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Abbreviated Pathway for Biosynthesis of 2-Thiouridine in *Bacillus subtilis*

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ABSTRACT

The 2-thiouridine (s^2U) modification of the wobble position in glutamate, glutamine, and lysine tRNA molecules serves to stabilize the anticodon structure, improving ribosomal binding and overall efficiency of the translational process. Biosynthesis of s^2U in *Escherichia coli* requires a cysteine desulfurase (IscS), a thiouridylase (MnmA), and five intermediate sulfur-relay enzymes (TusABCDE). The *E. coli* MnmA adenylates and subsequently thiolates tRNA to form the s^2U modification. *Bacillus subtilis* lacks IscS and the intermediate sulfur relay proteins, yet its genome contains a cysteine desulfurase gene, *yrvO*, directly adjacent to *mnmA*. The genomic synteny of *yrvO* and *mnmA* combined with the absence of the Tus proteins indicated a potential functionality of these proteins in s^2U formation. Here, we provide evidence that the *B. subtilis* YrvO and MnmA are sufficient for s^2U biosynthesis. A conditional *B. subtilis* knockout strain showed that s^2U abundance correlates with MnmA expression, and *in vivo* complementation studies in *E. coli* IscS- or MnmA-deficient strains revealed the competency of these proteins in s^2U biosynthesis. *In vitro* experiments demonstrated s^2U formation by YrvO and MnmA, and kinetic analysis established a partnership between the *B. subtilis* proteins that is contingent upon the presence of ATP. Furthermore, we observed that the slow-growth phenotype of *E. coli* Δ*iscS* and Δ*mnmA* strains associated with s^2U depletion is recovered by *B. subtilis yrvO* and *mnmA*. These results support the proposal that the involvement of a devoted cysteine desulfurase, YrvO, in s^2U synthesis bypasses the need for a complex biosynthetic pathway by direct sulfur transfer to MnmA.

IMPORTANCE

The 2-thiouridine (s^2U) modification of the wobble position in glutamate, glutamine, and lysine tRNA is conserved in all three domains of life and stabilizes the anticodon structure, thus guaranteeing fidelity in translation. The biosynthesis of s^2U in *Escherichia coli* requires seven proteins: the cysteine desulfurase IscS, the thiouridylase MnmA, and five intermediate sulfur-relay enzymes (TusABCDE). *Bacillus subtilis* and most Gram-positive bacteria lack a complete set of biosynthetic components. Interestingly, the *mnmA* coding sequence is located adjacent to *yrvO*, encoding a cysteine desulfurase. In this work, we provide evidence that the *B. subtilis* YrvO is able to transfer sulfur directly to MnmA. Both proteins are sufficient for s^2U biosynthesis in a pathway independent of the one used in *E. coli*.

Posttranscriptional RNA modifications are found among all organisms and are essential for their cellular function. More than 100 RNA modifications have been identified thus far which have a variety of physiological functions (1). In particular, posttranscriptional modifications of tRNA are often necessary for translational fidelity and efficiency, especially those found in anticodon bases. The third base in the anticodon is deemed the wobble position, as it is bound more loosely to the ribosome than the first two. Consequently, wobble bases have greater flexibility, allowing them to make noncanonical base pairs, leading to a greater chance of amino acid misincorporation into a growing peptide chain. An explanation of how this phenomenon is overcome is provided by the modified wobble hypothesis, which states that certain base modifications have evolved to adjust the anticodon shape, either to limit or to enable the occurrence of wobble pairing (2).

The modification of the wobble (34th position) in glutamate (Glu), glutamine (Gln), and lysine (Lys) tRNA molecules produces 5-methyl-2-thiouridine derivatives (xm^5s^2U) (Fig. 1). Thiolation of the wobble uridine places the base in an anti, C-3′-endo, gauche conformation, thus forming a rigid structure, which increases its affinity for pairing with adenosine and thereby yields higher accuracy in the corresponding peptide sequence. Additionally, this modification stabilizes the anticodon structures and confers the tRNA molecule's ability to bind to the ribosome. Tighter ribosomal binding to tRNA subsequently improves reading frame maintenance and deficiencies in the translational process (2–5). Lack of the 2-thiouridine (s^2U) modification in tRNA causes growth defects in bacteria attributed to accumulation of frameshifting during translation (6). In humans, mutations within the gene coding for the 2-thiouridylase TrmU, responsible for the sulfur insertion step, has been associated with acute infantile liver failure and respiratory defects that lead to dysfunction of the auditory system (7).

The biosynthesis of xm^5s^2U modification involves two distinct

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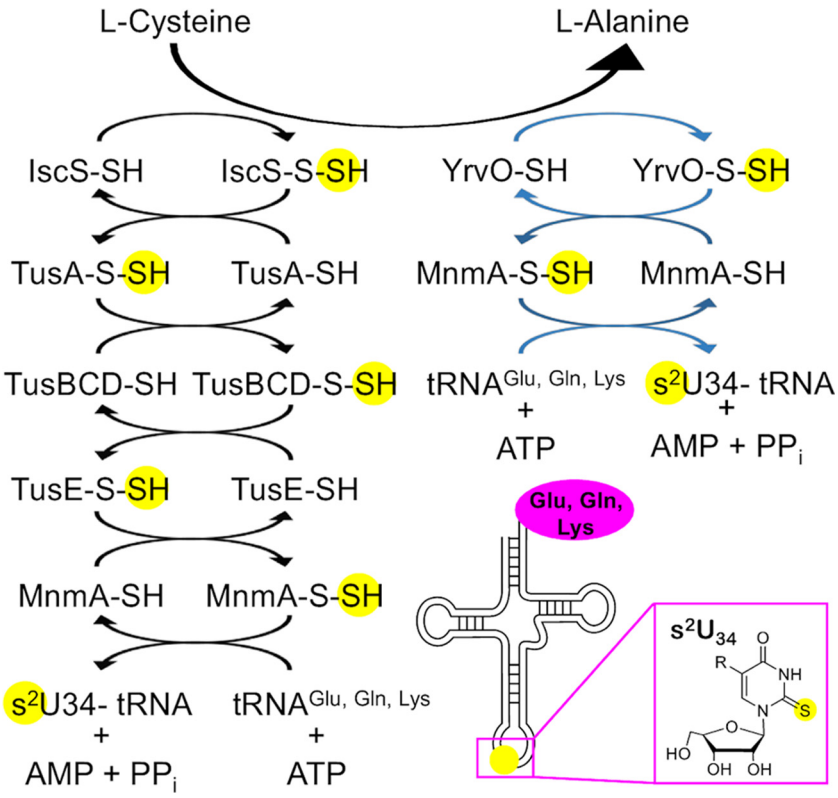

FIG 1 Biosynthetic pathway for tRNA s^2U formation in *E. coli* (black arrows) and proposed pathway in *B. subtilis* (blue arrows). (Inset) Position of the $tRNA^{Glu}$, $tRNA^{Gln}$, and $tRNA^{Lys}$ wobble uridine (U_{34}) and structure of modified 2-thiouridine. The R group at position 5 of U34 can occur as H, CH_2NH_2, CH_2NHCH_3, $CH_2NHCH_2CO_2^-$, or $CH_2CO_2CH_3$.

branches of U34 modification at C-5 and C-2 positions. The synthesis of 5-methylaminomethyl-uridine (mnm^5U) is mediated by the MnmEG complex, which catalyzes the insertion of the aminomethyl (nm) or carboxymethylaminomethyl (cmnm) group to the C-5 position of U34, and by the bifunctional enzyme MnmC, which is involved in the conversion of nm^5U or $cmnm^5U$ into mnm^5U tRNA (8, 9). In a parallel pathway, the thiolation of C-2 of U34 in *Escherichia coli* has been shown to involve seven proteins: IscS, TusA, the TusBCD complex, TusE, and MnmA (Fig. 1) (3, 6, 10, 11). Gene inactivation studies showed that elimination of the thiolation branch involving s^2U formation results in bacterial growth defects and consequently accumulation of the mnm^5U intermediate, revealing the essentiality of each component for *in vivo* functionality of this thiolation pathway (3, 11).

