO; lane 5, 2 μg YrrO plus 0.125 eq YrrN; lane 6, YrrO plus 0.25 eq YrrN; lane 7, YrrO plus 0.5 eq YrrN; lane 8, YrrO plus 1 eq YrrN; lane 9, YrrO plus 2 eq YrrN. Arrows show position of major (A) and minor (B) potential YrrNO complexes.

mutant and *E. coli yegQ* mutant will be useful to confirm potential tRNA selectivity of this pathway.

Peptidase U32 protein expression and initial characterization: YrrN and YrrO form a complex that is Fe-S cluster independent. Heterologous expression of *yrrN* and *yrrO* in *E. coli* BL21(DE3) cells gives highly soluble protein in each case. The cell pellet containing overproduced His_6-YrrO is a deep brown color while the pellet for His_6-YrrN is not. After purification under aerobic conditions, His_6-YrrO shows a deep brown colored band on a Ni-nitrilotriacetic acid (Ni-NTA) affinity column and retains its color after G-50 buffer exchange. Initial experiments with UV-visible (UV-Vis) spectroscopy under aerobic conditions of the purified YrrO shows absorbance characteristics consistent with an Fe-S cluster protein but the amount of iron is quite low, indicating that the bound iron is oxygen sensitive. Further characterization will be required under anaerobic conditions. Conversely, His_6-YrrN is overproduced at a high level but is a colorless protein through each step of the purification process. This is consistent with the amino acid alignment of the two proteins in Fig. 2B that shows mutation of three of the four conserved cysteines. Unlike the different properties of YrrN and YrrO in *B. subtilis*, overproduction and purification of the four peptidase U32 proteins in *E. coli* shows that all four give brown colored proteins. This is intriguing, since YhbU and YhbV both appear to lack one or more of the highly conserved cysteines shown in the amino acid sequence alignment. Thus, these proteins may contain an alternative type of Fe-S cluster or have insertions or deletions that bring in cysteines to structural positions analogous to those of YegQ or RlhA.

Because the *yrrN* and *yrrO* genes are adjacent in an operon in *B. subtilis* and they are homologous, it is possible that they form a complex. To assess this hypothesis, mixtures of purified His_6-YrrO and His_6-YrrN were analyzed by native polyacrylamide gel electrophoresis under aerobic conditions. Figure 7 shows that YrrN migrates as a single band, while YrrO shows two major bands in the presence of 5 mM dithiothreitol (DTT). Incubating YrrO in the presence of increasing concentrations of YrrN shows the appearance of new bands of slower mobility that increase in proportion to the amount

of YrrN added with a decrease in the two major YrrO bands. This suggests the formation of a YrrN-YrrO complex by possibly two YrrO species observed in the native gel. Addition of the iron-chelating agent 2,2′-dipyridyl (100 uM) had no effect on complex formation (data not shown), which is a further indication that the YrrN-YrrO complexes are not dependent on iron binding.

The *yrrNO* and *yegQ* genes cannot heterologously complement the function of each other *in vivo*. Unlike the ability of *B. subtilis yrrM* to complement *E. coli cmoB* mutants (22), heterologous expression of *yrrN* and *yrrO* either individually or together in the *E. coli yegQ* deletion strain had no effect on cmo^{5}U levels (Fig. 3B). Similarly, when *yegQ* is cloned into the pLIKE-rep plasmid, its expression in the *B. subtilis yrrNO* deletion strain in the presence of bacitracin is unable to affect mo^{5}U levels (Fig. 4B). This suggests that additional species-specific proteins are likely required for the modification reaction. One possible explanation is that the ferredoxin YfhL is not able to shuttle electrons to the FeS cluster of the putative YrrN-YrrO complex when it is expressed in *E. coli*. Similarly, a protein with function analogous to YfhL in *B. subtilis* may be unable to productively interact with YegQ in that organism. The coexpression of both *yegQ* and *yfhL* in *B. subtilis* has not been attempted but may provide an answer for this hypothesis. It is also reasonable to expect that there could be other proteins involved in the pathway to ho^{5}U synthesis that are required for complementation and do not have active orthologs or they are not active with YegQ. The possibility of tRNA substrate specificity being an issue is perhaps less likely due to high homology between the tRNA substrates in the two organisms.

