Sulfur availability impacts the accumulation of the s^2U tRNA modification in *Bacillus subtilis*

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Abstract

Post-transcriptional modifications to tRNA are critical elements for the folding and functionality of these adaptor molecules. Sulfur modifications in tRNA are installed by specialized enzymes that act on cognate tRNA substrates at specific locations. Most studied organisms contain a general cysteine desulfurase to mobilize sulfur for the synthesis of S-tRNA and other thio-cofactors. *Bacillus subtilis* and other Gram-positive bacteria encode multiple cysteine desulfurases that partner with specific sulfur acceptors in the biosynthesis of thio-cofactors. This metabolic layout suggests an alternate mode of regulation in these biosynthetic pathways. Here, tRNA modifications were exploited as a readout for the functionality of pathways involving cysteine desulfurases. These analyses showed that the relative abundance of 2-thiouridine modified tRNA (s^2U) responds to sulfur availability in the growth medium in a dose-dependent manner. This study found that low sulfur concentrations lead to decreased levels of the s^2U cysteine desulfurase YrvO and thiouridylase MnmA, without altering the levels of other cysteine desulfurases, SufS, NifS, and NifZ. Analysis of pathway metabolites that depend on the activity of cysteine desulfurases indicates that sulfur nutrient availability specifically impacts s^2U accumulation while having no effect on the level of other S-modified tRNA or activity levels of Fe-S enzymes. Collectively, these results support a model in which s^2U tRNA serves as a marker for sulfur availability in *B. subtilis*.

Importance

The 2-thiouridine (s^2U) tRNA modification is found ubiquitously across all domains of life. YrvO and MnmA, the enzymes involved in this modification, are essential in *B. subtilis*, confirming the well-established role of s^2U in maintaining translational efficiency and, consequently, cellular viability. Herein, we show that in the model Gram-positive organism *Bacillus subtilis*, the levels of s^2U are responsive to sulfur availability. Downregulation of the s^2U biosynthetic components leads to lower s^2U levels, which may serve as a signal for the slowing of the translational apparatus during cellular nutrient insufficiency. Our findings provide the basis for the identification of a

potential bacterial mode of regulation during S-metabolite depletion that may use s^2U as a marker of suboptimal metabolic status.

Introduction

tRNA provides the physical link for the decoding of genetic information during protein synthesis. The maturation of tRNAs involves a series of post-transcriptional modifications that alter the structure and function of these adaptor molecules, thereby guaranteeing the accuracy and efficiency of translation of the genetic code. More than a hundred modifications have been described throughout the structure of tRNA (1). Chemical alterations to anti-codon bases have long been defined as critical elements for recognizing degenerated codons within mRNA (2). It is also known that select tRNA modifications are essential for interactions with other components of the translational apparatus, including tRNA synthetases, elongation factor Tu (EF-Tu), and the ribosome (3-5). Thus, given the importance of these modifications, it is no surprise that defects in enzymes known to catalyze these modifications lead to either lethal phenotypes or metabolic defects resulting in compromised cellular viability (6-8).

The installment of tRNA modifications requires specialized enzymes that introduce expanded chemical functionalities within the bases and 2’OH of tRNA at predictable locations. These enzymes recognize their cognate tRNA substrates and install chemical modifications through substitutions or addition reactions with complexities varying from simple methylations to the insertion of highly intricate structures like the queuosine (Q34) full base replacement and geranyl moieties (9, 10). Biosynthetic pathways responsible for these modifications are diverse in nature, number of enzymatic components, and in some cases diverge across species and even in organelles within the same species. These various pathways display not only distinct mechanistic strategies for modifying tRNA, but also offer an opportunity for alternate regulatory mechanisms of these enzymes, and consequently, modulation of tRNA functionality.

In addition to its canonical role in translation, tRNAs in their fully and partially modified forms are also proposed to serve as regulatory entities and sensors of metabolic cellular status (11). These alternate roles are attributed to the fact that tRNA modifications vary in type and degree of modification in response to their growth phase, environmental signals, and nutritional conditions (12). These conditions are known to affect tRNA modifications either directly, by reacting with modified tRNA bases such as UV radiation and alkylating agents, or indirectly, by down-regulating expression or inactivating tRNA modifying enzymes (13, 14). Thus, tRNA and their associated modified nucleosides can be exploited as markers of metabolic changes and potentially serve as intermediates in signaling events that allow cells to adjust translational capacity under suboptimal growth conditions.

Sulfur-containing modifications to tRNA are found in all organisms studied thus far (15, 16). In bacteria and eukaryotes, these modifications are dependent on the activity of cysteine desulfurases. These enzymes are known to utilize the free amino acid cysteine as the initial sulfur source for the biosynthesis of S-containing tRNA (17-19). They directly participate as sulfur donors for the biosynthesis of thio-modified tRNA, as observed for the 4-thiouridine (s^4U8), 2-thiouridine (s^2U34), and 2-methylthioadenosine (ms^2A37) modifications (19). These enzymes are also involved in the synthesis of other thio-cofactors, including Fe-S clusters, thiamin, biotin, lipoic acid, and the molybdenum cofactor (20). Because of their role in Fe-S cluster formation, cysteine desulfurases also indirectly affect the synthesis of certain tRNA modifications that do not contain sulfur, but rather are dependent on the activity of Fe-S cluster-containing tRNA modifying enzymes, as in the case of the 2-methyladenosine (m^2A37), epoxyqueuosine (oQ34), and Q modifications (19). Therefore, analysis of tRNA modifications across distinct cellular contexts provides an attractive metabolic probe to assess the direct and indirect participation of cysteine desulfurases and tRNA modifying enzymes in sulfur trafficking pathways.

The involvement of cysteine desulfurases in pathways involving tRNA modifying enzymes has been previously explored in bacteria. In *Escherichia coli* and *Salmonella enterica*, IscS is the master cysteine desulfurase that acts as the primary sulfur donor to all sulfur-containing modifications in tRNA, in addition to serving as the sulfur source for other thio-cofactors (17, 21, 22). The general involvement of IscS in several biochemical pathways, not limited to Fe-S clusters as initially described, stems from its ability to interact with a suite of sulfur acceptors with diverse folds and functions (23). Conversely, *Bacillus subtilis* and other Gram-positive organisms do not encode one master cysteine desulfurase like IscS. Instead, their genomes code for multiple cysteine desulfurases.