In *Salmonella enterica* and *E. coli*, the s^2U thiolation pathway is initiated by the cysteine desulfurase IscS (6, 11), which catalyzes the pyridoxal-5′-phosphate-dependent cleavage of a carbon-sulfur bond from the amino acid cysteine. During catalysis, the enzyme forms a covalent-persulfide intermediate, which serves as a hub for sulfur delivery in the biosynthesis of several thio-cofactors not limited to s^2U formation (12). The versatility of IscS, which participates in multiple pathways, has been associated with its ability to interact with and transfer sulfur to a variety of sulfur acceptor molecules. In fact, inactivation of *iscS* in *E. coli* dramatically decreases the growth rate in rich medium and leads to a null-growth phenotype in minimal medium. It has been established that pleiotropic defects associated with *iscS* gene deletion are attributed to severe defects in synthesis of Fe-S clusters, thiamine, lipoic acid, molybdenum cofactor, and biotin, in addition to all thio-nucleosides (13). In the s^2U pathway, IscS is capable of persulfide sulfur transfer to a cysteine residue of TusA, which is then transferred to another cysteine residue within TusD when in a complex with TusC and TusB. In this sulfur relay scheme, TusE is also proposed to serve as a persulfide intermediate, since it is able to interact with the TusBCD complex and to form a ternary complex with MnmA and tRNA (14). The final sulfur insertion event is catalyzed by the thiouridylase MnmA in a reaction analogous to that of ThiI in the biosynthesis of s^4U (15). The bifunctional enzyme MnmA promotes the ATP-dependent activation of C-2 of U34 tRNA and subsequent sulfur transfer from its persulfurated form to the tRNA substrate (14). Structural and mutagenesis studies have provided insight into the mode of tRNA binding as well as the MnmA residues involved in substrate activation and thiolation. Interestingly, despite the requirement of all seven components for the *in vivo* formation of s^2U, *in vitro* reconstitution of this pathway showed that only IscS and MnmA are indispensable components, while TusA, TusBCD, and TusE are utilized for maximum activity levels (3). Furthermore, bioinformatic analysis of sequenced genomes showed that the Tus proteins are not as ubiquitously distributed as the occurrence of s^2U, suggesting the existence of alternative mechanisms for the biosynthesis of this modification in most species.

The model Gram-positive bacterium *Bacillus subtilis* does not contain the master cysteine desulfurase IscS, but instead its genome encodes four functionally active cysteine desulfurases: SufS, NifZ, NifS, and YrvO (16). Their genomic locations provide in-

sight into their functions in sulfur metabolism. In previous work, we and others showed that *sufS* is adjacent to its sulfur acceptor gene, *sufU* (17, 18), coding for a zinc-dependent sulfur transfer protein (19). Both proteins are involved in the formation of Fe-S clusters. Likewise, *nifZ* is located immediately upstream of the sulfur acceptor gene *thiI*, and both gene products are dedicated partners in the biosynthesis of s^4U in tRNA (20). Interestingly, the *yrvO* coding sequence is 31 bases away from the *mnmA* orthologous gene, suggesting that they are cotranscribed and possibly indicating the involvement of both proteins in the biosynthesis of s^2U tRNA. Furthermore, genome sequence searches failed to identify sequences coding for the Tus proteins participating in the sulfur relay pathway, the only exception being YrkF protein, which shows 38% identity to TusA. The genomic synteny of *yrvO* and *mnmA* and the lack of the Tus ortholog proteins led us to hypothesize that the presence of a devoted cysteine desulfurase promotes the direct sulfur transfer from cysteine to MnmA, dispensing with the need for a sulfur relay path as observed in *E. coli*.

Here, we describe the functional investigation of *Bacillus subtilis* YrvO and MnmA in the biosynthesis of s^2U tRNA. Biochemical analysis shows that YrvO is a cysteine desulfurase able to transfer sulfur to a cysteine residue within MnmA only when in the presence of ATP. Together, YrvO and MnmA are capable of forming s^2U tRNA *in vitro*. Using an *in vivo* complementation approach in *E. coli*, we demonstrate that YrvO and MnmA constitute a two-component pathway in which both proteins are mutually specific and competent in synthesizing s^2U using a distinct strategy from that of *E. coli*. Coexpression of *B. subtilis* YrvO and MnmA in an *E. coli iscS* deletion strain restores s^2U synthesis and partially recovers growth defects associated with loss of the master cysteine desulfurase, revealing the importance of this modification in cellular maintenance.

MATERIALS AND METHODS

Media, media additions, and chemicals. LB and M9 media (21) were used with the following concentrations of antibiotics and medium additives, unless otherwise specified: ampicillin (100 μg/ml), kanamycin (40 μg/ml), erythromycin (0.5 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside; 10 μg/ml), L-arabinose (2 mg/ml), L-isoleucine (1 mM), L-leucine (1 mM), L-methionine (0.25 mM), and L-cysteine (1 mM). MS-I medium consists of 17.5 mM NH_4SO_4, 80.3 mM K_2HPO_4, 44.1 mM KH_2PO_4, 3.4 mM trisodium citrate dehydrate, 0.81 mM $MgSO_4$, 0.5% glucose, 0.02% Casamino Acids, and 0.1% yeast extract. MS-II medium consists of MS-I medium with 0.125 mM $MgSO_4$ and 0.0125 mM $CaCl_2$. Unless otherwise specified, all chemicals were purchased from Fisher Scientific and Sigma-Aldrich Inc. Restriction enzymes were purchased from New England BioLabs.

Plasmid construction. All genes were amplified from *B. subtilis* strain PS832 genomic DNA, which was prepared using a commercial DNA extraction kit (QuickExtract; Epicentre). Amplification PCRs were performed using a Fail Safe PCR kit (Epicentre). The PCR products were all previously cloned into TopoTA vector (Invitrogen) for subcloning purposes. The correct sequences of all plasmids used in this study was confirmed by DNA sequencing (Genewiz). A comprehensive list and descriptions of primers and plasmids used in this work are shown in Table S1 in the supplemental material.

***B. subtilis* strain construction.** All strains of *B. subtilis* listed in Table S1 in the supplemental material were derivatives of strain 168 (strain PS832). The transformation protocol was as previously described (20). Transformed cells were selected for erythromycin resistance and blue/white selection in the presence of X-Gal and 1 mM IPTG.

Growth curve analysis. Cell growth was measured by monitoring absorbance at 650 nm (A_{650}) while strains were incubated with shaking at 37°C. Individual colonies were outgrown in 5 ml of the appropriate medium overnight. Overnight cultures were diluted to an optical density of 0.1, and subsequently, 5 μl of cell suspension was used to inoculate 195 μl of the appropriate medium in each well of a 96-well microtiter plate. Growth at 37°C with periodic shaking at intensity level 2 was monitored using a microplate spectrophotometer (Bio-Tek Instruments).

Isolation of tRNA from *B. subtilis* and *E. coli* cells. *B. subtilis* wild-type strain PS832 and strain J1235, kindly provided by T. J. Larson, were grown in 500 ml LB medium. *B. subtilis* strain DD19 was grown in the presence of 0 to 1 mM IPTG in 500 ml LB medium with erythromycin at 37°C to an optical density at 600 nm (OD_{600}) of 0.8 to 1.2. *E. coli* strain CL100 transformed with pBAD, pDS22, or pDS145 and *E. coli* strain JW1119 transformed with pBAD, pDS151, or pDS145 were grown in LB medium containing ampicillin and arabinose at 37°C to an OD_{600} of 0.8 to 1.2. Cells were harvested by centrifugation at 8,200 × *g* for 10 min and frozen at −20°C until further use.