DISCUSSION

The data presented in this work show that, in both *E. coli* and *B. subtilis*, a biosynthetic route similar to ho^{5}C synthesis in rRNA exists that requires the action of peptidase U32 proteins and prephenate as a possible source of the hydroxyl group. In *E. coli*, the data are supportive for a single peptidase U32 gene, *yegQ*, involved in ho^{5}U biosynthesis. The 50% reduction in cmo^{5}U levels in the *yegQ* mutant is unchanged at extended growth times, and no further reduction in cmo^{5}U is observed in a mutant that lacks all four of the *yegQ*-like proteins. In *B. subtilis*, the roles of the two peptidase U32 genes *yrrN* and *yrrO* are different. Each gene reduces mo^{5}U formation by the same 50% when deleted, and the double mutant has no additional reduction in the modification level. There is also *in vitro* evidence that the two proteins form a complex that is independent of iron binding. Whereas *E. coli* YegQ (and presumably YrrO in *B. subtilis*) has four conserved cysteines that are each required for wt cmo^{5}U (and hence ho^{5}U) synthesis *in vivo*, some of the other homologs have mutations in these positions that result in either alternative Fe-S cluster structures (YhbU, YhbV) or the loss of cluster forming ability (YrrN). Deletion of *aroD*, *iscS*, *yegQ*, and the ferredoxin *yfhL* in *E. coli* and combination mutants of these genes produces a similar level of reduction in cmo^{5}U in each case, which suggests that a non-Fe-S cluster and non-peptidase U32 protein-dependent pathway that utilize a different source for the hydroxyl than prephenate likely coexist. The alternate pathway likely shows unique tRNA substrate specificity, at least in *B. subtilis*.

While this paper was in review, Suzuki and coworkers reported the discovery of the alternative ho^{5}U biosynthesis pathway via an elegant systematic approach to the use of bioinformatics (31). In addition to their finding that *yegQ* and *yrrNO* in *E. coli* and *B. subtilis*, respectively, are required for prephenate-dependent anaerobic hydroxylation, they also identified a rhodanese-like protein YceA (named TrhO) in *E. coli* (YbfQ in *B. subtilis*) that utilizes O_2 as the substrate to synthesize ho^{5}U. Their extensive phylogenetic analysis showed a surprisingly diverse arrangement of ho^{5}U biosynthesis genes in nature, with some organisms harboring only one of the two pathways, while others, including *E. coli* and *B. subtilis*, utilize both pathways to enable ho^{5}U synthesis under both aerobic and anaerobic conditions. The data presented in this work are in complete agreement with their findings on the prephenate-dependent pathway.

One of the unique findings in this work is the discovery of the novel ferredoxin YfhL

