Functional analysis of *Bacillus subtilis* genes involved in the biosynthesis of 4-thiouridine in tRNA

Lauren J. Rajakovich, John Tomlinson, and Patricia C. Dos Santos*

Department of Chemistry, Wake Forest University, Winston-Salem, NC, USA, 27109

Running title: The biosynthesis of 4-thiouridine in *Bacillus subtilis*

*To whom correspondence should be addressed: dossanpc@wfu.edu, 336-758-3144 (phone), 336-758-4656 (fax).
ThiI has been identified as an essential enzyme involved in the biosynthesis of thiamine and the tRNA thionucleoside modification, 4-thiouridine. In *Escherichia coli* and *Salmonella enterica*, ThiI acts as a sulfurtransferase, receiving the sulfur donated from the cysteine desulfurase IscS and transferring it to the target molecule or additional sulfur carrier proteins. However, in *Bacillus subtilis* and most species from the Firmicute phylum, ThiI lacks the rhodanese domain that contains the site responsible for the sulfurtransferase activity. The lack of the gene encoding for a canonical IscS cysteine desulfurase and the presence of a short sequence of ThiI in these bacteria pointed to mechanistic differences involving sulfur trafficking reactions in both biosynthetic pathways. Here, we have carried out functional analysis of *B. subtilis* thiI and the adjacent gene, nifZ, encoding for a cysteine desulfurase. Gene inactivation experiments in *B. subtilis* indicate the requirement of ThiI and NifZ for the biosynthesis of 4-thiouridine, but not thiamine. *In vitro* synthesis of 4-thiouridine by ThiI and NifZ, along with labeling experiments suggest the occurrence of an alternate transient site for sulfur transfer, thus obviating the need for a rhodanese domain. *In vivo* complementation studies in *E. coli* IscS or ThiI deficient strains provide further support for specific interactions between NifZ and ThiI. These results are compatible with the proposal that *B. subtilis* NifZ and ThiI utilize mechanistically distinct and mutually specific sulfur transfer reactions.
INTRODUCTION

Post-transcriptional modifications in tRNA are widely represented in all three domains of life (21). In particular, the incorporation of new chemical functionalities to tRNA allows additional intra- and inter-molecular interactions that support proper folding to more thermodynamically stable conformations, that guarantee fidelity in translation, and that activate their ability to sense environmental and nutritional changes (1, 17, 31). Nearly a hundred tRNA modifications have been described (22), however the exact inventory of covalent modifications is not fully established. Even less understood are the identity and mechanisms of modifying enzymes as well as the impact of these modifications on the various cellular functions performed by individual tRNA molecules.

One of the best characterized tRNA modifications is the C-4 thiolation of the uridine base at position 8 of tRNA (s4U8) (5, 8, 10, 17). This thionucleoside acts as a photosensor of near-UV radiation (18). The absorption of UV light induces a photochemical cross-linking cycloaddition reaction between s4U8 and the cytosine at position 13 of tRNA. This event induces a conformational change that prevents aminoacylation of tRNA molecules, resulting in an accumulation of uncharged tRNA ultimately leading to the stringent response (4). In Escherichia coli, the UV-induced stringent response, manifested as growth delay, is disrupted in strains lacking s4U8 modification. Genetic studies mapped thiI, an essential gene involved in thiamine biosynthesis (29), as the s4U8 biosynthetic and UV-photoprotector gene (18, 29).

In E. coli, the formation of s4U8 is performed by Thil in conjunction with the cysteine desulfurase IscS (7). This tRNA modification begins with the conversion of cysteine to alanine by IscS with concomitant formation of a cysteine persulfide bond at the enzyme’s active site cysteine residue. The next step consists of a persulfide sulfur transfer from IscS to the Cys456 residue of Thil and the activation of C-4 of the uridine base at position 8 of tRNA, involving a Thil-
dependent adenylation reaction (32). The proposed catalytic cycle is completed upon transfer of the persulfide sulfur from Cys456 to form s^4U8 with the concomitant release of AMP and formation of a disulfide bond between Cys344 and Cys456 (19, 20).

Structural and amino acid sequence analyses show that *E. coli* and *Salmonella enterica* ThiI proteins contain three distinct domains: a THUMP domain involved in tRNA binding, a PP-loop pyrophosphatase domain likely involved in the formation of the tRNA adenylated-uridine intermediate, and the rhodanese domain (Rhd domain), which provides the sulfurtransferase site Cys456. *In vivo* and *in vitro* data points to the participation of all three domains in the formation of s^4U8 (11, 20, 28). A recent report by the Downs’ group has demonstrated that, in *S. enterica*, the Rhd domain alone is sufficient for the sulfur incorporation into the thiazole moiety of thiamine (14). This discovery led to the proposal that ThiI is a bifunctional enzyme carrying distinct tRNA adenylation and sulfurtransferase activities. Interestingly, a subset of species coding for sequences similar to *E. coli* and *S. enterica* thiI lacks the Rhd domain (28), thus raising the question of how these species synthesize s^4U8 and thiamine.

We have investigated the role of *Bacillus subtilis* ThiI and NifZ in the biosynthesis of s^4U8 and thiamine. In this organism and several other bacteria, the thiI gene encodes for a protein lacking the Rhd domain while retaining the THUMP, the PP-loop pyrophosphatase domains and a Cys residue at an equivalent position of the Cys344 in *E. coli* ThiI. NifZ is a cysteine desulfurase, which potentially serves as the initial sulfur donor in these pathways. Interestingly, the nifZ gene coding sequence is located four bases upstream from thiI.

In this report, we demonstrate that NifZ and ThiI are sufficient and crucial for the formation of s^4U8 modification in tRNA, while their inactivation does not impose a requirement for thiamine upon this bacterium. In addition, *in vivo* and *in vitro* experiments described here support the
model for NifZ and ThiI interactions in the s^4U8 pathway involving a specific sulfur transfer reaction.