The protocol for tRNA isolation was adapted from a method described elsewhere (20) with the following changes. Phenol for extraction was saturated with 1 M sodium acetate (pH 4.5). Following ethanol precipitation and centrifugation, the pellet was washed with 70% ethanol and then allowed to dry for 5 to 10 min. The pellet was resuspended in H_2O, and the concentration and purity (determined by the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230}) were analyzed using a Nanodrop spectrophotometer (Fisher Scientific).

***In vitro* generation of s^2U34 in *in vivo* isolated tRNA.** The pH of *in vivo*-isolated tRNA dissolved in H_2O was adjusted using 20 mM HEPES (pH 7.5). tRNA samples were diluted to 187 μM and folded in an 80°C water bath, and 1 mM $MgCl_2$ was added at 65°C. Assay reaction mixtures (1 ml) containing 5.6 μM tRNA, 1.7 μM YrvO or *Azotobacter vinelandii* IscS, 0.5 mM cysteine, and 5 mM $MgCl_2$ in the presence or absence of 34 μM *B. subtilis* or *E. coli* MnmA and 1 mM ATP were incubated at 37°C for 1 h. When indicated, dithiothreitol (DTT) was added to a final concentration of 2 mM. To isolate the tRNA following incubation, samples were extracted with 300 μl of phenol saturated with 1 M sodium acetate (pH 4.5) and precipitated with the addition of 2 M ammonium acetate and 2 ml of cold ethanol overnight at −20°C.

Analysis of modified tRNA nucleosides. Unfractionated tRNA isolated from *B. subtilis* and *E. coli* cells was digested into individual nucleosides for analysis. Approximately 60 μg tRNA dissolved in H_2O was heated at 100°C for 5 min and subsequently chilled on ice for 5 min. Once the mixture was cooled, 10 μl of 0.1 M ammonium acetate (NH_4OAc) and 10 μl of nuclease P1 (1 mg/ml) were added and incubated at 50°C for 3 h. Next, 20 μl of 0.1 M ammonium bicarbonate, 10 μl of FAST alkaline phosphatase (Life Technologies), and 20 μl phosphatase buffer were added and incubated at 37°C for 2 h. The sample was then centrifuged at 15,700 × *g* for 5 min, and the supernatant was transferred to a new tube. Methanol and formic acid were added to the sample to final concentrations of 2% and 0.1%, respectively, and a 20-μl sample was analyzed by high-performance liquid chromatography (HPLC) using electron spray ionization-mass spectrometry (ESI-MS) and/or a UV detector.

Digested tRNA samples were analyzed by HPLC using a C_{18} column (Supelcosil), coupled with a mass spectrometer (Orbitrap). Solvent A was Optima liquid chromatography-mass spectrometry (LC-MS)-grade water with 0.1% formic acid, and solvent B was Optima LC-MS grade methanol with 0.1% formic acid. The HPLC conditions included a solvent B gradient of 2% from 0 to 15 min, 5% at 22 min, 75% at 37 min, 100% at 38 min, and 2% for 7 min at a flow rate of 0.3 ml/min with 20-μl injections. The mass spectrum was recorded in the positive mode under the following conditions: nebulizer pressure of 2 torr, a drying gas flow of 53 liters/min at 350°C, and a capillary voltage of 27 V. Standards of s^4U (Sigma) and s^2U (Carbosynth) were run as controls.

Protein expression and purification. Plasmid pDS22 containing *yrvO* was transformed into *E. coli* CL100 competent cells and selected for am-

picillin resistance. Single colonies were inoculated in 3 liters of LB medium containing ampicillin and grown overnight (18 h) at 30°C in the presence of L-arabinose (0.2%). Cells were harvested by centrifugation at 8,200 × *g* for 10 min and resuspended (3 ml/g of cells [wet weight]) in 25 mM Tris-HCl (pH 8), 10% glycerol (buffer A). Cells were lysed using an Emulsiflex C5 high-pressure homogenizer (Avestin) and centrifuged for 15 min at 12,857 × *g*. Supernatant was incubated with 1% (wt/vol) streptomycin on ice for 15 min then centrifuged for 15 min at 12,857 × *g*. The supernatant was loaded using a fast protein liquid chromatography (FPLC) system (AktaPurifier; GE Healthcare) onto a 5-ml Q-Sepharose column (GE Healthcare) preequilibrated with buffer A. The column was washed with buffer A, and the sample was eluted at a flow rate of 2 ml/min with a 0-to-70% gradient of 25 mM Tris-HCl (pH 8), 1 M NaCl, 10% glycerol (buffer B) over 20 column volumes. The collected eluent was analyzed by SDS-PAGE, and the fractions that eluted with 40% buffer B, containing the desired protein of 41.2 kDa, were combined. Pooled fractions were treated with 75% saturating concentrations of ammonium sulfate with incubation on ice for 15 min, followed by centrifugation at 31,209 × *g* for 15 min after each step. The 75% ammonium sulfate pellet containing the desired protein was resuspended in 10 ml of 25 mM Tris-HCl (pH 8), 300 mM NaCl, 10% glycerol (buffer C) and loaded using an FPLC onto a 2.5-cm by 60-cm Sephacryl S-200 column (GE Healthcare) preequilibrated with buffer C, at a flow rate of 1 ml/min. The eluent was analyzed by SDS-PAGE, and the desired fractions were pooled and dialyzed against 25 mM Tris-HCl (pH 8), 0.1 M NaCl, 10% glycerol (buffer D) overnight at 4°C. The dialyzed sample was pelleted in liquid nitrogen for storage at −80°C.

Plasmid pDS151 containing *mnmA* was transformed into *E. coli* Arctic Express competent cells (Stratagene) and selected for ampicillin resistance. Plasmids containing *mnmA* variants were transformed into *E. coli* Rosetta 2(DE3) (Merck Millipore) competent cells. For expression of all *mnmA* variants, single colonies were inoculated into 3 liters of LB medium containing ampicillin and grown to an OD_{600} of 0.5 to 0.6 at 25°C. Cell cultures were induced with lactose (0.2%) and grown overnight (16 h) at 15°C, followed by centrifugation at 8,200 × *g* for 10 min. Cells were resuspended (5 ml/g of cells [wet weight]) in buffer C, lysed using an Emulsiflex C5 high-pressure homogenizer (Avestin), and centrifuged for 15 min at 12,857 × *g*. The crude extract was loaded onto a 5-ml HisTrap column preequilibrated with buffer C and isolated through nickel-nitrilotriacetic acid (Ni-NTA) chromatography by elution at 50% buffer E (25 mM Tris-HCl [pH 8], 300 mM NaCl, 500 mM imidazole). The eluent was analyzed by SDS-PAGE. The desired fractions were treated with 2 mM DTT, dialyzed overnight against degased buffer D, and then pelleted in liquid nitrogen for storage at −80°C. All protein concentrations were determined by the method of Bradford (22), using a Bio-Rad protein assay kit and bovine serum albumin as a standard.

Cysteine desulfurase activity. Cysteine desulfurase activity was assessed through formation of S^{2-} and alanine as previously reported (23). Assays were performed with fixed concentrations of YrvO (0.05 mg, 1.5 μM), varying the concentrations of L-cysteine (0.0125 to 0.5 mM) and in the presence of 2 mM DTT and/or MnmA (1 mg, 29 μM).

^{35}S transfer assays. Assay reactions (60 μl) contained 9 μg (3.6 μM) YrvO and 18 μg (6.9 μM) MnmA, 1 mM ATP, 5 mM $MgCl_2$ in 50 mM Tris-HCl (pH 8) with 200 μM L-cysteine and 10 μCi ^{35}S-cysteine (PerkinElmer). Reaction mixtures were incubated at 37°C for 1 h and quenched with 0.4 mM *N*-ethylmaleimide for 5 min. Reactions were evaluated by nonreducing SDS-PAGE. Protein size and migration were observed by Coomassie brilliant blue staining, and ^{35}S-persulfurated proteins were visualized using PhosphorImager (Bio-Rad).