as an additional requirement for the prephenate-dependent ho^{5}U pathway in *E. coli*. The *yfhL* mutant (as well as a *yfhL yegQ* double mutant) showed the same reduced level of cmo^{5}U in bulk tRNA as that in the *yegQ* and *aroD* mutants, which suggests that it is essential for the prephenate-dependent ho^{5}U pathway. Ferredoxins are ubiquitous throughout nature yet generally are ill-defined in terms of their physiological roles and specific redox partners (32). YfhL is a member of the "Alvin" ferredoxin family, first identified in *Allochromatium vinosum* (35). These novel proteins contain two [4Fe-4S] clusters of widely differing and very low redox potential ($E^o_1 \sim -650$ mV; $E^o_2 \sim -450$ mV) due to unique structural motifs (36). Elsen et al. found evidence that the essential Alvin ferredoxin in *Pseudomonas aeruginosa* was likely not involved in the catabolism of aromatic compounds but instead that its gene had the constitutive expression characteristics of a housekeeping gene (37). It's essentiality in that organism showed that other redox paralogs could not substitute for its essential function. Since many of these low potential ferredoxins exist in human pathogenic bacteria, they could be suitable as antibiotic targets. Since deletion of *yfhL* in *E. coli* appears to abolish prephenate-dependent ho^{5}U synthesis, it is possible that this is a specific function of *yfhL in vivo*. Mechanistically, it is not clear why YegQ (TrhP) would require electrons to catalyze what is formally an oxidation reaction, unless the mechanism is similar to a number of radical-mediated oxidation reactions. Recent work by Booker and coworkers has shown that in *Thermotoga maritima*, a ferredoxin together with a ferredoxin reductase efficiently provide electrons to the Fe-S cluster bound to the radical-SAM enzyme MiaB *in vitro* to support its methylthiolation of tRNA (38). The role for the reduced ferredoxin in these enzymes is to transfer an electron to facilitate formation of the 5′-adenosyl radical that initiates thiolation at the C-2 of adenosine. Thus, the role for YfhL in ho^{5}U synthesis may be to reduce an Fe-S cluster in YegQ for the formation of a radical species, such as a uridine-5-yl radical, that can combine with a hydroxyl radical from prephenate. Analysis of the *E. coli* flavodoxin/ferredoxin NADP reductase (*fpr*) gene for a possible requirement in ho^{5}U formation has not yet been assessed but could be the physiological source of reducing equivalents to *yfhL* via NADPH. Additional studies under anaerobic conditions will be required to further elucidate the role of a YfhL-YegQ interaction and possible electron transfer between the two proteins as well as the *in vitro* reconstitution of the hydroxylation step.

In *B. subtilis* and other bacteria without *yfhL* orthologs, ferredoxins of a different type or flavodoxins may act analogously to provide electrons for ho^{5}U formation. *B. subtilis* has one dedicated ferredoxin (Fer) and two flavodoxins (YkuN and YkuP) that could serve in this role, although all three proteins have previously been implicated in fatty acid desaturase activity *in vivo* (39) and they likely have additional redox roles. Interestingly, the ferredoxin/flavodoxin reductase is required for viability in *B. subtilis* (26). It is also possible that a unique redox protein exists that is specifically required for ho^{5}U synthesis in this organism. Identification of the specific redox factors may facilitate *in vitro* reconstitution of ho^{5}U activity.

Finally, during the search for evidence for an alternative independent pathway for ho^{5}U synthesis in bacteria, a few organisms that lack peptidase U32 genes were screened for the presence of ho^{5}U and its known derivatives in their tRNA. Data on modified nucleosides in tRNA for most organisms are lacking. However, recent work by Cao and Limbach using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (40) has shown that *Streptomyces griseus*, which has no peptidase U32 genes in its genome, also lacks ho^{5}U derivatives in its tRNA. A similar result has been confirmed in our laboratory for the closely related *Streptomyces coelicolor* A3(2) (my unpublished data). These organisms also appear to lack the newly discovered hydroxylase *trhO*, so they are expected to lack ho^{5}U-related modifications. Initial results indicate that *Lactobacillus casei* (ATCC 393), which also lacks both *trhP* and *trhO* homologs, was found to contain no ho^{5}U-related tRNA modifications. The alphaproteobacterium *Novosphingobium capsulatum* (ATCC 14666) has also been analyzed. This organism lacks peptidase U32 genes but does contain *trhO* as well as a homolog of the *trmR* methyltransferase. However, no mo^{5}U could be detected. Analysis of larger quantity