**MATERIALS & METHODS**

**Media, media additions, and chemicals.** LB medium was used with the following concentrations of antibiotics and media additives, unless otherwise specified: ampicillin (100 μg/mL), kanamycin (40 μg/mL), erythromycin (0.5 μg/mL), X-Gal (40 μg/mL), IPTG (10 μg/mL), L-arabinose (2 mg/mL). MS-I medium consists of 17.5 mM NH₄SO₄, 80.3 mM K₂HPO₄, 44.1 mM KH₂PO₄, 3.4 mM trisodium citrate dehydrate, 0.81 mM MgSO₄, 0.5% glucose, 0.02% casamino acids and 0.1% yeast extract. MS-II medium consists of MS-I medium with 0.125 mM MgSO₄ and 0.0125 mM CaCl₂. Spizizen’s minimal medium was prepared as previously described (2). Unless 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* PS832 strain genomic DNA prepared using a commercial DNA extraction kit (QuickExtract™, Epicentre). PCR amplification reactions were performed using Fail Safe PCR kit (Epicentre™). The PCR products were all previously cloned into TopoTA® vector (Invitrogen) for sub-cloning purposes. The correct sequence of all plasmids used in this study was confirmed by DNA sequencing (Wake Forest DNA Sequencing Laboratory). A comprehensive list and description of primers and plasmids used in this work are shown in Table S1.

**B. subtilis strain construction.** All strains of *B. subtilis* listed in Table S1 were derivatives of strain 168 (PS832 strain). Transformation protocol was adapted from previously described (2): *B. subtilis* PS832 was grown on 2x SG plates for 15 h at 30°C yielding individual colonies. A
single colony was inoculated in 5 mL MS-I medium at 37°C for 4.5 h. A 10-fold dilution of the
"pre-competent" cell culture was prepared in 5 mL of MS-II medium and incubated with shaking
at 37°C for 1.5 h. Plasmid pDS54 or pDS19 (50 µl) was added to 300 µl competent cells and
incubated with shaking at 37°C for 30 min. Transformed cells were selected for erythromycin
resistance and blue/white selection in the presence of X-gal and IPTG.

Detection of *thiI* transcript from total RNA samples. Total RNA purification from *B. subtilis*
strains PS832, DD5 and DD14 grown in the absence or presence of 500 µM IPTG was
performed according to the Qiagen RNeasy Kit bacteria protocol, using RNAProtect Bacteria
Reagent (Qiagen) and DNase I (Qiagen). RT-PCR reactions, using SuperScript III One-Step
RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen), were performed to amplify
*thiI* with *BstHindIII* and *BstBglII* primers, *sufC* with *BssufC-HindIII* and *BssufC-BamHI*5
primers and *suf* promoter region with *Bssufpro5* and *Bssufpro3* primers. RT-PCR products
were visualized by agarose ethidium bromide gel electrophoresis.

Thiamine feeding experiments. *B. subtilis* wild-type PS832 and DD14 (±/− 500 µM IPTG) and
1A603 strains were inoculated at an initial OD_{600nm} of 0.01 in Spizizen minimal medium (2) in the
presence and absence of 0.8 µg/mL thiamine. Cultures were grown at 300 RPM at 37°C for 14
h.

Sporulation experiments. *B. subtilis* strains PS832 and DD14 were inoculated in 5 mL LB
medium at an initial OD_{600nm} of 0.01 and incubated at 37°C, 300 RPM. Fifty microliter aliquots
were removed at time points between 10-24 hours. Serial dilutions (10^{-1} - 10^{-6}) of each aliquot
were spotted (5 µl drops) on LB agar plates. Dilutions were incubated at 65°C for 20 min and
spotted onto the same LB agar plates. The relative log of spores from heated samples was
compared to the total number of viable cells.
B. subtilis PS832 and DD14 sporulation experiments were performed without stress and under UV radiation, iron, and oxidative stress conditions. Cultures at an OD$_{600\text{nm}}$ of 0.5 were exposed to UV radiation of 350 nm for 30 min. Aliquots were taken at 1, 4, and 16 h after exposure. Cultures were challenged with 1 mM, 1.5 mM, 2 mM, 10 mM and 100 mM Fe(NO$_3$)$_3$ or 1 mM H$_2$O$_2$ added to LB medium.

Isolation of tRNA from B. subtilis and E. coli cells. B. subtilis wild-type strain PS832 was grown in 500 mL LB medium. B. subtilis strains DD5 and DD14 in the presence and absence of 500 µM IPTG were grown in 500 mL LB medium with erythromycin at 37°C to an OD$_{600\text{nm}}$ of 1.2-1.5. E. coli strain CL100 transformed with pAra13, pDS21, or pDS115 and E. coli strain JLD26501 transformed with pAra13, pDS108, or pDS115 were grown in LB medium containing ampicillin and arabinose at 37°C to OD$_{600\text{nm}}$ of 1.2-1.5. Cells were harvested by centrifugation at 8200 $g$ for 10 min and frozen at -20°C until further use.