In organisms expressing multiple cysteine desulfurases, the genomic neighborhood of cysteine desulfurases gives insight into their distinct functions during the synthesis of thio-cofactors. *B. subtilis,* for example, expresses four functionally active cysteine desulfurases: SufS, NifZ, YrvO, and NifS. In previous work, we have investigated the selective reactivity of these enzymes and their corresponding sulfur acceptors. We established that NifZ and the thiouridylase ThiI are involved in the biosynthesis of s^4U tRNA (24). Similarly, we showed that YrvO can transfer sulfur directly to the MnmA thiouridylase during s^2U tRNA formation, utilizing an abbreviated pathway that dispenses the need for a sulfur relay system observed in other species (25). We and others determined that SufS partners with SufU, a zinc-dependent sulfurtransferase, in the general synthesis of Fe-S clusters (26, 27). Lastly, inactivation of the cysteine desulfurase NifS leads to defects in NAD biosynthesis (28), a phenotype that is justified by the proposed role of NifS in the activation of the Fe-S enzyme quinolinate synthase, NadA. Taken together, biochemical and genetic studies have demonstrated that these cysteine desulfurases partner with sulfur acceptors to perform dedicated roles. This metabolic layout suggests that the recruitment of multiple enzymes provides an alternate strategy for directing sulfur supply for thio-cofactor synthesis by uncoupling routes of sulfur trafficking.

In this investigation, we sought to gain insight into how sulfur source and concentration would impact the accumulation of these biosynthetic enzymes and their pathway metabolites. Herein, we analyzed the relative levels of tRNA modifications as a metabolic readout of the functionality of biosynthetic pathways involving three cysteine desulfurases in *B. subtilis*: YrvO, SufS, and NifZ. We show that sulfur availability provided in the media selectively impacts the levels of the s^2U biosynthetic components, YrvO and MnmA, the accumulation of this thionucleoside, and select tRNAs targeted for this modification. Given the essentiality of these enzymes and the well-documented role of s^2U in protein synthesis, we propose that down-regulation of the s^2U modification during varying sulfur availabilities leads to changes in translational capacity in this organism.

Results

Bacillus subtilis* tRNA epitranscriptome is modulated by nutritional conditions. In a recent study, 29 modifications were reported at varying positions in *B. subtilis* tRNA (29). Analysis of genes encoding tRNA modifying enzymes in the Colombos database showed variation in their expression across different growth conditions and phases. Most of these enzymes displayed a unique pattern of expression across different stimuli, suggesting that the landscape of tRNA modifications possibly adjusts its composition in response to cellular changes. Therefore, we first surveyed the *B. subtilis* tRNA epitranscriptome in an effort to expand upon these previous findings and determine the presence of tRNA modifications in *B. subtilis* cells cultured at different growth stages and media. This initial analysis ensured our ability to add to the evolving body of literature surrounding *B. subtilis* tRNA modifications. Using LC-MS analysis of purified RNA nucleosides from *B. subtilis* cultures, we were able to confirm the prevalence of all base modifications recently reported by Crecy-Lagard (29). The varying levels of these modifications were compared in rich and minimal media cultures at early, mid, and late exponential phases, corresponding to OD_{600nm} of 0.5, 1, and 1.5, respectively (**Table S1 and S2**). The relative levels of several modifications exhibited varying patterns of accumulation (**Table I)** These analyses also matched previous findings that show increased relative levels of 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A37) over its precursor form, N^6-isopentenyladenosine (i^6A37), as cells enter the stationary phase (30). However, we do not attribute this result to the apparent increase of the hypermodified base ms^2i^6A, but instead to the decreased levels of i^6A at the later growth stages, which decreases 2 and 10-fold in minimal and rich media, respectively. Another modification showing marked fluctuation is the Q hypermodification and its precursor nucleoside, oQ. Total RNA isolated from cells cultured in rich medium showed no detectable levels of oQ, and accumulation of this intermediate was only observed in minimal medium. Additionally, the relative levels of the Q modification were over 10-fold higher in rich medium. These variations are compatible with the requirement of cobalamin as a cofactor during the conversion of oQ to Q by the epoxyqueuosine reductase, QueG (31). The ratio of 5-methyaminomethyl-2-thiouridine (mnm^5s^2U34) to 5-carboxymethyaminomethyl-2-thiouridine ($cmnm^5s^2U34$) remains mostly balanced across growth stages in minimal medium, and was in agreement with previous reports (32), but drops at later growth stages in rich media. Overall, these initial analyses indicated that tRNA modifications fluctuate across growth stages and that the availability of nutrients, as assessed by rich and minimal media, also impacts the relative accumulation of these metabolites, some of which have been previously described. Therefore, when performing a comparative study on the effects of nutritional and environmental factors on the tRNA epitranscriptome, it is important to assess cultures grown under controlled conditions and analyzed at the same growth stage.

S^2U-modified tRNA levels respond to sulfur availability. Utilizing this workflow, we specifically analyzed tRNA modifications dependent on cysteine desulfurases using cultures grown in chemically defined media. Cells were harvested at mid-exponential phase (OD_{600nm} of 0.5), as this represents a growth stage in which overall nutrient levels have not yet been depleted. We hypothesized that the occurrence of multiple cysteine desulfurases provides a strategy for differential regulation of these enzymes, and consequently, differential accumulation of metabolites dependent on their activities. Therefore, we determined the relative abundance of tRNA modifications as a readout of cysteine desulfurase functionality and ultimately assessed their ability to either directly or indirectly promote chemical modifications within tRNA nucleosides. The relative degree of modification to tRNA was determined by analyzing individual digested tRNA species, in which the mass abundance of each modification was normalized by the mass abundance of dihydrouridine (D). This modification serves as an excellent internal normalizing standard due to its high abundance, stability, and virtual exclusivity in prokaryotic tRNA (33). We specifically interrogated whether varying sulfur availability would impact the accumulation of tRNA modifications. Interestingly, under sulfur replete conditions, the source of sulfur did not affect growth (**Fig. S1**) or the accumulation of sulfur-containing nucleosides (**Table S3**), indicating that *B. subtilis* can adjust its metabolism to multiple sulfur sources.