TABLE 1 Strains used in this study

Strain[a]	Genotype or characteristic(s)	Source(s)
BW25113	*E. coli* Keio reference strain	CGSC
JW2066	*yegQ*::*kan*	Keio collection/CGSC
JW1431	*rlhA*::*kan*	Keio collection/CGSC
JW3127	*yhbU*::*kan*	Keio collection/CGSC
CL5120	*yegQ*::*cat*	This work
CL5131	*yegQ*<>*frt*[b]	This work
CL5133	*yhbV*::*cat*	This work
CL5136	*yegQ*::*cat yhbU*::*kan*	This work
CL5140	*yegQ*::*cat rlhA*::*kan*	This work
CL5142	*yhbU*::*cm rlhA*::*kan*	This work
CL5150	*yegQ*<>*frt yhbUV*::*cat rlhA*::*kan*	This work
JW2514	*iscS*::*kan*	Keio collection/CGSC
JW1683	*aroD*::*kan*	Keio collection/CGSC
CL5161	*aroD*::*kan yegQ*::*cat*	This work
JW2546	*yfhL*::*kan*	Keio collection/CGSC
CL5164	*yfhL*::*kan yegQ*::*cat*	This work
Bacillus subtilis 168	*trpC2* (*B. subtilis* reference strain)	BGSC
BKE27360	*yrrM*::*erm*	BGSC
BKK27360	*yrrM*::*kan*	BGSC
BKE27350	*yrrN*::*erm*	BGSC
BKK27350	*yrrN*::*kan*	BGSC
BKE27340	*yrrO*::*erm*	BGSC
BKK27340	*yrrO*::*kan*	BGSC
CL5145	*yrrNO*::*kan*	This work
CL5149	*yrrNO*::*erm*	This work
BKK25660	*aroD*::*kan*	BGSC

[a]Strains below reference strains are the same organism.
[b]<frt> refers to FLP-mediated excision of the antibiotic resistance cassette, as per reference 15.

digests showed a very small amount (~0.01 mol/mol tRNA) of cmo^{5}U in its tRNA. This is an order of magnitude lower cmo^{5}U modification level than that found in the tRNA of *E. coli*. Homology searches show that the *N. capsulatum* genome contains two weakly homologous genes to *cmoA*, in addition to *trmR*, but no close homolog of *cmoB*, which is required for cmo^{5}U synthesis. Thus, one of the three methyltransferases is likely performing the function of the product of *cmoB* in this organism. These initial results, although limited, show that it may be difficult to predict based on homology alone which ho^{5}U derivative is produced by an organism and to what degree each tRNA will be modified. Further identification and quantitation of these modifications in a variety of organisms will be useful to further refine the annotation of the genes required for their biosynthesis.

MATERIALS AND METHODS

General methods. All strains were grown in LB medium (5 g/liter NaCl; Lennox) at 250 rpm and 37°C unless otherwise indicated. The following antibiotics were used with concentrations indicated: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (10 μg/ml for *B. subtilis* mutant strains and 30 μg/ml for *E. coli* plasmid maintenance), and erythromycin (1 μg/ml, used with 25 μg/ml lincomycin). Electrocompetent *E. coli* cells were prepared by harvesting cells from a 50-ml culture grown in LB to an A_{600} of 0.6 (4,000 rpm for 10 min at 4°C; SH-3000 rotor) and resuspending the cells at least three times in 40 ml of ice cold 10% glycerol followed by centrifugation at 4°C each time. The final cell pellet was suspended in 0.35 ml cold 10% glycerol, and 50 μl of cells were aliquoted into 1.5-ml tubes and stored at −80°C. Electroporation was performed using a BTX electroporation system with 1-mm gap cuvettes (BTX model 610). Electroporation instrument settings were 2.5 kV resistance, 50 μF capacitance timing, 129 Ohm resistance timing, and a charging voltage of 1.4 kV. Cells were suspended in expression recovery medium (Lucigen) immediately after electroporation and grown at the desired temperature for 1 h prior to plating on selective medium. *Bacillus subtilis* competent cells were prepared and transformed with DNA according to Koo et al. (26).