Frozen cells were resuspended in 10 mL of 0.3 M sodium acetate, 10 mM EDTA (pH 4.5), after which 3.5 mL of Tris-saturated phenol was added. The solution was vortexed for 1 min and after 30 s was vortexed again for 1.5 min, then centrifuged at 3214 $g$ for 15 min. The upper layer was transferred to a clean tube and 3.5 mL of Tris-saturated phenol was added. The resulting solution was vortexed for 1 min and centrifuged at 3214 $g$ for 15 min. The upper layer was transferred to a clean tube followed by addition of 0.5 g NaCl and 10 mL of chloroform:isoamyl alcohol (24:1). The mixture was vortexed for 5 s and centrifuged at 3214 $g$ for 3 min. The upper portion was removed to a clean tube, filled to 50 mL with 100% cold ethanol and stored at -20°C for 2 h to overnight. The solution was centrifuged at 3214 $g$ for 20 min and the pellet was resuspended in 1.5 mL of 0.3 M sodium acetate, 10 mM EDTA (pH 4.5). The solution was vortexed for 3 s, heated at 90°C for 5 min and centrifuged at 15,700 $g$ for 5 min. The absorbance at 260 nm was measured and a spectrum from 200-800 nm was recorded. An aliquot of the sample was diluted to 500 µl with 0.3 M sodium acetate, 10 mM
EDTA (pH 4.5) to an equivalent A_260 nm of 40 and cold ethanol (100%) filled to a volume of 1.5 mL. The sample was stored at -20°C until further use.

**Synthesis and isolation of synthetic tRNA.** *E. coli* tRNA^{Met} was synthesized *in vitro* as previously described (24) with the following protocol. The *E. coli* tRNA^{Met} DNA template was synthesized in a 2 mL reaction containing 4 µM Ec^{tRNA^Met5} and Ec^{tRNA^Met3} primers, 400 µM dNTPs, 50 U Klenow exo- DNA polymerase, 1x buffer 2 from New England Biolabs. The reaction mixture was incubated for 20 cycles using a Thermocycler at 10°C for 10 s and 37°C for 30 s. Transcription reaction (8 mL) contained 2 mL of DNA template, 5 mM of each NTP, 20 mM spermidine, 40 mM DTT, 250 mM HEPES (pH 7.5), 30 mM MgCl_2, 20 µg bovine serum albumin, 40 µg/mL T7 RNA polymerase, 60 U RNase inhibitor. The reaction was incubated while rotating at 37°C for 8-18 h. DNase (81 U) was added to the reaction followed by 1.5 h incubation at 37 °C while rotating and centrifuged for 20 min at 2,057 g. Eight milliliters of 3 M NH_4OAc, pH 6 and 32 mL of isopropanol were added to the supernatant and stored at -20°C for 2 h to overnight. Solution was centrifuged for 30 min at 17,555 g and isopropanol was removed. Pellet was resuspended in 800 µl RNase-free water and 1:1 (v/v) of 90% formamide was added to sample and boiled for 5 min. tRNA^{Met} was isolated from a 12% acrylamide (19:1), 8 M urea gel electrophoresis with 100 mM Tris, 100 mM Boric acid, 2 mM EDTA buffer (1x TBE running buffer). Sample was purified from gel using an EluTrap Electrophoresis chamber (Schleicher & Schuell) at 150 V. tRNA was precipitated by adding 1:10 (v/v) 3 M NaOAc, pH 5.2 and 2:3 (v/v) of 100% cold ethanol and stored overnight at -20°C. Sample was centrifuged for 30 min at 17,555 g and pellet was resuspended in 100 µl 20 mM HEPES, pH 7.5 and stored at -20°C.

**In vitro generation of s^U8 in synthetic tRNA.** Assay reactions (150 µl) contained 100 µM L-cysteine, 4 µM NifZ or IscS, 4 µM Thil, 100 µM synthetic *E. coli* tRNA^{Met}, 1 mM ATP and 5 mM MgCl_2 in 50 mM Tris-HCl, pH 8. Reactions were incubated at 37°C for 2 h, adding 1 mM DTT.
after 1 h. To isolate the tRNA, samples were extracted with 45 µL of Tris-saturated phenol and precipitated with 1 mL of cold ethanol.

**Analysis of modified tRNA nucleosides.** Unfractionated tRNA isolated from *B. subtilis* and *E. coli* cells, or synthetic tRNA from *in vitro* experiments was digested into individual nucleosides for analysis. Each solution was centrifuged at 15,700 g for 20 min and the pellet was resuspended in 150 µl of 40 mM sodium acetate, 1 mM ZnCl₂, pH 5.2 and heated at 100°C for 5 min. Once cooled, 20 µl Nuclease P1 (1 mg/ml) was added and incubated at 50°C for 3 hrs. Then, 20 µl of 1 M Tris, pH 8, 10 µl of shrimp alkaline phosphatase (Thermo) and 10 µl of phosphatase buffer were added and incubated at 37°C for 1.5 hrs. The sample was centrifuged at 15,700 g for 5 min and analyzed by HPLC using Electron Spray Ionization Mass spectrometry (ESI-MS) and/or UV detector.

Digested tRNA samples were analyzed by HPLC using a C-18 column (Supelcosil), coupled with a mass spectrometer and diode array detector (1100 Series LC/MSD Trap, Agilent) or a UV/Vis detector (Waters). For analysis of modified tRNA transcripts, a short HPLC method was employed; method A used Optima LC-MS grade water with 0.1% formic acid as solvent A and 40:60 water:acetonitrile as solvent B. The HPLC conditions for method A included a gradient of solvent B—5% from 0-15 min, 75% at 25 min, 100% at 26 min, 5% from 28-33 min at a flow rate of 0.75 mL/min with 20 µl injections. Analysis of modified tRNA from *E. coli* and *B. subtilis* cells were performed using a longer HPLC protocol; method B used 0.1% (w/v) ammonium acetate, pH 5.3 as solvent A and 60:40 water:acetonitrile as solvent B. Method B included a gradient of solvent B—2-5% from 0-10 min, 5-25% from 10-25 min, 25-50% from 25-30 min, 50-75% from 30-34% and 75-100% from 34-40 min. The flow rate was kept at 0.5 mL/min with 10 µL injections. The diode array detector and UV/vis detector monitored the UV absorbance at 260 nm and 330 nm. The mass spectrum was recorded in the negative mode under the following conditions: nebulizer pressure of 60 psi, drying gas flow of 11 L/min at 350°C and capillary
voltage of 3500 V. Standards of s\textsuperscript{4}U and pseudouridine (Berry & Associates) were run as controls.