However, varying concentrations of the sulfur source showed an impact on the rate of growth (**Fig. 1)**. *B. subtilis* cells cultured under low sulfur concentrations (*e.g.* 0.1 mM sulfate) displayed prominent lag phase extension when compared to the 1 mM and 50 mM “sulfur replete” growth rates. This growth profile suggests the presence of regulatory mechanisms under nutrient deprived conditions. Relevant to the analysis of this study, we found that the relative levels of mmn^{5}s^{2}U and cmnm^{5}s^{2}U responded drastically to varying sulfur concentrations (**Fig. 2**). In fact, the accumulation of hypermodified s^{2}U derivatives showed a dose-dependent accumulation upon increasing concentrations of sulfur in the growth medium. Under these conditions in the same tRNA samples, the levels of sulfur-containing modifications whose synthesis depends on the activity of other cysteine desulfurases remained unchanged, suggesting a potential alternate mechanism for regulation of thio-cofactor synthesis (**Fig. 2**). It is worth mentioning that *B. subtilis* does not contain 2-thiocytidine (s^{2}C32), and its genome does not encode the s^{2}C-modifying biosynthetic enzyme, TtcA. Additionally, another S-modification reported in this organism, 2-methylthio-N^6-threonylcarbamoyladenosine (ms^{2}t^{6}A37), does not accumulate to high enough levels that permit a semi-quantitative assessment of its presence under the tested conditions. Collectively, these results show that the accumulation of s^{2}U tRNA is responsive to nutritional growth conditions.

***Levels of s^{2}U biosynthetic enzymes respond to sulfur availability*.** The decreased levels of s^{2}U tRNA in response to sulfur limitation could be attributed to decreased levels of thiolation substrates (i.e., cysteine) and/or their biosynthetic enzymes. To investigate these possibilities, we determined intracellular levels of reduced cysteine, YrvO, and MnmA from cells cultured under distinct sulfur concentrations. Cell extracts from washed cell pellets were derivatized with monobromobimane, followed by HPLC separation and quantification of the fluorescently-labeled Cys-bimane adduct. These analyses showed steady levels of cysteine across various conditions (**Table S4**); that is, under controlled growth conditions, reduced cysteine varied from 0.35-0.39 mM, which is similar to previously reported intracellular reduced cysteine concentrations in *B. subtilis* (0.13-0.58 mM) (34, 35). Notably, changes in sulfur concentrations in the media did not dramatically impact intracellular levels of reduced cysteine, further iterating the tight regulatory network imposed on cysteine biosynthesis and degradation as previously described. While the levels of cysteine did not change under these varying conditions, the levels of s^2U biosynthetic enzymes, YrvO and MnmA, responded to sulfur availability in the growth medium in a dose-dependent manner. Further quantification of these enzymes in soluble cell extracts showed that cells cultured under low sulfur concentrations displayed reduced levels of both enzymes (**Fig. 3**). We then investigated if the decrease in accumulation of these enzymes was a result of down-regulation at the transcriptional level. However, qPCR analysis showed that the levels of *yrvO* and *mnmA* transcripts remain unaltered in cells cultured under both high and low sulfur conditions (**Fig. S4**). Interestingly, previous kinetic analysis reporting the reactivity of *B. subtilis* cysteine desulfurases towards their cysteine substrate revealed that YrvO has the lowest K_M for cysteine when compared to the other desulfurases in this organism (24-26). Since the availability of intracellular cysteine is tightly controlled, regulation of the s^2U pathway component's expression or activity provides a rationale for the low levels of its final pathway metabolite, s^2U tRNA.

Accumulation of other cysteine desulfurases and their metabolites do not respond to sulfur availability. This regulatory response was not observed for the other three *B. subtilis* cysteine desulfurases. Analysis of the same crude extracts showed that the levels of NifS, NifZ, and SufS did not vary with sulfur concentration (**Fig. 3**). In fact, metabolites dependent on NifZ and SufS activity also remain unchanged under these conditions. Supporting this result, the levels of s^4U, whose synthesis depends on NifZ and ThiI, did not vary with sulfur concentration (**Fig. 2**). Likewise, the metabolites presumably dependent on pathways involving SufS also remained unaltered under these varying conditions. In *B. subtilis*, the general synthesis of Fe-S clusters is proposed to involve the Suf system (36). The functionality of this system can be assessed through the activity of Fe-S enzymes and through quantification of the levels of tRNA modifications that require Fe-S enzymes in their biosynthetic schemes (19). Under low sulfur concentrations, the activity of Fe-S enzymes aconitase (ACN), glutamine:2-oxoglutarate amidotransferase (GOGAT), and isopropyl malate dehydratase (LeuCD) remained unchanged. The same trend was also observed for enzymes that do not require Fe-S clusters or the activity of SufS, such as isocitrate dehydrogenase (IDH) and fumarase (FUM) (**Fig. 4**). Furthermore, relative levels of the tRNA modifications ms^2i^6A, oQ, Q, and m^2A, which are synthesized by Fe-S enzymes proposed to rely on the Suf system, also remained unresponsive to these varying sulfur concentrations (**Figs. 2 and 5**). Lastly, as expected, the levels of NifS did not change. The expression of this cysteine desulfurase has been shown to depend on the availability of nicotinic acid in a regulatory system dependent on the transcriptional regulator NadR, and its activity is known to impact the synthesis of NAD in *B. subtilis* (28).

***Low levels of tRNA thiolation lead to low levels of tRNA ^{Lys} and tRNA^{Glu}*.** It has been previously demonstrated that hypomodified tRNA is targeted for degradation (2, 37, 38). Therefore, we sought to determine whether conditions that led to downregulation of the synthesis of s^2U tRNA would ultimately lead to lower levels of its targets substrates, tRNA^{Glu,Gln,Lys}. Northern blot analyses of total RNA samples isolated from cells cultured under low sulfur concentrations showed lower levels of accumulated tRNA^{Lys} and tRNA^{Glu}, and a modest decrease of tRNA^{Gln} (**Fig. 6**). Importantly, under sulfur limiting conditions, the extent to which these tRNA substrates are depleted was not nearly as dramatic as the effect observed in mnm^5s^2U under the same nutrient limitations. Control experiments included tRNA^{Thr}, which does not carry the s^2U

modification, but is transcribed in the same operons as these selected tRNAs. While mechanisms triggering degradation of hypomodified tRNA have been described for s^2U in *E. coli* (39), it is unknown if *B. subtilis* evokes a specific RNA nuclease under conditions of nutrient limitation or if this process fits under the umbrella of the *B. subtilis* general tRNA quality control system. Nevertheless, it is worth noting that the lower abundance of the s^2U-modified tRNA under sulfur depleted conditions is not accompanied by proportionally increased levels of the partially modified 5-methylaminomethyluridine (mnm^5U34) and 5-carboxymethyaminomethyluridine ($cmnm^5U34$) precursor tRNAs (**Fig. S5).** Taken together, these results support the presence of lower levels of these tRNA species under low sulfur conditions and further highlight the importance of s^2U for the stability of tRNAs carrying this modification.