Generation and verification of deletion strains. A complete list of deletion strains obtained or generated in this study is found in Table 1. *Bacillus subtilis* single-gene deletion strains were obtained from the Bacillus Genetic Stock Center. The *yrrNO* double-knockout strain was prepared by replacing the *yrrNO* genes with a kanamycin resistance cassette amplified from plasmid pDR240a using the procedure of Koo et al. (26). Single gene deletion strains of *E. coli* (with the exception of the *yhbV* mutant) were from the Keio collection (27) obtained through the *E. coli* Genetic Stock Center. The *yhbV* single mutant and all multiple deletion strains were prepared by the method of Datsenko and Wanner (28) using either

TABLE 2 Plasmids used in this study

Plasmid	Characteristic(s)	Source or reference[a]
pBAD33	L-Arabinose-induced expression	41
pETiteN-His	N-terminal His_6 fusion, T7 promoter	Lucigen
pRhamN-His	N-terminal His_6 fusion, rham-induced	Lucigen
pKD3	Source of *cat* gene	CGSC (28)
pKD46	λ red recombinase/*bla* gene	CGSC (28)
pCL433	*E. coli yegQ*/pETiteN-His	This work
pCL455	*E. coli yegQ*/pBAD33	This work
pCL502	*B. subtilis yrrM*/pBAD33	This work
pCL512	*B. subtilis yrrN*/pRham	This work
pCL513	*B. subtilis yrrO*/pBAD33	This work
pCL496	*E. coli yfhL*/pBAD33	This work
pLIKE-rep	*B. subtilis* replicative plasmid	BGSC
pCL504	*yrrN*/pLIKE-rep	This work
pCL550	*yrrO*/pLIKE-rep	This work
pCL506	*yegQ*/pLIKE-rep	This work

[a]CGSC, Coli Genetic Stock Center (http://cgsc2.biology.yale.edu); BGSC, Bacillus Genetic Stock Center (www.bgsc.org).

chloramphenicol or kanamycin gene replacement using pKD46-mediated double crossover. Removal of the antibiotic resistance cassette was mediated by pCP20 as described (28). All mutant strains were verified by PCR using both internal and external primers to either the antibiotic resistance genes or the wt genes to ensure loss of the wt gene in the genome as well as correctly placed insertion of the antibiotic resistance gene. Primers for all mutant generation and verification are listed in Table 2.

Cloning and complementation analysis of genes required for ho^{5}U synthesis. For complementation of *E. coli* xo^{5}U-deficient mutant strains, *E. coli yegQ* and *yfhL*, as well as *B. subtilis yrrN* and *yrrO*, were each cloned into plasmid pBAD33 (41) using KpnI and HindIII (primers for all cloning and mutant verification are given in Table 2). Electrocompetent *E. coli* mutant strains were transformed with the corresponding plasmid and streaked on LB agar plates with chloramphenicol (30 μg/ml). Colonies were grown in LB with chloramphenicol and gene expression was initiated by addition of 0.1% L-arabinose at early log phase (A_{600} = 0.1). When cells reached early stationary phase (A_{600} = 0.8 to 1.0), they were harvested by centrifugation and tRNA was isolated and analyzed for xo^{5}U levels as described above. For complementation of *B. subtilis* mo^{5}U-deficient mutant strains, the pLIKE system was used (25). The pLIKE-rep plasmid contains a beta-lactamase gene for maintenance with ampicillin in *E. coli* and an erythromycin resistance cassette for propagation in *B. subtilis*. The *yrrN* and *yrrO* genes from *B. subtilis* and the *yegQ* gene from *E. coli* were cloned into pLIKE-rep using XbaI and HindIII as N-terminal and C-terminal primers, respectively (see Table 2).