**Protein expression and purification.** Plasmid pDS21 containing \textit{nifZ} was transformed into \textit{E. coli} CL100 competent cells and selected for ampicillin resistance. Single colonies were inoculated in 3 L of LB medium containing ampicillin and grown overnight (20 h) at 30°C in the presence of L-arabinose (0.2%). Cells were harvested by centrifugation at 8200 g for 10 min and resuspended (3 mL/g of cell wet weight) in 25 mM Tris-HCl, pH 8 (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% (w/v) 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 Q-Sepharose column (GE Healthcare) pre-equilibrated 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 0-70% gradient of 25 mM Tris-HCl, pH 8, 1 M NaCl (buffer B) over 20 column volumes. The collected eluant was analyzed by SDS-PAGE and the fractions eluting with 10-15% buffer B, containing the desired protein of 41.4 kDa, were pooled. Pooled sample was treated with 40% and 50% 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 50% ammonium sulfate pellet containing the desired protein was resuspended in 10 mL of 25 mM Tris-HCl, pH 8, 300 mM NaCl (buffer C) and loaded using an FPLC onto a 2.5 cm x 60 cm Sephacryl S-200 column (GE Healthcare) pre-equilibrated with buffer C, at a flow rate of 1 mL/min. The eluant was analyzed by SDS-PAGE and the desired fractions were pooled and pelleted in liquid nitrogen for storage at -80°C.

\textit{B. subtilis} Thl protein was expressed by transforming pDS108 into \textit{E. coli} Arctic Express competent cells (Stratagene). Single colonies were inoculated in 3 L of LB medium with ampicillin and grown to an OD\textsubscript{600nm} of 0.5 at 25°C. Cell cultures were induced with L-arabinose
(0.2%) and grown overnight (16 h) at 15°C, then harvested by centrifugation at 8200 g for 10 min. Cell pellets were resuspended (5 mL/g cells wet weight) in 25 mM Tris-HCl, pH 8, 20% glycerol (buffer D) and lysed using an Emulsiflex C5 High Pressure homogenizer (Avestin). Cell lysate was centrifuged for 15 min at 12,857 g, then the supernatant was treated with 1% (w/v) streptomycin on ice for 15 min and centrifuged for 15 min at 12,857 g. The soluble protein was treated with 50% and 70% saturating concentrations of ammonium sulfate, incubating on ice for 15 min followed by centrifugation for 15 min at 31,209 g after each step. The desired protein of 45.4 kDa was contained in the 70% ammonium sulfate pellet, which was resuspended in 10 mL of 25 mM Tris-HCl, pH 8, 300 mM NaCl, 20% glycerol (buffer E) and loaded onto a 2.5 cm x 60 cm Sephacryl S-200 column (GE Healthcare) pre-equilibrated with buffer E. Fractions containing the desired protein were pooled and pelleted in liquid nitrogen for storage at -80°C.

Azotobacter vinelandii IscS was produced from E. coli BL21(DE3) cells containing pDB943 and isolated as previously described (33). E. coli ThiI was produced from E. coli BL21(DE3) and isolated through Ni-NTA chromatography as previously reported (18). All of the protein purifications were monitored by SDS-PAGE (9), and the protein concentrations were determined by the method of Bradford et al (3), using BioRad protein assay kit and bovine serum albumin as standard. Pyridoxal-5'-phosphate bound to NifZ and IscS was quantified as described previously (27).

Cysteine desulfurase activity. Cysteine desulfurase activity was determined through formation of S²⁻ and alanine as previously described (23). Assays were performed with fixed concentrations of NifZ (0.01 mg, 0.3 µM), varying the concentrations of L-cysteine (0.0125-0.5 mM), and in the presence and absence of 2 mM DTT and/or 6 µM ThiI.

³⁵S-Sulfur transfer assays. Assay reactions (40 µl) contained 4.26 µM NifZ or IscS and 3.89 µM ThiI, 1 mM ATP, 5 mM MgCl₂ in 50 mM Tris-HCl, pH 7.5 with 50 µM L-cysteine and 10 µCi
35S-cysteine (Perkin Elmer). Reactions were incubated at 37°C for 1 h and quenched with 0.4 mM N-ethylmaleimide for 5 min. Reactions were analyzed by non-reducing SDS-PAGE. Protein size and migration were observed by Coomassie Brilliant Blue staining and 35S-persulfurated proteins were visualized using Phosphoimager (BioRad).

32P-adenylation assays. B. subtilis ThiI and E. coli ThiI (10 µM) were incubated with 100 µM synthetic E. coli tRNA\textsuperscript{Met} in 30 µl 50 mM Tris-HCl, pH 8, 1 mM MgCl\textsubscript{2}. The reaction was started with 10 µCi of [\(\alpha\text{-}32\text{P}\)]-ATP (Perkin Elmer) followed by incubation at 37°C for 2 h. Reaction components were separated by 12% acrylamide, 8 M urea gel electrophoresis in 1x TBE buffer. The migration pattern of tRNA was observed by UV after ethidium bromide staining, while the [\(\alpha\text{-}32\text{P}\)]-adenylated tRNA intermediate was visualized using Phosphoimager (BioRad).