Western blots. Crude extract aliquots (40 μg of protein) obtained from cell lysates from the appropriate cell culture were separated on 12% SDS-PAGE gels for 1.5 h at 150 V and transferred to polyvinylidene difluoride (PVDF) Immobilon membranes (Millipore) at 100 V for 1 h. Polyclonal primary rabbit antiserum raised against purified *B. subtilis* MnmA (Thermo Scientific) was used to probe expression of MnmA in *B. subtilis* crude extracts. Anti-rabbit antibody conjugated with alkaline phosphatase produced in goat (Sigma) served as the secondary antiserum. LumiPhosWB (Pierce) was used as the substrate for chemiluminescent detection following the manufacturer's protocol.

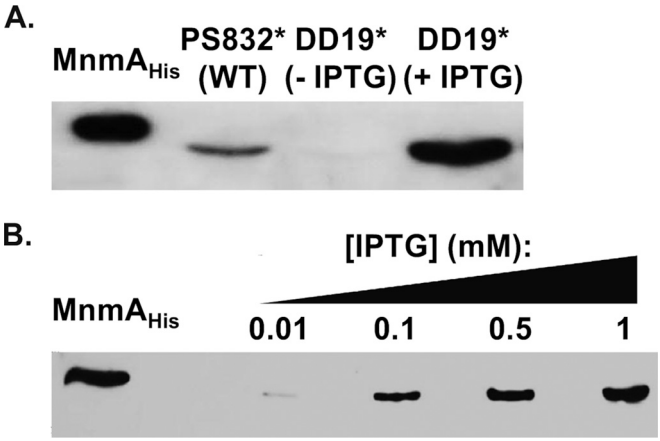

FIG 2 Western blots displaying MnmA expression in 40 μg of crude protein extracts using an antibody against *B. subtilis* MnmA. (A) MnmA expression in the *B. subtilis* wild-type strain PS832 and in the *mnmA* conditional knockout strain DD19, in the absence of the IPTG inducer (−IPTG) or in the presence of 1 mM IPTG (+IPTG). See also Fig. S1 in the supplemental material. (B) MnmA expression patterns in DD19 in the presence of variable IPTG concentrations.

RESULTS

Levels of expression of MnmA affects abundance of s^2U formation in *Bacillus subtilis*. Although the s^2U modification affects cellular viability in *E. coli*, it is not an essential modification (11). Therefore, we hypothesized that this modification would also not be essential in *B. subtilis* and strains lacking YrvO and/or MnmA would be viable. However, despite numerous attempts, we were unable to inactive *yrvO*. Likewise, similar strategies for inactivating *mnmA* have also failed. These results are also supported by a previous report suggesting that both YrvO and MnmA are among the select group of essential genes in *B. subtilis* (24). As an alternative approach, we constructed a conditional knockout strain (DD19) by inserting an IPTG-inducible promoter (P_{SPAC}) (25) upstream of *mnmA* while retaining an intact copy of *yrvO*. This genetic construct allowed controlled expression of MnmA when culturing DD19 strain in different concentrations of IPTG (Fig. 2), but it did not completely eliminate the expression of MnmA. Longer exposure of the Western blot film showed detection of trace amounts of MnmA (see Fig. S1 in the supplemental material).

The involvement of MnmA in the biosynthesis of s^2U was then assessed by quantification of modified nucleosides from purified tRNA samples. In studied systems, the absence of the thiolation pathway leads to accumulation of 5-methylaminomethyl uridine precursor (mnm^5U) (see Fig. S2 in the supplemental material), therefore the ratio of fully modified mnm^5s^2U to mnm^5U precursor was used as a normalized readout for s^2U abundance (11, 26). Since standards of both mnm^5U and mnm^5s^2U are not commercially available, we have optimized our protocol for tRNA extraction and digestion for separation and detection using HPLC/MS analysis. The resolution of this technique allows quantification of the specific mass abundance of nucleosides with high resolution

TABLE 1 tRNA modification levels in *B. subtilis* strains

B. subtilis strain (relevant genotype)	IPTG concn (mM)	mnm^5s^2U/mnm^5U (SD)[a]
PS832 (wild type)	0	12.7 (2.9)
J1235 (*yrkF*::*cat*)	0	15.1 (1.3)
DD19 (P-$_{SPAC}$-*mnmA*)	0	5.0 (0.12)
	0.01	7.1 (1.6)
	1	24.7 (2.2)

[a] The mass abundance ratio of mnm^5s^2U to its precursor nucleoside, mnm^5U, is shown. Values are means. Standard deviations were obtained from at least three independent experiments.

TABLE 2 tRNA modification levels in *E. coli* strains expressing *B. subtilis* MnmA and/or YrvO

E. coli strain (relevant genotype)	Expression vector	mnm^5s^2U/mnm^5U (SD)[a]
BW25113 (wild type)	pBAD	**30.2 (1.4)**
MG1655 (wild type)	pBAD	**30.2 (2.3)**
JW1119 (Δ*mnmA*)	pBAD	0.14 (0.04)
	pBAD-*mnmA*	0.05 (0.04)
	pBAD-*mnmA*(*C51A*)	0.02 (0.01)
	pBAD-*mnmA*(*C66A*)	0.03 (0.02)
	pBAD-*mnmA*(*C104A*)	0.06 (0.01)
	pBAD-*mnmA*(*C200A*)	0.09 (0.02)
	pBAD-*mnmA*(*C304A*)	0.04 (0.02)
	pBAD-*mnmA*(*C352A*)	0.07 (0.01)
	pBAD-*yrvO-mnmA*	**31.7 (5.7)**
	pBAD-*yrvO-mnmA*(*C51A*)	0.18 (0.03)
	pBAD-*yrvO-mnmA*(*C66A*)	**35.8 (3.45)**
	pBAD-*yrvO-mnmA*(*C104A*)	0.13 (0.001)
	pBAD-*yrvO-mnmA*(*C200A*)	0.11 (0.02)
	pBAD-*yrvO-mnmA*(*C304A*)	**28.1 (1.4)**
	pBAD-*yrvO-mnmA*(*C352A*)	**7.0 (0.6)**
CL100 (Δ*iscS*)	pBAD	0.04 (0.03)
	pBAD-*yrvO*	0.002 (0.08)
	pBAD-*yrvO-mnmA*	**64.7 (4.1)**
	pBAD-*yrvO*(*C325A*)-*mnmA*	0.03 (0.01)
JW3435 (Δ*tusA*)	pBAD-*yrvO*	0.006 (0.003)
	pBAD-*mnmA*	0.035 (0.013)
	pBAD-*yrvO-mnmA*	**35.0 (4.25)**
JW0952 (Δ*tusE*)	pBAD-*yrvO*	0.003 (0.001)
	pBAD-*mnmA*	0.069 (0.023)
	pBAD-*yrvO-mnmA*	**23.1 (2.49)**

[a] The mass abundance ratio of mnm^5s^2U to its precursor nucleoside, mnm^5U, is shown. Values are means. Standard deviations were obtained from at least three independent experiments. Values in bold indicate modification levels resulting from a fully functional pathway.

(<5 ppm) (see Fig. S2 in the supplemental material). Analysis of the mnm^5s^2U/mnm^5U ratio of nucleoside samples extracted from cells containing different levels of MnmA (Fig. 2) shows that the accumulation of s^2U modification reflects the levels of the thiouridylase MnmA (Table 1). These results provided direct evidence for the involvement of MnmA in s^2U biosynthesis. Since YrkF showed sequence similarity to TusA, the effect of *yrkF* inactivation was also determined. For this experiment, the *B. subtilis* J1235 strain carrying inactivation of the four rhodanese domain-containing proteins, including YrkF, was used (Table 1). When cultured in minimal medium, this strain showed no growth defects or changes in the levels of s^2U modification (Table 1). Despite its similarity to TusA, YrkF is either not involved in or not required for sulfur transfer for this pathway.