Isolation and purification of total tRNA. Isolation of tRNA from both *B. subtilis* and *E. coli* was performed using the TRIzol reagent. Cell pellets from 50-ml cultures were resuspended in 10 mM Tris, pH 8, and treated with 15 mg of lysozyme in a 13-ml polypropylene tube at 37°C with shaking for 20 min. TRIzol (4 ml) was added, and the mixture was vortexed until homogeneous and allowed to stand at room temperature for 10 min. Chloroform (0.8 ml) was then added, and the mixture was shaken vigorously for 15 s and allowed to stand for an additional 10 min. After centrifugation (20 min at 10,000 rpm Sorvall SS-34 rotor at 4°C), the top aqueous layer was carefully transferred to a new polypropylene tube. Aqueous sodium acetate (0.35 ml of 3M) was added followed by cold ethanol (~9 ml to top of tube), and the tube was vortexed and stored at –20°C for 2 h to overnight to precipitate RNA. The precipitated RNA was washed with 2 ml of cold 70% aqueous ethanol, air dried for 10 min, and then dissolved in 2 ml of R200 buffer (10 mM Tris-phosphate [pH 6.3], 200 mM KCl, 15% ethanol). The crude tRNA was purified on an AX 500 column (Macherey-Nagel) as described (15) with the following modifications. The column was first washed with R1500 buffer and then equilibrated with R200 buffer. The RNA solution was added to the equilibrated column, which was then washed with 6 ml of R200 buffer and 4 ml of R600 buffer, and the tRNA was eluted with 7 ml R1000 buffer into a 13-ml polypropylene tube. Isopropanol (4.9 ml) was added, and the tube was vortexed and allowed to stand at room temperature (RT) for 30 min. The tRNA was isolated by centrifugation (Sorvall SS-34 rotor at 14,000 rpm at 4°C) for 30 min, and the pellet was washed with 2 ml of cold 70% ethanol, dried, and dissolved in water. A typical yield of tRNA from a 50-ml culture at late log phase was 0.5 mg. Denaturing gel electrophoresis showed no significant rRNA contamination. The tRNA purified in this manner was the starting material for the enrichment of single tRNA species.

Analysis of total tRNA from wt and mutant strains. Modification levels of AX 500-purified tRNA from the different strains were analyzed by enzymatic digestion and HPLC analysis of the digests. The method of Gehrke and Kuo (42) was used to digest tRNA. Two different protocols were used. To analyze for cmo^{5}U, 50 μg of tRNA was digested with 3 units of nuclease P1 (Millipore-Sigma) in a total volume of 90 μl of 30 mM sodium acetate (NaOAc), 0.2 mM zinc diacetate [$Zn(OAc)_2$] at 37°C for 1.5 h, and then 10 μl of morpholinepropanesulfonic acid (MOPS) (or Tris) buffer, pH 8.3, and 0.5 unit of bacterial alkaline phosphatase (Worthington) were added. The mixture was incubated at 37°C for 4 h to fully hydrolyze mcmo^{5}U to cmo^{5}U. For the analysis of mo^{5}U and ho^{5}U, a "quick" digestion protocol was used that employed 50 μg tRNA in 100 μl of 30 mM NaOAc, 0.2 mM $Zn(OAc)_2$, pH 6, with the above amounts of

both nuclease P1 and alkaline phosphatase incubated at 37°C for 1.5 h. This quick digestion avoided the production of a previously described degradation product that comigrates with ho^{5}U in the HPLC analysis (15).

HPLC analysis was essentially as previously described (15) using a Develosil RP-Aqueous C_{30} column (Phenomenex) with the following modifications. A binary gradient was used with buffer A consisting of 50 mM ammonium phosphate, pH 5.3, and buffer B consisting of buffer A with 20% (vol/vol) methanol. UV detection was used at 280 nm, and quantitation was based on peak integration with standardization by comparing the peak areas of known quantities of synthetic standards of mo^{5}U (Millipore Sigma) and ho^{5}U and cmo^{5}U (synthesis described below). Certain nucleosides (mo^{5}U, cmo^{5}U) isolated from mutant strains were also confirmed for correct mass using mass spectrometry of isolated HPLC peak fractions. Isolated ho^{5}U, cmo^{5}U, and mo^{5}U were also analyzed by coinjection with synthetic standards in a different chromatographic system (Supelco LC-18S column using binary gradient with 5 mM ammonium acetate as buffer A and 40% aqueous acetonitrile as buffer B).