RESULTS

Gene inactivation of nifZ and thiI in Bacillus subtilis. The chromosomal location of thiI coding sequence is 4 bases downstream of the nifZ gene encoding for a cysteine desulfurase and 94 bases upstream of the sspB gene encoding for the small, acid soluble spore protein (16). Transcript sequence analysis in Bacillus anthracis indicated that, along with nifZ and sspB, thiI is part of a polycistronic operon (13). Therefore, in order to avoid any potential polar effect on the downstream genes, pMutin integrational plasmid containing fragments of ‘nifZ’ or ‘nifZ’ were used in the generation of knockout strains. For construction of B. subtilis strain DD14, pDS54, an internal ‘nifZ’ fragment cloned into pMutin4 (25), was inserted into the chromosome, simultaneously disrupting nifZ expression and placing thiI under control of pSPAC, an IPTG-inducible promoter. A similar strategy was used for construction of B. subtilis strain DD5; pMutin containing a ‘nifZ’ fragment (pDS19) was inserted into B. subtilis chromosome placing thiI and sspB under control of the pSAPC promoter.
In order to confirm that the strain DD14 was able to express thiI upon the addition of IPTG, RT-PCR analysis was performed on purified total RNA samples of strains PS832 and DD14. Amplification of cDNA generated from the RNA samples was used to determine the presence of thiI transcripts in the wild type and the DD14 samples in the presence and absence of IPTG (Figure 1). The sufC gene amplification was used as an internal positive control (Figure 1, lanes 2, 4, 6), while suf promoter region amplification was used as negative control of any potential DNA contamination in purified total RNA samples (data not shown). As shown in the gels from RT-PCR experiments (Figure 1), B. subtilis strain DD14 only expresses ThiI when cultured with 0.5 mM IPTG.

B. subtilis strains lacking nifZ and/or thiI display a specific disruption in the s^4U8 synthesis. The analysis of modified nucleosides from isolated tRNA samples was performed using HPLC-MS and/or UV detection. B. subtilis strains PS832, DD5, and DD14 were cultured in the absence and in the presence of 0.5 mM IPTG. The samples were analyzed for the presence of s^4U by monitoring the A_330nm. The peak associated with s^4U in these samples was verified by comparison to s^4U standard and by the observance of the 259 M/Z^-, corresponding to the mass of s^4U-H (Figure S1). For every sample, the intensity of s^4U signal was normalized to the intensity of the pseudouridine (ψ) peak, which was used as an internal control. Cells lacking nifZ and/or thiI displayed dramatically lower levels of s^4U (Table I, Figure S1), thus indicating the participation of both proteins in this thiolation pathway.

In B. anthracis and B. subtilis, genetic defects influencing the stringent response pathway can be manifested by defects in sporulation (26). Because of the association between UV-dependent stringent response and s^4U8 tRNA in E. coli (18), we hypothesized that the lack of s^4U8 could lead to defects on spore formation or spore resistance to UV radiation. Interestingly, the sspB gene located downstream of thiI is involved in spore resistance to heat and UV radiation (16). The spore count was estimated on cultures exposed to UV radiation, iron, and...
H$_2$O$_2$ challenges. Under these conditions, the pattern of sporulation of \textit{B. subtilis} strain DD14 was affected but not significantly different than the wild-type (data not shown). We have also not detected any differences on spores isolated from cells lacking NifZ and/or ThiI to survive upon exposure to UV radiation or heat.

In addition to the $s^4$U8 synthesis in \textit{E. coli}, IscS and ThiI also participate in sulfur mobilization during the formation of the thiazole moiety in the biosynthesis of thiamine (6). Strains lacking IscS or ThiI do not synthesize $s^4$U8 and they are thiamine auxotrophs (12, 18, 29). In \textit{B. subtilis}, however, strain DD14 showed no growth defects when cultured in minimal medium in the absence of thiamine (Figure S2). Although this experimental approach does not completely rule out the involvement of NifZ and ThiI in sulfur transfer reactions for the synthesis of thiazole, it does provide an indication that these steps can be bypassed or substituted by other cellular components \textit{in vivo}. For example, \textit{B. subtilis} genome encodes for NifZ and three additional cysteine desulfurases (YrvO, NifS, and SufS) which could act as alternative sulfur donors in strains lacking NifZ, thus providing functional replacement in the initial sulfur transfer step in thiazole biosynthesis. The lack of a specific sulfur donor in thiazole biosynthesis is also manifested under certain growth conditions in \textit{S. enterica}; the thiamine auxotrophy of a \textit{thiI} mutant strain is suppressed in cysteine enriched cultures (14).

\textbf{ThiI is a sulfur acceptor of NifZ cysteine desulfurase.} The purified form of NifZ showed identical catalytic activity in the standard cysteine desulfurase assay for sulfide (Figure 2) and alanine formation (Figure 4, left). The affinity of the enzyme for cysteine ($K_M$ = $29.7 \pm 8.7 \mu$M), was similar to the values determined for other cysteine desulfurases (15, 23, 30). The pH-dependence activity profile (Figure 2, inset) was suggestive that, at lower pH values, the rate limiting step is controlled by the deprotonation of the active site cysteine residue that performs the nucleophilic attack onto the substrate-activated thiol. The calculated $pK_a$ of $7.19 \pm 0.09$ for...
NifZ was slightly lower for a cysteine residue, but within the expected range of thiol-dependent enzymes.

SDS-PAGE analysis showed that in the presence of $^{35}$S-cysteine, NifZ becomes covalently modified with $^{35}$S. In addition, NifZ was able to covalently modify ThiI under non-reducing conditions (Figure 3). These results provide further support for involvement of the NifZ active site cysteine in forming a persulfide intermediate during NifZ’s catalytic cycle and the occurrence of a persulfide sulfur transfer step from NifZ to ThiI during the biosynthesis of $s^4$U8.

Recently, we have described the kinetic mechanism of the bisubstrate cysteine:SufU sulfurtransferase reaction catalyzed by the *B. subtilis* SufS enzyme (23). The presence of the sulfur acceptor SufU increases the catalytic rate of the SufS reaction by more than 100 fold. In addition, *E.coli* ThiI was able to elicit a two-fold enhancement of IscS cysteine desulfurase activity under reducing conditions (our unpublished results and (7)). The *B. subtilis* ThiI, however, was not able to enhance NifZ activity of sulfide or alanine formation (Figure 4, left). It is possible that in some representatives of class I cysteine desulfurases, such as NifZ, the active site cysteine, contained in a longer structural loop, is easily susceptible to cleavage by reducing agents (i.e. DTT could compete with ThiI for the enzyme’s persulfide-sulfur).