Analyses of modified nucleosides indicated that the DD19 genetic construct did not completely eliminate s^2U synthesis. This result suggested that the P_{SPAC} promoter had residual activity without the inducer, providing minimal expression of MnmA, and/or that, in the absence of MnmA, thiolation may be provided through an alternate mechanism. In a previous study, we reported the use of the pMUTIN vector for the inactivation of the nonessential proteins NifZ and/or ThiI, involved in the biosynthesis of s^4U tRNA (20). In the latter case, cells cultured in the absence of the inducer showed only 10% of wild-type s^4U levels. However, the inherent instability of P_{SPAC} and potentially leaky expression of its associated promoter in *B. subtilis* have been reported for the essential thioredoxin and RNase P genes (27, 28). It is possible that similar challenges were encountered for the DD19 strain if *mnmA* is in fact essential. Interestingly, the DD19 strain did not display significant growth defects in minimal medium lacking the IPTG inducer (data not shown). Considering the MnmA expression as observed in Western blots (see Fig. S1 in the supplemental material) and the residual s^2U levels in the absence of inducer, it is likely that the P_{SPAC} promoter in this *B. subtilis* strain allows just enough MnmA expression to circumvent growth defects associated with s^2U depletion. Similarly, lack of a growth phenotype was also reported for a strain of *B. subtilis* containing the essential RNase P gene under the control of P_{SPAC}. Our failed attempts to inactivate *yrvO* and *mnmA*, along with similar challenges with residual expression of the P_{SPAC} promoter, suggest that both genes are essential in *B. subtilis*. Furthermore, these observations highlight the genetic plasticity of *B. subtilis* and experimental challenges when essential genes in this bacterium are manipulated.

YrvO and MnmA are functional partners in the biosynthesis of 2-thiouridine tRNA. In *E. coli*, s^2U modification is not essential, and strains carrying inactivation of biosynthetic components do not produce s^2U (3, 11). Therefore, this model bacterium offered an ideal platform to test the functionality of the *B. subtilis* candidate biosynthetic enzymes. The *B. subtilis* proteins YrvO and MnmA show 42% and 55% sequence identity to the *E. coli* proteins IscS and MnmA, respectively. The initial presumption was that YrvO could provide a functional complement to the ortholog cysteine desulfurase IscS and that the *B. subtilis* MnmA could complement the loss of the endogenous *E. coli* MnmA. This hypothesis was tested through complementation studies using *E. coli* strains defective in the s^2U pathway. *E. coli iscS* and *mnmA* deletion strains were transformed with plasmids expressing *B. subtilis* YrvO and/or MnmA. *In vivo* functionality of YrvO and MnmA was assessed by quantification of modified nucleosides from purified tRNA from *E. coli* cells lacking either IscS or MnmA but expressing the *B. subtilis* enzymes. Contrary to our initial predictions, the individual *B. subtilis* enzymes were not able to complement their *E. coli* orthologs. Namely, YrvO was not able to perform IscS' function in s^2U synthesis, since ratios of mnm^5s^2U/mnm^5U were similar to those in the deletion strain (Table 2). Likewise, *B. subtilis* MnmA was not able to complete the thiolation of tRNA. The lack of functional complementation of individual

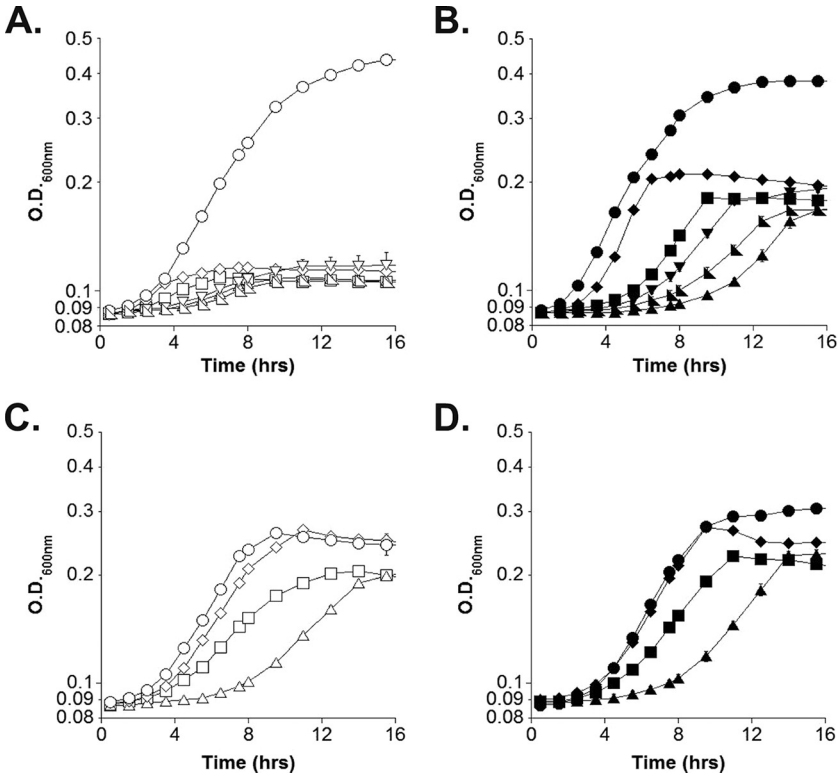

FIG 3 Growth profile of *E. coli* wild-type and mutant strains expressing *B. subtilis* YrvO and/or MnmA. (A and B) *E. coli* MG1655 (circles) and an *E. coli* Δ*iscS* strain expressing either *B. subtilis yrvO* (triangles), *yrvO*(*C325A*) (inverted triangles), *yrvO* and *mnmA* (diamonds), or *yrvO*(*C325A*) and *mnmA* (right triangles) or the empty expression vector (squares) were cultured in minimal medium, and optical density measurements were taken as indicated. (C and D) *E. coli* BW25113 (circles) and an *E. coli mnmA*-deficient strain expressing *B. subtilis mnmA* (triangles) or *yrvO* and *mnmA* (diamonds) or the empty expression vector (squares) were cultured in minimal medium, and optical density measurements were taken as indicated. For growth analysis of all strains tested, cultures were grown in M9 minimal medium in the absence (A and C) or presence (B and D) of isoleucine, leucine, methionine, cysteine, thiamine, and nicotinic acid at 37°C and 300 rpm. When not visible, error bars are smaller than the symbols.

proteins indicated that neither YrvO or MnmA was able to interact with the Tus proteins, mediating the sulfur transfer reactions from the cysteine desulfurase to the final thiouridylase enzyme (Fig. 1). However, s^2U synthesis was restored to wild-type levels when both YrvO and MnmA were coexpressed in *E. coli* strains lacking either IscS or MnmA (Table 2). Furthermore, the ability of YrvO and MnmA to form s^2U in *E. coli tusA* and *tusE* deletion strains was assessed by individual or coexpression of *yrvO* and *mnmA*. This experiment demonstrated that even in the absence of the *E. coli* proteins which interact with *E. coli* IscS and MnmA, *B. subtilis* YrvO and MnmA can synthesize s^2U only when coexpressed (Table 2). Bioinformatic analysis using Gram-positive sequenced genomes (80 species) showed the occurrence of a cysteine desulfurase gene either immediately upstream of or up to two genes away from the *mnmA* coding sequence in 65 genomes (see Table S2 in the supplemental material). These bacterial species lacked a complete set of *tus* genes; however, most of them showed the presence of a gene similar to *B. subtilis yrkF*, which was found to have no role in U34 thiolation in this study. Together, these results provided evidence for the catalytic competency of YrvO and MnmA enzymes in synthesizing s^2U. In addition, the lack of cross-reactivity between individual ortholog enzymes suggests that *B. subtilis* and likely other Gram-positive bacteria utilize a pathway independent of the one employed by *E. coli*.