Enrichment of single tRNA species from wt and mutant strains. Individual tRNAs were isolated from AX 500-purified tRNA using the batchwise method of Tsurui et al. (33) with the buffer system of Suzuki and Suzuki (34), as modified by Kohrer et al. (43). Biotinylated oligonucleotides complementary to residues 1 to 37 of the targeted tRNA were used to bind individual tRNAs. These oligonucleotides were immobilized by adding 27 nmol biotinylated DNA to 0.3 ml (settled volume) of streptavidin-agarose (85-nmol/ml binding capacity; EMD) in binding buffer of 10 mM HEPES, pH 7.5, 400 mM NaCl, and 5 mM EDTA. The mixture was rotated at RT for 1 h and then transferred to an empty Bio-Rad Econo-Column with frit and washed with 4 × 1 ml binding buffer to remove unbound oligonucleotide. From the A_{260} of the washes, the amount of bound oligonucleotide was estimated at 95%. The resin was then washed with 1× binding buffer (34) consisting of 30 mM HEPES (pH 7.5), 1.2 M NaCl, and 15 mM EDTA. A total of 4 mg of purified bulk tRNA was used as the starting material for the isolation. Thus, 4 mg AX 500-purified tRNA from either wt or mutant strain was dissolved in binding buffer and added to the 0.25 ml DNA-bound resin in a total volume of 1.2 ml. The mixture was heated to 70°C in a heating block for 30 min and then slow cooled over 30 min to 30°C. After incubation at 30°C for 30 min, the resin was transferred to a column and washed with 4 × 1 ml 0.5× binding buffer preequilibrated to 37°C (34). The first two 1-ml washes were precipitated with 2.5 vol cold ethanol and reserved for subsequent tRNA isolations. The resin in the column was then further washed with 2 × 0.4 ml of 0.05× (1/20) binding buffer and transferred to a 1.5-ml tube. The tube was centrifuged for 30 s (10,000 rpm in microcentrifuge at RT), and the supernatant was removed. The tRNA-bound resin was then eluted with 4 × 0.45 ml of 0.017× (1/60) binding buffer preequilibrated to 65°C. To each elution fraction (~0.4 ml) was added 40 μl of 5 M NaCl and 1 ml of cold ethanol, and the eluted tRNA was stored at −20°C overnight. The typical yield of tRNA was 20 to 40 μg in fraction 1 and 10 to 20 μg in fraction 2. After each round of tRNA isolation, the isolated and precipitated flow through and washes containing the nonbinding tRNA were pooled and precipitated for the next round of tRNA isolation. Purity of the individual tRNAs was estimated by PAGE analysis and HPLC modification profile. For the gel analysis, 0.5 μg of tRNA was loaded onto denaturing polyacrylamide minigels using urea as denaturant (SequaGel; National Diagnostics). Gels were stained with ethidium bromide in Tris-acetate-EDTA (TAE) buffer, and RNA was visualized by UV transillumination. Unmodified individual tRNA transcripts were used as estimates of migration standards. HPLC analysis of tRNAs as described above was used to assess if the isolated individual tRNAs showed only the expected modification profile (7, 8), which was also an indication of purity. Initial analysis showed that a significant amount of the DNA oligonucleotides coelute with the eluted tRNA; therefore, 15 μg of each tRNA was treated with 3 units of RQ1 DNase (Promega) in recommended buffer in a total volume of 20 μl for 1 h at 37°C. The tRNA was then applied to a 0.8-ml Sephadex G-50 spin column using empty Micro Bio-Spin columns (Bio-Rad). The G-50 columns were centrifuged at 7,000 rpm in an Eppendorf microcentrifuge for 1 min to remove water, and then the reaction mixture was loaded onto the center of the resin and the column was centrifuged again to separate the tRNA from the buffer and hydrolyzed DNA. The purified tRNA was then digested and analyzed by HPLC as described above. For each enriched tRNA species, xo^{5}U species were quantified by absorbance at 280 nm and the ratio of xo^{5}U/Ψ was recorded for mutant strains relative to that of the wt.