Therefore, we determined the activity profile of NifZ in the presence and absence of ThiI under non-reducing conditions (without DTT). Figure 4 (right panel) shows the linear steady-state rate of alanine formation catalyzed by NifZ in the absence of DTT ($80 \pm 1.4$ nmol Ala/min/mg). The activity is lower than that observed in the standard assay with DTT ($354 \pm 12$ Ala nmol/min/mg), which is in agreement with the slower catalytic formation of polysulfide by cysteine desulfurases under non-reducing conditions (33).

In contrast, under non-reducing conditions, the NifZ reaction in the presence of ThiI showed a biphasic profile of alanine formation (Figure 4, right). The activity associated with the initial
reaction rate was calculated to be $250 \pm 9.0$ nmol Ala/min/mg. Interestingly, the activity in the first phase in a reaction containing 20 molar equivalent of Thil (4.8 nmol) generated nearly stoichiometric amount of alanine (~4 nmol, 16 turnovers). The fast consumption of Thil led to the second phase of the reaction ($84 \pm 5.5$ nmol Ala/min/mg), which displayed a rate similar to the one observed for polysulfide formation (i.e. activity of NifZ without DTT and Thil).

NifZ and Thil are sufficient and capable of synthesizing $s^4U_8$ in vitro. The proposed catalytic cycle involving the $s^4U_8$ formation can be divided in three discrete steps: sulfur transfer from the cysteine desulfurase to Thil, the adenylation of tRNA by Thil, and the final transfer of the persulfide sulfur to the activated C-4 of the uridine 8 of tRNA to form $s^4U_8$ (17). We have investigated these three steps by probing: (i) the persulfide formation on Thil by NifZ (as described above), (ii) the [$\alpha$-$^{32}$P]-ATP Thil dependent adenylation of tRNA (Figure 5, left), and (iii) the overall synthesis of $s^4U_8$ in unmodified tRNA transcripts (Figure 5, right). The in vitro reaction required the presence of DTT (i.e. no $s^4U_8$ was formed under non-reducing conditions), suggesting the participation of an additional, as yet unidentified molecule serving as a reducing agent in this pathway.

Specific partnership between NifZ and Thil. The occurrence of analogous steps in the biosynthesis of $s^4U_8$ in B. subtilis and E. coli led us to investigate if the B. subtilis NifZ and/or Thil were capable of replacing E. coli IscS and/or Thil in vivo. Initial complementation studies were performed for individual components: (1) E. coli cells lacking IscS and expressing NifZ (E. coli CL100 strain transformed with pDS21 and cultured in the presence of arabinose) and (2) E. coli cells lacking Thil and expressing B. subtilis Thil (E. coli JLD26501 strain transformed with pDS108 and cultured in the presence of arabinose). In both cases, analysis of nucleosides isolated from tRNA samples showed no detectable $s^U$ (Figure 6). However, in vivo $s^4U_8$ formation was observed when these strains co-expressed both B. subtilis proteins NifZ and Thil (E. coli JLD26501 or CL100 strains transformed with pDS115 and cultured in the presence of...
arabinose) (Figure 6). The ability of B. subtilis NifZ and ThiI to heterologously synthesize s\textsuperscript{4}U8 provides additional in vivo evidence for specific interactions promoted during sulfur transfer, and discards the potential involvement of an additional B. subtilis specific component participating in sulfur trafficking in this pathway.

Despite general similarities between enzymes performing analogous steps in this pathway for E. coli and B. subtilis, the mechanism of sulfur transfer from the cysteine desulfurase to ThiI likely follows a different route. This observation is evident by the lack of individual-protein complementation with the E. coli system, which could be justified by the absence of the sulfur-receiving Rhd domain in the B. subtilis ThiI.

**DISCUSSION**

We have demonstrated the specific involvement of ThiI and NifZ in the biosynthesis of s\textsuperscript{4}U8 in B. subtilis. Despite similarities of the general scheme involving s\textsuperscript{4}U8 formation, this study provides the first experimental evidence that, in some species, tRNA modification involving ThiI dispenses the need for a sulfur-acceptor Rhd domain. This observation seems to be linked with the direct involvement of a “devoted” cysteine desulfurase that provides specific partnership during sulfur transfer. A sequence search on the microbial database of fully sequenced genomes indicated the presence of 229 genes encoding proteins similar to ThiI, 148 of which were sequences lacking the Rhd domain. In species representing the Firmicutes (87 genomes), ThiI sequences always lacked the Rhd domain and the thiI genomic location was always adjacent to a cysteine desulfurase gene similar to nifZ; the only two exceptions were Enterococcus faecalis and Lactococcus lactis.

Interestingly, none of the thiI genes encoding a ThiI-Rhd fusion protein were located next to a cysteine desulfurase gene. One possible function of the sulfur-acceptor Rhd-handle of ThiI is to interact more easily with general cysteine desulfurases. In E. coli and S. enterica, IscS is a
general cysteine desulfurase that interacts with a suite of different sulfur acceptors in the biosynthesis of most, if not all, thio-cofactors (17). Therefore, the activity of this enzyme has to be partitioned among the various physiological partners including ThiI. In these cases, the Rhd-sulfurtransferase domain seems to be more suitable for competing with other sulfur-accepting biomolecules. Conversely, ThiI sequences lacking this handle do not compete well in a one-donor/multi-acceptor environment. In *B. subtilis*, for example, this biological problem is circumvented with the presence of a devoted cysteine desulfurase.