Discussion

The synthesis and reactivities of modified tRNA are known to be affected by nutritional and environmental changes (12). Here, we report that the levels of the essential s^2U modification in tRNA vary with different sulfur concentrations provided in the growth medium. This phenomenon was specific to both s^2U derivatives, namely mnm^5s^2U and $cmnm^5s^2U$, and was not observed in other abundant thio-tRNA modifications. The near exclusivity of this dose-dependent response is relevant given the essential nature of this modification, and the broader implications of these findings highlight the complex nature of cellular physiological checkpoints. These findings provide a potential link between s^2U's long understood and highly conserved role in directly influencing the speed and accuracy of translation (40-42), and its ability to respond to the available pool of cellular S-metabolites in a manner that is independent of other known *B. subtilis* sulfur trafficking systems.

Interestingly, the sulfur-mediated, dose-dependent effect impacting this modification did not show a proportionally inverse relationship to the partially modified forms of mnm^5s^2U and $cmnm^5s^2U$. In fact, the levels of these modifications only slightly increased at higher sulfur concentrations and not to the degree their hypermodified counterparts did (**Fig. S5**). While modest increases were observed, a direct comparison between the levels of these hypomodified tRNAs and their thiolated forms is convoluted, as it has been shown that the *E.coli* Mnm machineries, MnmEG and MnmC, are able to use $tRNA^{Leu,Arg,Gly}$ as substrates for their methylation and decarboxylation reactions in addition to the thiolation substrates, $tRNA^{Lys,Glu,Gln}$ (43). These results, however, support previous *in vitro* experiments promoting the synthesis of singly modified s^2U, and an absence of mnm^5s^2U formation when using bulk *in vivo* isolated tRNA containing both U34 and partially modified mnm^5U34 tRNA (25). Furthermore, structural analysis of *E.coli* MnmA bound to the adenylated tRNA shows the presence of conserved, bulky residues that would hinder the binding of a partially modified tRNA substrate containing mnm^5U or $cmnm^5U$ at the active site (44). Together with previously demonstrated structural and enzymatic data, the results presented herein support a model in which the s^2U modification precedes installation of additional modifications at C5 of U34 in $tRNA^{Lys,Glu,Gln}$. Notably, our tRNA analyses yielded no detectable levels of 5-aminomethyluridine (nm^5U34) or 5-aminomethyl-2-thiouridine (nm^5s^2U34) intermediates. It is known that *B. subtilis* lacks an MnmC ortholog, the enzyme responsible for the synthesis of these respective modified tRNAs. However, *B. subtilis* has been shown to efficiently catalyze the formation of mnm^5s^2U (32). Although the proposed pathway involves the transient formation of a nm^5s^2U intermediate, this species has not been detected in *B. subtilis* tRNA samples, suggesting the high efficiency of this proposed MnmC-like reaction.

Analysis of tRNA also showed that low sulfur availability does not impact the levels of other tRNA modifications dependent on the activity of other cysteine desulfurases. The occurrence of distinct cysteine desulfurases in *B. subtilis* provides alternate points of regulation for the synthesis and use of thiocofactors. The levels of SufS were unchanged under these conditions, indicating that its pathway components remain relatively stable. SufS is expressed with other Suf components and is proposed to be the primary, if not sole, Fe-S cluster biosynthetic system in this organism (36). The activities of Fe-S enzymes were unchanged; likewise, the levels of nucleosides whose syntheses are known to involve Fe-S enzymes were also unperturbed under identical conditions, suggesting that *B. subtilis* potentially prioritizes Fe-S metabolism to sustain critical biochemical reactions, even during sulfur limiting conditions.

The occurrence of multiple cysteine desulfurases in *B. subtilis* and the involvement of a dedicated enzyme for the synthesis of s^2U that is uncoupled from the general synthesis of Fe-S clusters suggest an alternate strategy to mobilize and regulate thiocofactor biogenesis. The levels of these enzymes respond to sulfur availability and impact the accumulation of their pathway metabolites. Interestingly, the *E. coli* thiouridylase MnmA has been recently shown to coordinate an Fe-S cluster that provides the enzyme with enhanced reactivity (45). Whether the occurrence of this type of cofactor is also observed in the *B. subtilis* MnmA merits investigation. Yet, the utilization of an abbreviated s^2U pathway in this organism provides an exciting model for elucidating the mechanistic steps during sulfur acquisition and transfer, and potentially establishes additional points of regulation in s^2U formation.

The low levels of s^2U tRNA are attributed to the low availability of its biosynthetic enzymes. In this study, the quantification of cysteine desulfurases was determined in clear soluble cell extracts, allowing for accurate protein quantification of samples prior to western-blot analysis. The levels of other cysteine desulfurases in these samples did not vary across conditions, which unintentionally provided an internal standard for these analyses. Additionally, transcriptomic analyses revealed that levels of all *B. subtilis* cysteine desulfurases remained relatively consistent across all sulfur conditions, suggesting that regulation of YrvO and MnmA occurs at translational or post-translational levels (**Fig. S4**). While the mode of regulation of s^2U biosynthetic enzymes will be explored in subsequent investigations, it was outside the scope of this present study. However, it is known that genes *encoding B. subtilis* YrvO and MnmA are located immediately downstream of the master regulator of cysteine metabolism, CymR (46). The regulation of the promoter driving the expression of *cymR-yrvO-mnmA* operon has been identified to be under the control of Spx, and consequently *sigA*, which are known to respond to stressors affecting thiolredox homeostasis, such as diamide and carbonyl electrophiles. Spx functions in sulfate-dependent control of organosulfur utilization operons through stimulation of *cymR* expression. Spx could affect the concentration of a metabolite, perhaps an intermediate of sulfur assimilation or organosulfur utilization, which serves as an effector of *cymR* expression control (47). Spx was also found to negatively regulate the expression of these operons in sulfate medium, in part, by stimulating the expression of the *cymR* gene (48). In addition, *trmU* (*mnmA*) was the only tRNA-modifying gene affected by the RelA-dependent stringent response during norvaline-induced stress, suggesting that nutritional triggers other than sulfur availability may also lead to lower levels of s^2U accumulation. Therefore, the genomic location of s^2U biosynthetic genes and their co-expression with *cymR* provides us with a working model that links s^2U to *B. subtilis* sulfur metabolism.