The ability of *B. subtilis* YrvO and MnmA to replace biochemical pathways in *E. coli* was also assessed in growth experiments (Fig. 3). Inactivation of *iscS* results in a slow-growth phenotype in rich medium and a null-growth phenotype in minimal medium unless the medium is supplemented with nicotinic acid, thiamine, and branched-chain amino acids (13). This strong phenotype has been attributed to the involvement of IscS in sulfur transfer reactions for the biosynthesis of several cofactors, including all thionucleosides (10). Subsequent studies showed that the growth rate of a strain lacking *mnmA* was comparable to that of an Δ*iscS* strain, leading to the notion that the absence of s^2U was a major cause of the growth defects observed in rich medium (6). Therefore, we assessed the growth profiles of *E. coli* Δ*iscS* and Δ*mnmA* strains expressing the two *B. subtilis* proteins individually or together (Fig. 3). In agreement with thionucleoside analysis, restoration of the s^2U pathway was achieved only by coexpression of YrvO and *B. subtilis* MnmA, which completely recovered the growth rate in the *E. coli* Δ*mnmA* strain (Fig. 3D). Similarly, complementation of the s^2U pathway in the *E. coli* Δ*iscS* strain also resulted in growth improvement, but it did not fully recover the defects associated with lack of the master cysteine desulfurase (Fig. 3B). Thus, the partial recovery in growth rate of the *E. coli* Δ*iscS* strain containing fully modified mnm^5s^2U indicates that lack of this modification is one of the factors and not necessarily the major factor contributing to growth defects of *E. coli* Δ*iscS*. Furthermore, the inability of YrvO to recover growth defects associated

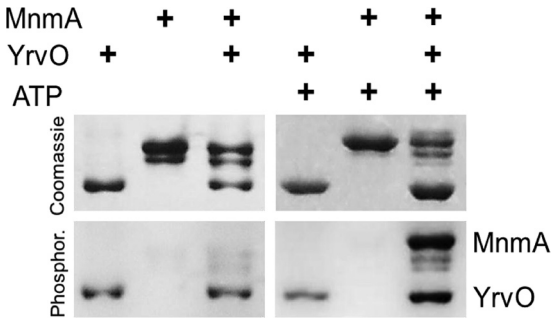

FIG 4 Sulfur transfer from YrvO to MnmA *in vitro*. YrvO (9 μg) and/or MnmA (18 μg) was incubated with ^{35}S-L-cysteine (10 μCi), L-cysteine (200 μM), and $MgCl_2$ (5 mM) in the presence (right) or absence (left) of ATP (1 mM) and quenched with *N*-ethylmaleimide (4 mM). Labeling of both YrvO and MnmA displays the presence of the ^{35}S covalent modification in the absence of DTT. The gels were stained with Coomassie (top), and radioactivity was imaged using a phosphorimager (bottom). See also Fig. S6 in the supplemental material.

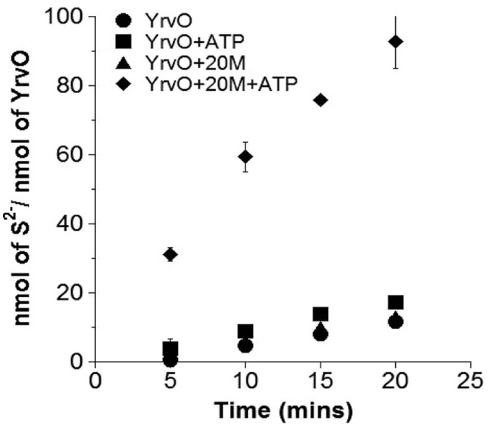

FIG 5 Cysteine desulfurase reaction profile of YrvO. The rate of sulfide production was determined through methylene blue assay as described in Materials and Methods. The standard reaction mixture contained 0.5 mM cysteine, 2 mM DTT, and 1.2 nmol YrvO (circle). The effects of 1 mM ATP (squares), 23.1 nmol of MnmA (triangles), and both ATP and MnmA (diamonds) were determined. When not visible, error bars are smaller than the symbols.

with lack of IscS suggests that YrvO is also not able to effectively interact with additional sulfur acceptors of this master cysteine desulfurase. In fact, auxotrophies observed in minimal medium cultures of *E. coli* Δ*iscS* were not rescued by YrvO regardless of the presence of its counterpart MnmA (Fig. 3A). This result is supported by amino acid sequence alignment between *E. coli* IscS and *B. subtilis* YrvO, which demonstrates that four of the IscS residues necessary for interaction with TusA, and thus formation of s^2U, are not conserved within YrvO (W45, E49, D65, and R340) (see Fig. S3 in the supplemental material). In addition, although some IscS residues which enable interaction with IscU, ThiI, IscX, and CyaY are conserved within YrvO, several that are critical for interactions with these proteins are not conserved in YrvO (29). This analysis supports the hypothesis that YrvO alone cannot replace other functions of *E. coli* IscS and suggests that it may in fact be capable of nonproductive interactions with IscS' sulfur acceptor proteins, potentially resulting in growth defects when overexpressed in *E. coli* Δ*iscS*.

YrvO is a cysteine desulfurase capable of sulfur transfer to MnmA only in the presence of ATP. The lack of complementation of individual proteins led us to probe individual reaction steps involving the formation of s^2U. The first step in sulfur mobilization involves the activation of cysteine by YrvO with formation of an enzyme-persulfide intermediate, as shown in ^{35}S-L-Cys labeling experiments (Fig. 4). The affinity of the enzyme for cysteine (K_m = 4.3 μM) is an order of magnitude greater than that of any other cysteine desulfurase found in *B. subtilis* (see Fig. S4 in the supplemental material) (17, 20). As reported for other cysteine desulfurases, the active-site cysteine (Cys325) is essential for YrvO activity (see Fig. S5 in the supplemental material), and the Cys325Ala YrvO variant is not able to rescue the levels of s^2U in *E. coli* (Table 2). The pH-dependent activity profile displays an ionization event with associated pK_a of 7.34, compatible with a proposed mechanism involving the active site cysteine residue (see Fig. S4 in the supplemental material).

The subsequent step in this proposed pathway is the persulfide sulfur transfer reaction from Cys325 of YrvO to a cysteine residue within MnmA. Interestingly, ^{35}S-L-Cys labeling of MnmA is observed only in the presence of ATP, suggesting that the enzyme undergoes a conformational change upon nucleotide binding to allow sulfur transfer. The dependency of ATP binding on persulfide sulfur transfer was also observed for the *E. coli* MnmA; however, ^{35}S labeling was reported when both ATP and tRNA were present (14). In the case of the *Bacillus* cysteine desulfurase and sulfur acceptor, the binding of tRNA to MnmA is not required for sulfur transfer (Fig. 4). Additional evidence for the catalytic competency of MnmA in serving as a sulfur acceptor was also assessed through the activity of YrvO in cysteine desulfurase assays. In the presence of ATP and under reducing conditions, MnmA caused a 6-fold increase in the rate of sulfide formation (Fig. 5), whereas, in the absence of ATP, MnmA did not affect YrvO's cysteine desulfurase activity. This activity enhancement has also been observed for cysteine desulfurases in the presence of their corresponding sulfur acceptors and has been attributed to the ability of sulfur acceptor substrates to accelerate the regeneration of the enzyme for the next catalytic cycle through a rapid persulfide sulfur transfer (19, 20, 30, 31). That is, the sulfur transfer step from YrvO to MnmA is faster than the reduction of YrvO's persulfide by DTT. Nevertheless, MnmA was able to enhance the turnover rate of sulfide formation only in the presence of ATP (Fig. 5). Labeling experiments along with kinetic analysis of YrvO in the presence of MnmA show that the ATP-bound state of the MnmA is the catalytically competent form of the sulfur acceptor protein.