Gel mobility shift experiments. The presence of a YrrN-YrrO protein complex was analyzed using nondenaturing PAGE with Tris-glycine buffer. Briefly, mixtures of 2 μg of N-terminal His_6 fused *B. subtilis* YrrO (His_6-YrrO) with increasing amounts of His_6-YrrN (0.125 to 2 molar equivalents) in 30 mM Tris-glycine, pH 7.5, and 10 mM DTT (10 μl total volume) were incubated at RT for 15 min. Each binding mixture was then loaded into an individual lane on a 10% polyacrylamide gel using 30 mM Tris-glycine, pH 7.5, as running buffer. Proteins were visualized using Coomassie brilliant blue R200 and destained with 10% (vol/vol) acetic acid and 10% (vol/vol) methanol in water. Native gels constructed for tRNA binding analysis consisted of 8% polyacrylamide-bisacrylamide (19:1) in either 1× Tris-borate-EDTA (TBE) or 30 mM Tris-glycine buffer at pH 7.5 containing 2 mM $MgCl_2$.

Preparation of synthetic ho^{5}U and cmo^{5}U. The synthesis of ho^{5}U used a minor modification of the procedure of for the preparation of 5-hydroxy-2′-deoxyuridine (44). The crude material was purified by silica gel chromatography using acetonitrile-water (9:1) as eluent. This material was used to prepare cmo^{5}U as follows. To a solution of 10.5 mg (40 μmol) ho^{5}U in 260 μl H_2O in a 1-ml brown glass vial was added 40 μl (80 μmol) of 2 N NaOH at room temperature to give a pH of >9. To this mixture, 7.5 mg (40 μmol) of iodoacetic acid was added, and the reaction was mixed by vortexing and allowed to stand overnight. Two additional aliquots of 7.5 mg iodoacetic acid and 40 μl 2 N NaOH were added at 24-h intervals to maintain the pH of >9. After a total of 72 h, HPLC showed the reaction was approximately 70% complete. The desired product was purified by HPLC on a Supelco LC-18 semiprep column (5 μm,

25 cm by 10 mm) using 5 mM ammonium acetate, pH 5.3, as buffer A and 40% acetonitrile in water as buffer B with the following step gradient: $t = 0$ to 13 min 0% B; $t = 13$ to 20 linear gradient of 0% to 100% B. Flow rate was 3 ml/min, and cmo^{5}U was detected at 280 nm. The final yield of cmo^{5}U was 55% and was >99% pure by analytical HPLC (t_R = 35.5 min; Supelco LC-18s column; same gradient as described for purification; t_R = 33.5 min RP-Aqueous C_{30} column, gradient described above for analysis of tRNA digests). UV λ_{max} = 278; ^{1}H NMR 500 MHz (D_2O) δ 7.53 (s, 1H), 5.98 (t, 1H, J = 4.0 Hz), 4.39 (s, 2H), 4.36 (m, 1H), 4.27 (m, 1H), 4.17 (m, 1H), 3.92 (AB part of ABX pattern, 2H). HRMS calculated for $C_{11}H_{14}N_2O_9(H^+)$: 319.07721. Found: 319.0765.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00433-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

I thank Daniel Ziegler of the Bacillus Genetic Stock Center for helpful discussions on preparing deletion mutants and using expression plasmids in *B. subtilis*.

I gratefully acknowledge the School of Pharmacy at the University of Wisconsin-Madison for financial support.

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