The dose-dependent accumulation of s^2U with varying sulfur concentrations supports a scenario in which s^2U serves as a marker of sulfur availability. Previous studies demonstrated that both *yrvO* and *mnmA* are essential genes in *B. subtilis*, indicating that s^2U tRNA is a critical modification enabling translational accuracy and cellular viability. In yeast, the degree of U34 thiolation reflects the availability of S-containing amino acids. This process ultimately utilizes phosphate homeostasis as a metabolic control point during amino acid insufficiency (8, 49). Notably, the yeast pathway involved in this modification is phylogenetically distinct from the ones described in bacteria (50). While the loss of U34 thiolation in yeast led to the characterization of broad metabolic defects and the downregulation of enzymes indispensable to central metabolism, this study explored the impact of sulfur nutrient limitation on sulfur trafficking pathways. Here, we showed that under conditions of sulfur limitation, *B. subtilis* cells display a growth delay phenotype accompanied by a remarkable decrease in the accumulation of YrvO and MnmA. This may be a result of a combination of not fully understood regulatory mechanisms in place during these nutrient conditions. It is also anticipated that other conditions and cellular processes may elicit the degradation of tRNA and regeneration of bases modified within tRNA molecules. For instance, *B.*

subtilis contains a putative ortholog of the 4Fe-4S cluster thiouracil desulfidase TudS, which is shown to catalyze the desulfurization of 4-thiouracil and 2-thiouracil (51).

The results shown in this study suggest that when cell growth and adequate translational efficiency is compromised through the combined loss of s^2U biosynthetic components, unmodified substrate tRNAs, and thiolated U34 tRNA, metabolic output is proposed to wane and mirror a manufactured “starvation state”. Under these conditions, lower levels of s^2U biosynthetic enzymes are proposed to result in lower levels of s^2U tRNA, leaving substrate $tRNA^{Lys,\ Glu,\ Gln}$ hypomodified (**Fig. 7**). These tRNAs are then likely targeted for degradation by a yet unknown cellular quality control system. It is possible that an s^2U-dependent response triggers a metabolic rewiring that allows for direct and indirect salvage of essential metabolites. Future studies aim to explore these interconnected regulatory pathways responsible for nutrient sensing in *B. subtilis*.

Methods

Media, media additions, and chemicals. All *B. subtilis* PS832 growths were cultured in Spizizen’s minimal media (52) consisting of 15 mM NH_4Cl, 80 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM trisodium citrate dehydrate, 0.5% sucrose, varying concentrations of $(NH_4)_2SO_4$ as the sulfur source, and trace elements (60μM $MgCl_2$, 5 μM $CaCl_2$, 5 μM $FeCl_2$, 0.5 μM $MnCl_2$, 1.25 μM $ZnCl_2$, 0.25 μM $CuCl_2$, 0.4 μM $CoCl_2$, 0.3 μM Na_2MoO_4, final concentration). Unless stated, all chemicals were purchased from Fisher Scientific and Sigma-Aldrich Inc.

B. subtilis growth and tRNA isolation. *B. subtilis* PS832 was grown in LB medium or Spizizen medium at 37°C at 300 RPM in 0.5 L cultures. Growth was monitored by optical density at 600 nm (OD_{600}). Unless otherwise indicated, cells were harvested at early log-phase (OD_{600} = 0.5), and cell pellets were obtained through centrifugation at 8,200 x *g* for 10 min and frozen at -20°C until further use. Total RNA was extracted from cell pellets as reported previously (53). Briefly, RNA was extracted from resuspended cells using sodium acetate-saturated phenol, pH 4.3, followed by an additional extraction using a 24:1 chloroform/isoamyl alcohol mixture, and precipitated overnight with 100% ethanol at 4°C. RNA pellets were washed with 70% ethanol, air-dried, and resuspended in Optima, LCMS grade water for further analyses.

Northern Blotting*.* An aliquot of 5 µg of total purified RNA was separated on a 10% TBE urea denaturing gel (10% acrylamide/Bis 19:1 w/v; 7 M urea). Electrophoresed RNA was transferred to a 0.45 µM Biodyne B Pre-Cut Modified Nylon Membrane at 250 mA for 2 hr, and then at 350 mA for 2 hr, all performed at 4°C. Membranes were twice cross-linked for 60 sec with 1,200 µJ UV light, followed by a pre-hybridization for 2 hr at 42°C with ULTRAhyb-Oligo Hybridization buffer. Membranes were subjected to 18 hr of hybridization at 42°C with 10 pmols of the appropriate Cy5-labeled tRNA-specific oligonucleotide probe (**Table S5**). Membranes were washed twice with 2X SSC/0.1% SDS (0.3 M NaCl; 0.03 M sodium citrate dihydrate; 0.1% sodium dodecyl sulfate; pH 7.0) low stringency buffer for 5 min at 42°C, followed by two additional 0.1X SSC/0.1% SDS high stringency buffer washes for 15 min at 37°C. Hybridized probe was detected using an Amersham AI600 imager (Amersham Biosciences) using the default Cy5 settings at 630 nm. All fluorescence intensities were quantified after digital capture using ImageJ software and normalized to *B. subtilis* PS832 grown in Spizizen's minimal medium supplemented with 17 mM $(NH_4)_2SO_4$.

Western Blotting**.** Protein crude extracts were prepared using a sonicator (Fisherbrand Model 120 Sonic Dismembrator 4X) for 10 sec at 50% amplitude, followed by centrifugation at 16,873 x *g* for 20 min. Protein concentration of soluble crude extracts were determined using the Bio-Rad Protein Assay and a bovine serum albumin (BSA) standard curve. Aliquots of 50 µg of protein were separated using a reducing 10% SDS PAGE. Electrophoresed protein was transferred to a 0.2 µm nitrocellulose membrane for 1 hr at 4°C, and then blocked with 4% milk in 1X TBST for 1 hr at room temperature. Blocked membranes were incubated agitating at 4°C with rabbit custom antibodies against *B. subtilis* YrvO, MnmA, NifZ, NifS, and SufS (Fisher scientific) used at a

1:1,000 or 1:20,000 dilution. Membranes were washed 6 times with 0.1% TBST at room temperature and then incubated at 4°C with horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad) secondary antibody for 90 min, followed by an additional set of 6 washes using 0.1% TBST at room temperature. Protein bands were detected by chemiluminescence using the automatic detection setting on an Amersham AI600 imager (Amersham Biosciences). Samples from cells cultures under varying sulfate concentrations were loaded in the same gel, along with a purified protein standard. Signal intensity was quantified using ImageJ software and normalized to the levels detected of each protein in soluble extracts of *B. subtilis* PS832 grown in Spizizen's minimal media supplemented with 17 mM $(NH_4)_2SO_4$.