Conserved cysteine residues are essential for MnmA function. In the proposed s^2U-biosynthetic scheme (14), MnmA serves as a sulfur intermediate from the cysteine desulfurase to the tRNA. Results from labeling experiments showed the ability of MnmA to directly receive sulfur from YrvO. Therefore, we sought to identify whether the formation of an S-covalent enzyme intermediate is a mandatory step prior to sulfur insertion into tRNA. In order to gain further insight into chemical steps involving the thiolation reaction catalyzed by MnmA, variant forms of this thiouridylase were generated and their *in vivo* functionality was assessed through complementation studies in *E. coli*. Sequence analysis using the Concise database with MnmA ortholog sequences from over 200 distinct species showed that among the six Cys residues of *B. subtilis* MnmA, three of them (Cys51, Cys104, and Cys200) were completely conserved, while the other three (Cys66,

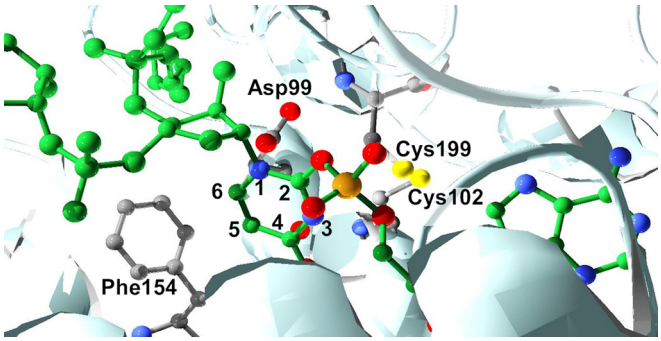

FIG 6 Active site of *E. coli* MnmA in the presence of the tRNA intermediate (PDB code 2DEU). The structure of *E. coli* MnmA (gray) is shown in complex with the tRNA adenylated intermediate (green). C-2 of U34 $tRNA^{Glu}$ is adenylated and positioned close to both catalytic residues Cys102 and Cys 199 (equivalent to Cys104 and Cys200 in *B. subtilis*). C-5 of U34 is located between Phe154 and Asp99, which are 3.6 Å and 3.3 Å from C-5, respectively.

Cys304, and Cys352) were not fully conserved. Expression plasmids containing individual Ala substitutions at each of the six Cys residues of MnmA were used for individual expression and coexpression with YrvO. As expected, none of the MnmA variants were able to complement *E. coli* Δ*mnmA* when expressed individually (Table 2). YrvO coexpression with MnmA Cys66Ala or Cys304Ala variants was able to restore ratios of mnm^5s^2U/mnm^5U to wild-type levels, while MnmA Cys352Ala showed only partial complementation (23%), suggesting that these three nonconserved Cys residues are not required for the thiolation reaction. Furthermore, radiolabeling experiments with YrvO, MnmA Cys352Ala, and ^{35}S-L-Cys confirmed that MnmA Cys352 is not required for thiolation of MnmA (see Fig. S6 in the supplemental material). Inspection of the MnmA-tRNA complex structures (14) shows that the *E. coli* equivalent residue is positioned at a location remote from the tRNA binding and adenylation site.

Individual alanine substitutions of the three conserved Cys residues (at positions 51, 104, and 200) completely hindered the ability of MnmA along with YrvO to synthesize s^2U *in vivo*. Previous structural studies in *E. coli* MnmA suggested that residues Cys102 and Cys199 (equivalent to *B. subtilis* MnmA Cys104 and Cys200) were located at the active site (Fig. 6) (14). *In vitro* synthesis of s^2U using *E. coli* Cys-to-Ser variants showed the requirement of both residues for the MnmA thiolation reaction. ^{35}S-labeling experiments of *B. subtilis* MnmA showed that the Cys104Ala variant was not competent for persulfide formation, while inactivation of Cys200 retained the capacity for ^{35}S modification (see Fig. S6 in the supplemental material). Interestingly, substitution of residue Cys51 also abolished persulfide sulfur transfer. In the structure of *E. coli* MnmA, this residue is located close to the surface, away from the active site and tRNA binding domain. The involvement of this residue in the regulation, structure, or function of this enzyme remains to be determined.

***In vitro* synthesis of s^2U tRNA by *B. subtilis* MnmA and YrvO.** The thiouridylation reaction of MnmA involves the ATP-dependent adenylation of the tRNA substrate and the sulfur transfer reaction from the cysteine desulfurase to the activated tRNA substrate. Incubation of MnmA, YrvO, ATP, Cys, and tRNA under reducing conditions resulted in the formation of s^2U modified tRNA, providing *in vitro* evidence that MnmA and YrvO are catalytically competent in completing this modification. The source of tRNA for *in vitro* s^2U synthesis reactions was tRNA purified from an *E. coli* Δ*mnmA* strain, which contained both precursor forms, unmodified U34 tRNA and partially modified mnm^5U34 tRNA. The use of native tRNA, rather than a synthetic transcript, allowed the investigation of substrate preferences in samples also containing nonsubstrate tRNA molecules. Thiolation of U34 of tRNA was determined by taking the ratio of MS signal intensities of mnm^5s^2U/mnm^5U and s^2U to another thionucleoside, s^4U, the biosynthesis of which should remain unaltered (Table 3). Furthermore, U34 thiolation was assessed by direct comparison of the signal intensity of a standard curve generated with s^2U (see Fig. S7 in the supplemental material). Complete reactions with YrvO/Cys and MnmA showed the formation of 43 pmol of s^2U (see Table S3 in the supplemental material), representing a >400-fold enhancement in the s^2U/s^4U ratio. In the same reactions, the ratio of mnm^5s^2U/mnm^5U increased only 1.7- or 1.4-fold for reactions in the absence or presence of DTT, respectively, compared to the control (Table 3). This apparent substrate preference was observed under all reaction conditions tested in this study. Inspection of the *E. coli* MnmA structure in complex with tRNA shows the presence of two conserved residues (Phe154 and Asp99) positioned 4 Å away from C5 of the U34 tRNA substrate (Fig. 6). The steric hindrance possibly imposed by the Phe and Asp side chains at the active site suggests that the partially modified mnm^5U34 tRNA may not fit into the active site, although validation of this hypothesis awaits experimental investigation. The results from *in vitro* synthesis reported here are in agreement with structural analysis, implying that MnmA's active-site conformation may restrict tRNA substrate to unmodified U34.

In vitro s^2U reactions were performed in the presence of an artificial reducing agent, DTT. Previous studies reporting the *in vitro* synthesis of s^2U using the *E. coli* biosynthetic proteins were also performed under reducing conditions. However, DTT is known to interact with the cysteine desulfurase persulfide intermediate, releasing free sulfide (Fig. 5). Supporting this observation, *in vitro* s^2U formation was detected when sulfide was used as the sulfur source. This result indicates that, in the presence of

TABLE 3 *In vitro* s^2U synthesis

Reaction component(s)	mnm^5s^2U/mnm^5U (10^3) (SD)[a]		s^2U/s^4U (10^3) (SD)[a]	
	−DTT	+DTT	−DTT	+DTT
Cysteine	0.22 (0.09)	0.70 (0.3)	1.8 (0.3)	1.5 (1.3)
Cysteine + YrvO + MnmA	0.37 (0.2)	0.94 (0.3)	381.3 (44.7)	628.5 (72.6)
Sulfide + MnmA	0.38 (0.3)	0.95 (0.3)	191.1 (18.2)	290.9 (27.6)
Cysteine + YrvO + *E. coli* MnmA	0.34 (0.03)		39.6 (3.5)	
Cysteine + *A. vinelandii* IscS + *B. subtilis* MnmA	0.35 (0.07)		12.2 (2.3)	

[a] Values are means. Standard deviations were obtained from at least three independent experiments.

DTT, YrvO and cysteine may serve as a source of sulfide and that the formation of a YrvO-MnmA-tRNA ternary complex is not mandatory for s^2U formation. Nevertheless, *in vitro* s^2U synthesis was also accomplished under nonreducing conditions, albeit at a reduced level (Table 3). It is worth noting that these experiments were performed with naturally produced bulk tRNA and in the presence of excess equivalents of MnmA, suggesting that *in vitro* synthesis of s^2U is likely the result of single-turnover reactions.