The genomic synteny observed in the Firmicutes provides a good indicator for the partnership between a cysteine desulfurase and ThiI. Several lines of experimental evidence presented here not only support for this proposal, but also provide additional insight into specific steps of sulfur trafficking. First, NifZ and ThiI are required for the biosynthesis of s^U8 in *B. subtilis* resulting from the phenotypes observed upon gene inactivation. Second, the *in vitro* synthesis of s^U8 is achieved in the presence of purified forms of NifZ and ThiI. Third, complementation studies in *E. coli* indicated that the sulfur transfer step from NifZ to *B. subtilis* ThiI are mutually specific: i) NifZ is not capable of persulfide sulfur transfer to *E. coli* ThiI (NifZ does not complement IscS *in vivo*) and ii) *B. subtilis* ThiI is not capable of accepting a persulfide sulfur from IscS (*B. subtilis* ThiI does not complement *E. coli* ThiI *in vivo*). Collectively, these results indicate that *B. subtilis* NifZ and ThiI take an alternate route for sulfur acquisition and delivery when compared to studied systems involving IscS and ThiI-Rhd enzymes.

The requirement of a reducing agent, such as DTT, for the *in vitro* synthesis of s^U8 by *B. subtilis* NifZ and ThiI indicates the participation of another yet unidentified reducing agent in the catalytic cycle. Unlike the *B. subtilis* ThiI, in the absence of reducing agent, the *E. coli* ThiI is able to undergo one turnover reaction thus generating one equivalent of s^U8. The proposed mechanism for this reaction involves the regeneration of *E. coli* ThiI through reduction of the Cys344-Cys456 disulfide bond at the end of each catalytic cycle (17, 19). It is possible that in
the NifZ-ThiI reaction, a reducing agent could also be involved in the reduction of the persulfide sulfur on the cysteine desulfurase or on Thil to release S^{2-}. While the identity of the physiological reductant as well as the biosynthetic step(s) affected by such molecule are still undefined, results from complementation studies rule out the participation of a species-specific molecule acting as the reducing agent.

It has been proposed that short Thil sequences similar to the one from B. subtilis would lack sulfurtransferase activity (i.e. the ability to accept a sulfur from the cysteine desulfurase and transfer it to the adenylated-tRNA intermediate) and this role would be fulfilled by a separate polypeptide (28). However, the results presented here do not support this proposal. Despite the lack of a Rhd domain, B. subtilis Thil is able to directly interact with NifZ as these proteins co-purify when isolated from cells co-expressing NifZ and Thil. However, we do not believe that a stable complex is mandatory for the functionality of this pathway since separately purified enzymes are equally competent of performing \textit{in vitro} s^{4}U8 synthesis.

Kinetic analysis of cysteine desulfurase under non-reducing conditions revealed the role of Thil as a direct sulfur acceptor NifZ. The high catalytic rate of the cys:Thil sulfurtransferase reaction, displayed in the first phase of the reaction, showed stoichiometric dependence of Thil (i.e. reactions containing 4.8 nmol of Thil generated \~4 nmol of alanine in the first phase). The involvement of \textit{B. subtilis} Thil in persulfide sulfur transfer from NifZ cysteine desulfurase, shown in \textit{35}S-labeling experiments and kinetic analysis of NifZ under non-reducing conditions, suggests the formation of a catalytic intermediate on Thil.

Nevertheless, the lack of the sulfur-acceptor Rhd-domain indicates that this protein possesses an alternate transient site for sulfur modification. The \textit{B. subtilis} Thil Cys344 residue holds an equivalent position to the \textit{E. coli} Thil Cys344, which is involved in the final step of sulfur transfer through formation of a disulfide bond with Cys456 located in the Rhd domain (19, 20). While
The B. subtilis ThiI Cys344 residue likely displays a function analogous to the Cys344 of E. coli ThiI, the identity of the residue carrying the Thi sulfurtransferase activity remains unidentified. The lack of an obvious Cys456 corresponding residue suggests the participation of another cysteine residue in ThiI fulfilling this role. The B. subtilis ThiI sequence contains three additional cysteine residues (Cys81, Cys229, and Cys345). Among them, only Cys344 is completely conserved in all ThiI sequences. Interesting to note is the presence of the adjacent Cys345 residue which is conserved only in ThiI sequences from Gram-positive species. Either the participation of an additional cysteine residue of ThiI or the occurrence of an alternate sulfur transfer step involving the direct participation of NifZ are plausible mechanisms for the synthesis of s^4U8 tRNA. These two hypotheses are currently being explored.

Acknowledgements

The authors would like to thank T. J. Larson for providing E. coli strains used in this work, as well as for helpful feedback and R. W. Alexander for assisting with the synthesis of tRNA. This project was supported by the National Science Foundation (MCB-1054623).

References


Table I. tRNA modification levels in *B. subtilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Media additive</th>
<th>Relevant genotype</th>
<th>s^4U/ψ (std dev)^a</th>
<th>Relative levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS832</td>
<td>-</td>
<td><em>nifZ</em>, <em>thi</em></td>
<td>0.117 (+ 0.017)</td>
<td>7%</td>
</tr>
<tr>
<td>DD5</td>
<td>-</td>
<td><em>nifZ</em>, <em>thi</em></td>
<td>0.008 (+ 0.002)</td>
<td>7%</td>
</tr>
<tr>
<td>DD5 IPTG</td>
<td>IPTG</td>
<td><em>nifZ</em>, <em>thi</em></td>
<td>0.113 (+ 0.012)</td>
<td>96%</td>
</tr>
<tr>
<td>DD14</td>
<td>-</td>
<td><em>nifZ</em>, <em>thi</em></td>
<td>0.012 (+ 0.007)</td>
<td>10%</td>
</tr>
<tr>
<td>DD14 IPTG</td>
<td>IPTG</td>
<td><em>nifZ</em>, <em>thi</em></td>
<td>0.014 (+0.001)</td>
<td>11%</td>
</tr>
</tbody>
</table>

^a Absorbance ratio of the s^4U peak area from A_{330} nm chromatogram by the ψ peak area from A_{260} nm.