Digestion of Total RNA and LC-MS Sample Preparation. Unfractionated tRNA isolated from *B. subtilis* cells was digested into individual nucleosides for analysis. An aliquot of 60 µg of total RNA was heat-denatured for 5 min at 95°C, digested for 4 hr at 50°C using 5 µL of 0.25 U/ µL nuclease P1 (Sigma Aldrich) and 17 µL of 0.4 M NH_4OAc, pH 5.3, Optima, LCMS grade, followed by a 2 hr dephosphorylation using recombinant shrimp alkaline phosphatase (NEB) at 37°C. Digested nucleosides were spun at 16,873 x g at room temperature. The supernatant containing digested RNA was transferred to LCMS glass vials and spiked with 0.1% formic acid/ 2% MeOH, Optima LCMS grade, prior to further analysis.

LC-MS Method Run and Tune Parameters*.* All LCMS nucleoside analyses were performed in ESI-positive mode using an Agilent Polaris C18-A HPLC column pre-equilibrated with 98% Solvent A (Water, Optima LCMS grade, 0.1% formic acid) and 2% Solvent B (Methanol, Optima LCMS grade, 0.1% formic acid) for 15 column volumes at 300 µL min^{-1} flow rate. Tune file parameters used during runs were as follows: Voltage (kV): 4.01; Sheath Gas Flow Rate (arb): 47.00; Auxiliary Gas Flow Rate (arb): 30.00; Sweep Gas Flow Rate (arb): 0.00; Capillary Voltage (V): 2.00; Capillary Temperature (C): 350.00; Tube Lens Voltage (V): 49.89. Digested nucleosides were separated and detected over the course of a 39-min run using the gradient of solvent B as follows: 0–4 min, hold at 2% B (v/v); 4–25 min, increase B from 2% to 100%; 25–33 min, hold at 100% B; 33–39 min switch from 100% B to 2% B to allow column re-equilibration at 2% B.

HPLC Detection of MBBR-labeled Cellular L-cysteine. The contents of reduced thiol L-cysteine were determined using monobromobimane (mBBr) described as in Hamilton et al. (34). In short, cell pellets from 0.5-liter culture harvested at $OD_{600\ nm}$ 0.5 were resuspended in 4.5 mL of 25 mM 4-(2-hydroxyl)-1-piperazineethanesulfonic acid (HEPES) pH 8. Aliquots of this resuspended pellet were used to determine the dry weight of the cell pellet, mBBr background, and cysteine bimane quantification. Controls were prepared by adding 5 mM *N*-ethylmaleimide (NEM) to block-free thiol, followed by derivatization with 8 mM mBBr in warm acetonitrile. Reduced thiol samples were prepared by resuspension in 25 mM HEPES, pH 8, followed by direct incubation with 8 mM mBBr in warm acetonitrile. The mixture was incubated at 65°C for 20 min and quenched by the addition of 20 µL of 0.1 M HCl. The reactions were then centrifuged for 5 min and 100 µL of each supernatant were diluted ten times with 2 mM HCl and 10 µL was injected for high-performance liquid chromatography (HPLC) analysis. The HPLC method was conducted using a Waters Spherisorb C18 column (4.6 × 250 mm, 5 µM, 80 Å) using the gradient as described in (34). The intracellular concentrations of L-cysteine derivatives were calculated based on the L-cysteine standard and normalized to cell dry weight.

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Table I - Relative levels of selected tRNA modifications in *B. subtilis* PS832 cells cultured in Luria Bertani (LB) and Spizizen's (MM) media[a]

Modification[b]	**LB**	**MM**
s^4U	0.08 (0.0)	0.13 (0.05)
mnm^5U *	0.02 (0.02)	0.06 (0.01)
mnm^5s^2U	0.29 (0.07)	0.24 (0.15)
i^6A	2.0 (0.48)	1.81 (0.64)
ms^2i^6A *	0.88 (0.23)	0.50 (0.06)
Q *	1.2 (0.38)	0.09 (0.03)
oQ	ND**	0.6 (0.26)
$cmnm^5s^2U$	0.3 (0.04)	0.25 (0.02)
$cmnm^5U$	0.04 (0.02)	0.07 (0.03)
mo^5U *	0.42 (0.09)	0.19 (0.09)
m^7G *	0.02 (0.03)	0.06 (0.01)

[a] tRNA isolated from cells cultured up to OD_{600nm} of 0.5

[b] Relative levels of each modification were determined by normalizing the mass abundance associated with each modification to the mass abundance of dihydrouridine in the same sample. The reported averages and associated standard deviations were calculated based on data acquired from at least three independent experiments

*Statistically significant differences between modification levels from total RNA isolated from cells cultured in LB versus MM ($p<0.05$)

**not detected

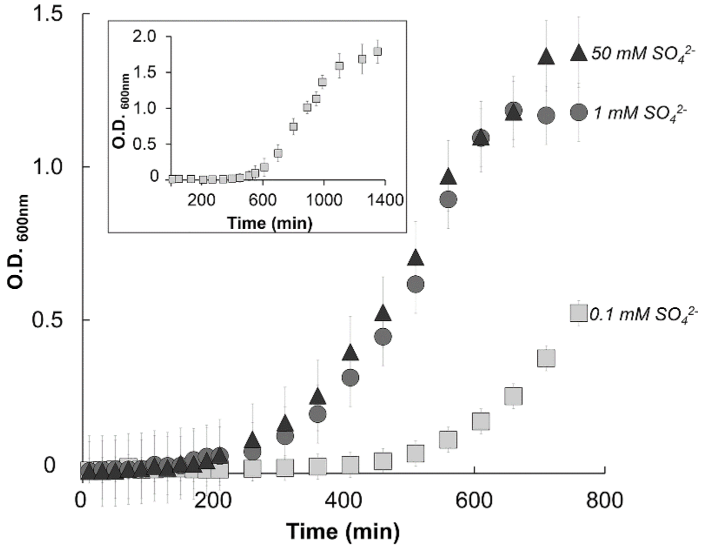

Figure 1. Growth profile of *B. subtilis* PS832 cultured in the presence of various sulfur concentrations of $(NH_4)_2SO_4$. Spizizen's minimal media containing as the sole sulfur source either 0.1 (square), 1 (circle), or 50 mM (triangle)$(NH_4)_2SO_4$. The full growth curve for 0.1 mM $(NH_4)_2SO_4$ is represented in the inset. Growth was monitored through optical density (600 nm) while shaking at 300 RPM, 37ºC. Values on the y-axis have been adjusted for a pathlength of 1 cm. The reported averages and associated standards deviations were calculated based on data acquired from at least three independent experiments.