To verify the result from complementation experiments that *B. subtilis* YrvO or MnmA could not directly complement the loss of *E. coli* IscS or MnmA, *in vitro* s^2U synthesis reactions were performed with YrvO and *E. coli* MnmA and with IscS and *B. subtilis* MnmA in the absence of DTT. Although both reactions demonstrated s^2U formation, the levels of s^2U were only 10% of those observed in reaction mixtures containing both *B. subtilis* proteins, confirming the specificity of the partnership between YrvO and MnmA.

DISCUSSION

Thiolation of U34 within $tRNA^{Lys}$, $tRNA^{Gln}$, and $tRNA^{Glu}$ is universally conserved in all three domains of life. Analysis of tRNA nucleosides confirms that *B. subtilis* accumulates the mnm^5s^2U modification while displaying no detectable levels of s^2U (data not shown). Biosynthetic components involved in tRNA substrate activation as well as in sulfur mobilization and insertion have been identified in eukaryotic, archaeal, and Gram-negative bacterial species (3, 32–35). However, the biosynthetic pathway involving thiolation of C-2 of U34 of tRNA has not been reported in Gram-positive bacteria. Bioinformatic analysis pointed to the presence of a gene coding for the MnmA thiouridylase adjacent to *yrvO*, which codes for a cysteine desulfurase, which combined with the lack of the Tus relay proteins led to the hypothesis that only YrvO and MnmA would be required for biosynthesis of s^2U in *B. subtilis*.

Inactivation of the s^2U pathway in *E. coli* is not lethal, but it causes growth defects associated with loss of reading frame maintenance (36, 37). Similarly, inactivation of the nonessential 2-thiouridylase gene *MTU1*, while leaving the organism viable, causes respiratory defects in yeast and humans (38, 39). On the other hand, the archaeal 2-thiouridylase gene *Ncs6* is essential in *Methanococcus maripaludis* (33) and necessary for growth at high temperatures in *Haloferax volcanii* (34). In the model Gram-positive bacterium *B. subtilis*, we and others failed when attempting to inactivate *yrvO* or *mnmA* (24), indicating that both proteins participate in essential cellular processes. Insertion of an IPTG-inducible promoter in front of *mnmA* coding sequence resulted in a viable strain, and levels of MnmA expression correlated with the accumulation of fully modified mnm^5s^2U, providing direct evidence for its involvement in the thiolation pathway.

Coexpression of YrvO and MnmA but not individual ortholog components rescued s^2U synthesis to wild-type levels in *E. coli* strains deficient in the s^2U pathway. Results from complementation studies support the model that, in *B. subtilis*, the cysteine desulfurase YrvO is capable of direct sulfur transfer to the final acceptor MnmA. This abbreviated pathway involves a dedicated cysteine desulfurase, which dispenses with the need for a bucket brigade of sulfur transport involving the Tus proteins, as observed in *E. coli*. Mutual specificity between a cysteine desulfurase and its cognate sulfur acceptor protein was previously reported by our group in the investigation of the sulfur transfer reaction involving NifZ and ThiI in the biosynthesis of s^4U tRNA in *B. subtilis* (20). Similar to the results reported in this study, the individual *Bacillus* enzymes NifZ and ThiI also failed to provide functional complementation of equivalent *E. coli* orthologs, while their coexpression provided a full complement of the pathway. For the biosynthesis of s^4U, the *B. subtilis* 4-thiouridylase ThiI is a shorter version of its *E. coli* ortholog as it lacks the rhodanese sulfurtransferase domain. It has been suggested that the lack of a sulfur acceptor rhodanese handle was compensated for by the recruitment of the dedicated cysteine desulfurase acting as the direct sulfur donor to *B. subtilis* ThiI. We propose that YrvO and MnmA exert a similar catalytic dependency for the formation of s^2U. In s^2U biosynthesis, the involvement of a dedicated cysteine desulfurase YrvO provides direct sulfur transfer to MnmA, thus dispensing with the need for a complex sulfur relay system.

Labeling experiments along with kinetic analysis of the YrvO cysteine desulfurase provides biochemical evidence for the requirement of ATP during the sulfur transfer event. MnmA was able to enhance the rate of sulfide formation by YrvO only when in the presence of ATP. Likewise, ^{35}S-labeling of MnmA by YrvO and ^{35}S-Cys also showed requirement for ATP suggesting that ATP binding precedes the sulfur transfer reaction. This observation makes sense mechanistically, since both persulfide-enzyme and adenylated tRNA are labile intermediates. It is possible that the requirement for ATP binding preceding the sulfur transfer event imposes coordination of the two reactions catalyzed by MnmA. This proposed sequential binding guarantees that once persulfurated, MnmA is able to bind tRNA and lead the reaction to completion.

Distinct mechanisms of s^2U in eukaryotic, archaeal, and Gram-negative model species have been reported. The unifying feature in all reported pathways is the participation of a pyrophosphatase enzyme involved in the adenylation of the C-2 position of U34 tRNA, enabling the subsequent thiolation reaction (3, 32–35). However, the mechanisms of sulfur activation and insertion vary and are not fully understood. In archaeal species, the 2-thiouridylase, although not orthologous to MnmA, catalyzes the two-step tRNA adenylation and sulfur transfer reaction; however, the identity of the sulfur donor remains unidentified. In *E. coli*, ^{35}S-labeling studies showed that a MnmA Cys102Ser variant, but not a MnmA Cys199Ser variant, retained the ability to be ^{35}S modified upon incubation with ^{35}S-Cys in the presence of IscS, TusA, TusBCD, TusE, ATP, and tRNA (14). These results led to a proposed catalytic mechanism of *E. coli* MnmA in which Cys199 is the site of S modification by the final sulfur donor TusE, and Cys102 would act as a resolving Cys residue, forming a disulfide bond with Cys199 at the end of each catalytic cycle. Although it is anticipated that *E. coli* and *B. subtilis* MnmA enzymes use similar chemical steps during sulfur transfer to the activated tRNA, the role of each Cys residue appears to be switched; i.e., the MnmA variant carrying Cys200Ala retained the ability to accept a sulfur from YrvO, while MnmA Cys104Ala did not. In addition, complementation studies showed that Cys51 is also necessary for the functionality of MnmA *in vivo*. A recent report on the *M. maripaludis* 2-thiouridylase also showed the requirement of three conserved cysteines necessary for sulfur transfer (33). Interestingly, mass spectrometry analysis showed that two of these Cys residues can form a trisulfide bond, possibly resulting from an oxidation event between a persulfide with another Cys thiol at the active site, while the role of the third Cys residue remains unknown. Likewise, labeling experiments along with *in vitro* synthesis of s^2U tRNA

showed that MnmA Cys51Ala is not competent to receive a sulfur from YrvO but is likely capable of adenylating tRNA, since MnmA Cys51Ala can complete s^2U synthesis in the presence of sulfide (data not shown). It is possible that this residue participates in the interaction between MnmA and its sulfur donor, YrvO; however, such a proposal awaits experimental validation.

YrvO and MnmA were fully functional in operating in the s^2U pathway in a heterologous system such as *E. coli*, while individual expression of these proteins did not provide a functional complement of their equivalent orthologs. Furthermore, individual or coexpression of YrvO and MnmA did not rescue the null-growth phenotype of IscS deletion in minimal medium (Fig. 3A). This result indicates that despite the high degree of sequence similarity to IscS (42% identity), YrvO is not able to effectively interact with other sulfur acceptors of IscS. Most importantly, individual expression of YrvO resulted in an apparent toxic phenotype in an *E. coli* Δ*iscS* strain. The slow-growth phenotype of the Δ*iscS* strain was exacerbated upon expression of YrvO but not when YrvO was expressed in conjunction with MnmA or when it was inactivated with a Cys325Ala substitution (Fig. 3B). It seems plausible to propose that YrvO, when expressed without its partner MnmA, is able to form nonproductive interactions with other sulfur acceptors, thus causing dysfunction among pathways involving sulfur transfer. We are currently investigating potential interactions and pathways in which YrvO may interfere.

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