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**Figure Legends**

**Figure 1.** IPTG-dependent expression of ThiI in the DD14 strain. *B. subtilis* PS832 wild-type (1-2), and DD14 (3-6) were cultured in LB in the absence (1-4) and presence of IPTG (5-6). Total RNA was extracted and used in RT-PCR analysis of *thiI* (1, 3, 5) and the control *sufC* (2, 4, 6) transcripts.

**Figure 2.** Activity profile of NifZ cysteine desulfurase. Substrate saturation curve of NifZ activity determined through quantification of sulfide production. Assays were performed with a fixed concentration of NifZ (0.01 mg, 0.3 µM) and varying concentrations of L-cysteine in the presence of 2 mM DTT. Catalytic rates were calculated by the slopes of three-time point reactions. Saturation curve was a best fit for the Michaelis-Menten equation, determining the kinetic constants $K_m = 29.7 \pm 8.7$ µM and $V_{max}$ of $350 \pm 26$ nmol/min/mg. **Inset:** pH profile of NifZ activity demonstrates pH-dependence of cysteine desulfurase activity. Assays detecting sulfide were conducted with fixed concentrations of NifZ (0.01 mg, 0.3 µM), L-cysteine (0.5 mM), and DTT (2 mM) over a range of pHs. The line is a best fit for the Henderson-Hasselbalch equation resulting in a pKa of 7.19 ± 0.09.

**Figure 3.** *In vitro* sulfur transfer between NifZ and ThiI. NifZ and/or ThiI (4 µM each) were incubated with $^{35}$S-L-cysteine (10 µCi), L-cysteine (50 µM), ATP (1 mM) and MgCl$_2$ (5 mM) and quenched with *N*-ethylmaleimide (0.4 mM). Non-reducing SDS-PAGE gels were stained with Coomassie Brilliant Blue (right) and exposed using PhosphoImager (left). Labeling of NifZ and ThiI shows the $^{35}$S -covalent modification in the absence of DTT.

**Figure 4.** NifZ activity is enhanced by sulfur acceptor ThiI in non-reducing conditions. Alanine formation monitored over time upon incubation with NifZ (0.01 mg, 0.3 µM) in the absence (▲) and presence of 6 µM of ThiI (■). (Left) The linear rate of alanine formation under reducing (2
mM DTT) conditions shows activity levels of 354 ± 12 nmol/min/mg (__, NifZ) and 262 ± 24
nmol/min/mg (----, NifZ + ThiI). (Right) NifZ activity under non-reducing conditions shows a
linear rate of alanine formation (80 ± 1.4 nmol/min/mg), while in the presence of ThiI, displays a
biphasic reaction profile for alanine production. The reaction velocity associated with the first
phase of the reaction (250 ± 9.0 nmol/min/mg) was faster than the rate calculated for the
second phase of the reaction (84 ± 5.5 nmol/min/mg). The graphs show representative curves
from individual experiments. The velocities were calculated from a linear fit of alanine formation
over time for each reaction or distinct phase within each reaction. The specific activities
indicated above are the average of at least three independent experiments.

Figure 5. In vitro synthesis of s^4U by B. subtilis ThiI and NifZ. (Left) Formation of adenylated-
tRNA was performed by incubation of synthetic tRNA transcripts (100 μM) with [α-^32P]-ATP (10
μCi) and MgCl₂ (1 mM). Reactions containing 10 μM E. coli or B. subtilis ThiI (+) were compared
to the reactions in the absence of ThiI (-). Samples were analyzed by urea-PAGE and
visualized in Phosphoimager. The lower MW ^32P-signal is attributed to degradation of tRNA-
adenylated during PAGE analysis. (Right) In vitro generation of s^4U was performed in
synthetic tRNA transcripts in the presence of NifZ and ThiI as described in the Materials and
Methods section. The A₃₃₀ nm chromatographic profiles of tRNA-digested nucleoside resulting from
reactions in the presence of separately expressed and purified NifZ and ThiI (- · · ·), and co-
expressed and co-purified NifZ-ThiI (- · · ·) were compared to the chromatographic profile of the
s^4U standard (—), and digested unmodified tRNAMet transcript (— — ).

Figure 6. In vivo synthesis of s^4U by B. subtilis ThiI and NifZ in E. coli. Complementation of E.
coli CL100, iscS deletion strain (— — ) or E. coli JLD26501, thiI deletion strain (*****- ) was
performed with plasmids expressing B. subtilis NifZ and/or B. subtilis ThiI. The HPLC A₃₃₀ nm
chromatograms of tRNA-digested nucleosides show the distinct presence of s^4U in cells co-
expressing both B. subtilis nifZ and thiI (pDS115, black lines). Chromatograms from individual
complementation experiments along with negative controls (both strains transformed with pAra13) displayed absorbances near detection limit (gray). The peak corresponding to s4U from complementation studies was compared to the s4U standard (inset) and to s4U from E. coli MG1655 (——). The levels of s4U, from at least three independent experiments, were determined from the ratio of absorbances from assigned peak areas, $A_{330\text{ nm}} (\text{s4U})/A_{260\text{ nm}} (\psi)$. The calculated average absorbance ratios of s4U/ψ were the following: E. coli MG1655 (wild-type), 0.712±0.279; E. coli JLD26501 - pDS115, 0.361±0.246; E. coli JLD26501 - pDS108, 0.007±0.004; E. coli JLD26501 – pAra13, 0.001±0.007; E. coli CL100 - pDS115, 0.464±0.234; E. coli CL100 - pDS21, 0.007±0.007; and E. coli CL100 – pAra13, 0.007±0.004.