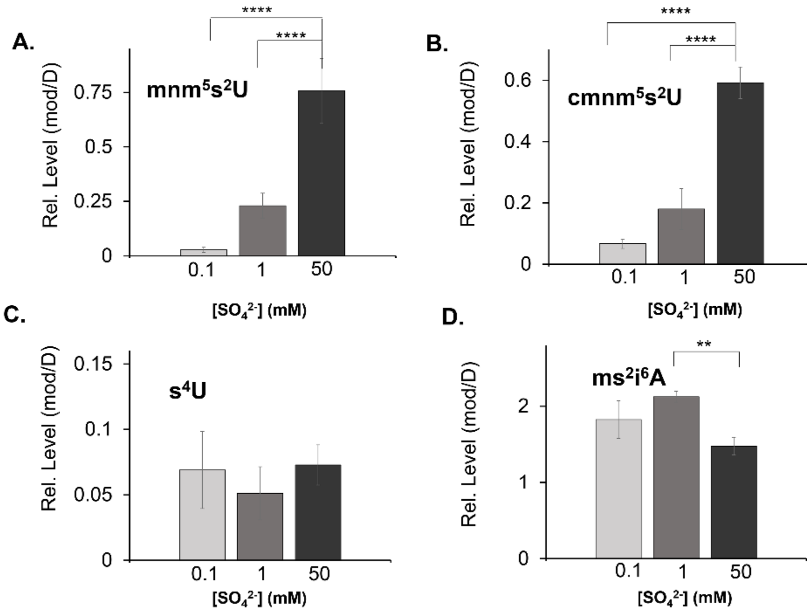

Figure 2. **Relative levels of S-tRNA modifications vary in *B. subtilis* cells cultured under varying sulfur concentrations**. *B. subtilis* PS832 was cultured to OD 0.5 in Spizizen minimal medium containing different concentrations of $(NH_4)_2SO_4$. Relative levels of modified tRNA nucleosides (mod) were analyzed by ultra-high-pressure liquid chromatography coupled mass spectrometry (UHPLC-MS) and normalized to the levels of dihydrouridine (D). tRNA modifications are shown for 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) (**A**), 5-carboxymethylaminomethyl-2-thiouridine (cmnm^{5}s^{2}U) (**B**), 4-thiouridine (s^{4}U) (**C**), and 2-methylthio-N^6-isopentenyl adenosine (ms^{2}i^{6}A) (**D**). A paired t-test compared samples at 0.1,1, and 50 mM $(NH_4)_2SO_4$ (** $p \leq 0.01$; **** $p \leq 0.0001$); Values without an asterisk are considered statistically insignificant.

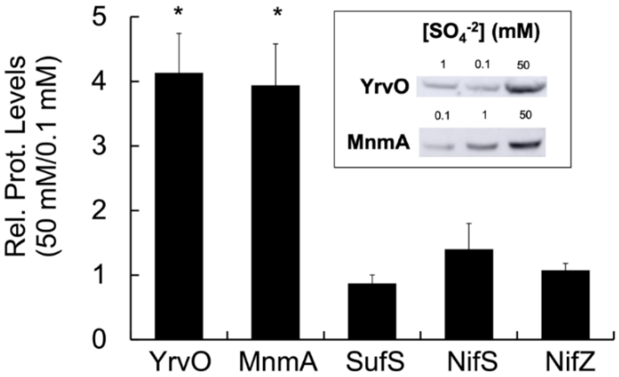

Figure 3. ***B. subtilis*** **cells cultured under low sulfur concentrations show increased accumulation of YrvO and MnmA in soluble extracts**. Western blots were used to determine the levels of *B,subtilis* YrvO, MnmA, SufS, NifS, and NifZ in 50 µg of soluble crude extracts prepared from *B.s.* PS832 cells were cultured to OD 0.5 in Spizizen's minimal medium containing 0.1, 1, 17, and 50 mM $(NH_4)_2$SO4. Changes in relative protein levels were determined by the signal intensity from samples cultured in 50 mM $(NH_4)_2SO_4$ over the signal intensity of samples cultured in 0.1 mM $(NH_4)_2$SO4 from the same gel. A paired t-test compared samples at 0.1 and 50 mM from the same gel (* $p<0.05$); values without an asterisk are considered statistically insignificant. A representative blot is shown in the inset and additional data sets are provided in **Fig. S2**.

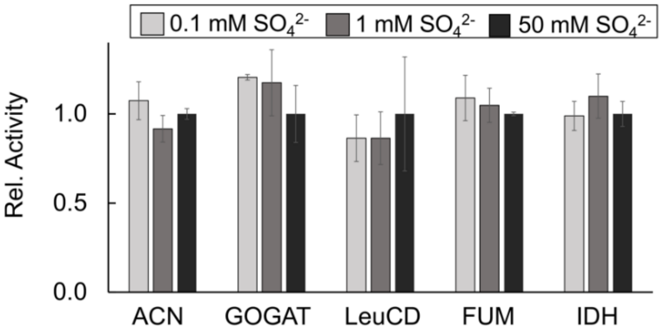

Figure 4. **Varying sulfur concentrations in the growth medium does not impact the activity of Fe-S enzymes.** Soluble extracts from cells cultured in different sulfur concentrations were prepared anoxically and were subsequently tested for the activity of Fe−S enzymes aconit50ase (ACN), glutamine:2-oxoglutarate amidotransferase (GOGAT), isopropylmalate isomerase (LeuCD), and non-Fe-S enzymes fumarase (FUM) and isocitrate dehydrogenase (IDH). The relative activities of ACN, GOGAT, LeuCD, FUM, and IDH were normalized to the activity values of cell extracts cultured in Spizizen's medium containing 17 mM $(NH_4)_2SO_4$ (254, 32, 35, 584, 210 nmol min^{-1} mg^{-1}, respectively). The reported averages and associated standard deviations were calculated based on data acquired from at least three independent experiments. A paired t-test compared samples at 0.1,1, and 50 mM $(NH_4)_2SO_4$. None of the conditions showed statistically significant differences.

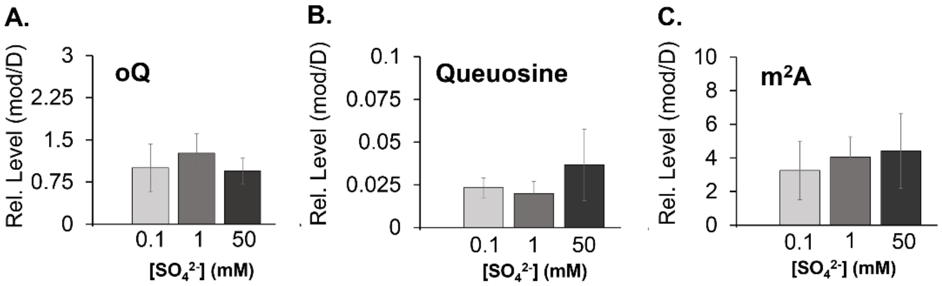

Figure 5. **Relative levels of** epoxyqueuosine (oQ) (**A**), queuosine (Q) (**B**) and 2-methyl adenosine (m^{2}A) (**C**) tRNA modifications from *B. subtilis* cells cultured under different sulfur concentrations. *B. subtilis* PS832 was cultured to OD_{600} 0.5 in Spizizen's minimal medium containing different concentrations of $(NH_4)_2SO_4$. Total RNA was purified from these cultures, and the relative levels of individual tRNA nucleosides were analyzed by mass spectrometry and normalized to the levels of dihydrouridine (D). A paired t-test compared samples at 0.1,1, and 50 mM $(NH_4)_2SO_4$. None of the conditions showed statistically significant differences.

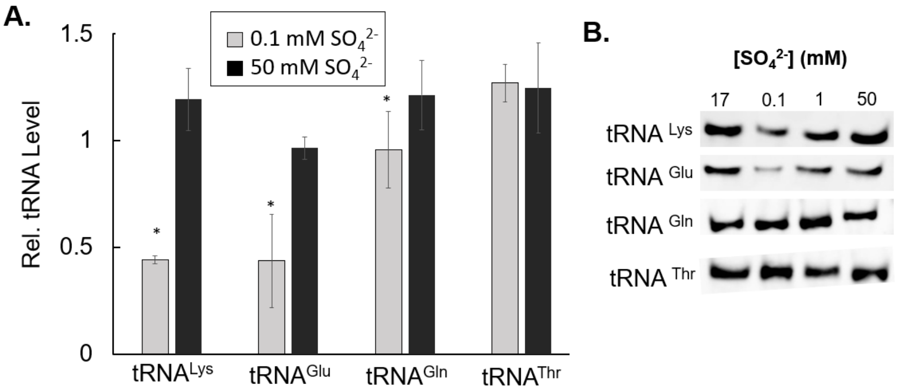

Figure 6. ***B. subtilis*** **cells cultured under low sulfur concentrations show reduced levels of** $\mathbf{tRNA^{Lys}}$ **and** $\mathbf{tRNA^{Glu}}$. tRNA levels from *B. subtilis* cells cultured under different sulfur concentrations. (**A**) *B. subtilis* PS832 was cultured to OD 0.5 in Spizizen's minimal medium containing different concentrations of $(NH_4)_2SO_4$. tRNA was purified from these cultures and the relative levels of tRNA known to contain s^2U modifications ($tRNA^{Lys,\ Glu,\ and\ Gln}$) was compared to the levels of a tRNA that is not modified at this position ($tRNA^{Thr}$). For each sample, 5 µg of total RNA was loaded in each gel lane, and representative samples of cells grown under varying sulfur sources were quantified in the same gel. The relative accumulation of each cognate tRNA was determined by normalizing the signal intensity of each sample by the signal intensity of a tRNA sample extracted from cells cultured in Spizizen's medium containing 17 mM $(NH_4)_2SO_4$. Averages of ratios and associated standard deviations were calculated from blots generated from at least three experimental replicates. A paired t-test compared samples at 0.1 and 50 mM from the same gel (* $p<0.05$). Values without an asterisk are considered statistically insignificant. **B)** A representative blot is shown for each tRNA cognate and additional data sets are provided in **Fig. S3**.

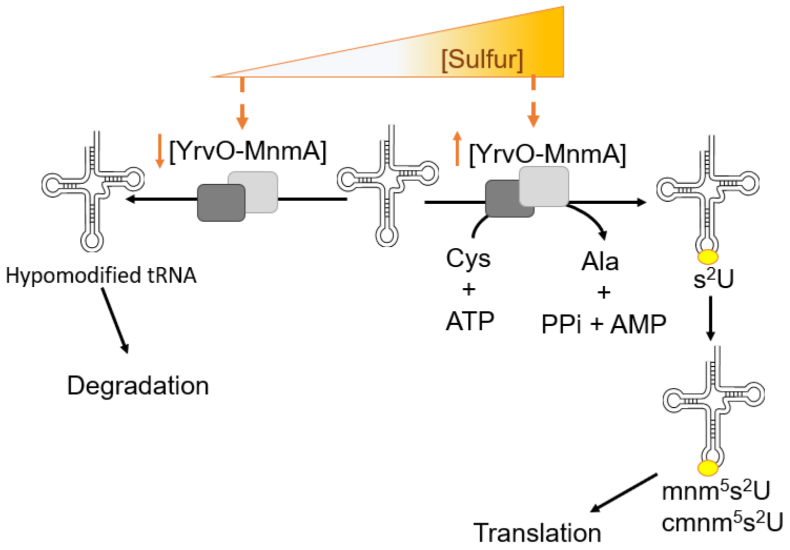

Figure 7. Working model for regulation of s^{2}U tRNA synthesis in *B. subtilis*. YrvO and MnmA catalyze the synthesis of s^{2}U at position 34 of tRNA^{Lys}, ^{Glu}, ^{Gln}. When cells are cultured under sulfur replete conditions, YrvO and MnmA promote the formation of s^{2}U tRNA on U34, which is subsequently modified to cmnm^{5}s^{2}U and mnm^{5}s^{2}U as fully functional tRNAs that are indispensable to translation. Under sulfur deplete conditions, the levels of YrvO and MnmA decrease significantly in a dose-dependent manner, leaving their thiolation substrate tRNAs hypomodified., These translationally inefficient surplus tRNAs are then proposed to be targeted for degradation, thereby impacting the overall translational apparatus.