## Supplemental Information

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

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**Supplemental Methods**

***qPCR analysis of B. subtilis transcripts.*** Reverse transcription (RT) assays were carried out per manufacturers recommendations (Promega M-MLV Reverse Transcriptase) using 1 µg template total RNA, 0.4 µM of forward and reverse gene-specific primers, and the reaction was brought up to 10 µL with ultrapurified, autoclaved water. Using a SimpliAmp thermal cycler, the reaction was denatured for 5 min at 80°C, followed by a return to 4°C. To each RT reaction, 25 µL of master mix was added containing a final concentration of 100 U Promega M-MLV Reverse Transcriptase, 1X M-MLV RT Reaction Buffer, and 357 mM DNTPs and incubated for 50 min at 50°C, followed by 5 min at 85°C. After RT, 1 µL of each RT reaction mixture was taken and subjected to Pfu-Ultra PCR using the previously mentioned gene-specific primers, 5 µL of the PFU mastermix. Each PCR reaction was analyzed using 3% agarose gel electrophoresis for the formation of cDNA products and corresponding absence in no reverse transcriptase controls. Using SYBR Select qPCR Master Mix, 1.5 µL of cDNA was used as the template for subsequent qPCR reactions using QuantStudio3 Real-Time PCR system. All experimentally determined *C*t values were normalized both to the abundance of each corresponding transcript level under standard WT [$NH_4SO_4$] (17 mM), and then normalized against the values of bacterial reference gene *B.subtilis icd* (isocitrate dehydrogenase). Values are reported as fold change in expression relative to ICD.

**Table S1.** Relative levels of tRNA modifications* in *B. subtilis* PS832 cells cultured in Spizizen's minimal medium at various growth phases.

| Modification | OD 0.5 | OD 1.0 | OD 1.5 |
|---|---|---|---|
| **U** | 23.8 (9.2) | 23.1 (10.9) | 24.99 (13.63) |
| **ψ** | 1.03 (0.51) | 2.25 (1.65) | 2.67 (2.16) |
| **$s^4U$** | 0.13 (0.05) | 0.08 (0.05) | 0.08 (0.06) |
| **$mnm^5U$** | 0.06 (0.01) | 0.08 (0.42) | 0.09 (0.05) |
| **$mnm^5s^2U$** | 0.24 (0.15) | 0.49 (0.42) | 0.52 (0.41) |
| **$i^6A$** | 1.81 (0.64) | 0.89 (0.49) | 0.81 (0.57) |
| **$ms^2i^6A$** | 0.50 (0.06) | 0.34 (0.03) | 0.52 (0.12) |
| **$t^6A$** | 0.14 (0.06) | 0.41 (0.14) | 0.5 (0.25) |
| **$m^s2t^6A$** | 0.003 (0.001) | 0.01 (0.001) | 0.006 (0.003) |
| **Q** | 0.09 (0.03) | 0.12 (0.07) | 0.06 (0.03) |
| **oQ** | 0.6 (0.26) | 1.2 (0.81) | 1.31 (1.41) |
| **$cmnm^5s^2U$** | 0.25 (0.02) | 0.39 (0.3) | 0.65 (0.35) |
| **$cmnm^5U$** | 0.07 (0.03) | 0.07 (0.06) | 0.10 (0.08) |
| **$ct^6A$** | 0.35 (0.12) | 0.93 (0.36) | 1.26 (0.44) |
| **$k^2C$** | 0.03 (0.02) | 0.12 (0.14) | 0.23 (0.21) |
| **$mo^5U$** | 0.19 (0.09) | 0.55 (0.44) | 0.72 (0.54) |
| **$m^5U$** | 1.18 (0.58) | 2.13 (1.43) | 2.6 (1.67) |
| **$m^7G$** | 0.06 (0.01) | 0.05 (0.01) | 0.05 (0.01) |
| **Inosine** | 0.42 (0.23) | 2.82 (1.88) | 2.95 (2.02) |
| **$ac^4C$** | 0.55 (0.17) | 3.33 (2.88) | 0.64 (0.38) |
| **$m^6A$** | 6.39 (1.2) | 3.69 (2.22) | 4.93 (2.52) |
| **$m^1A$** | 0.22 (0.13) | 0.6 (0.41) | 0.66 (0.49) |
| **$m^2A$** | 1.09 (0.4) | 3.29 (2.04) | 4.22 (2.88) |
| **$m^1G$** | 5.58 (1.2) | 7.02 (3.87) | 9.38 (0.08) |
| **Cm** | 0.3 (0.13) | 0.14 (0.1) | 0.12 (0.08) |
| **Gm** | 2.2 (0.4) | 2.87 (1.51) | 3.45 (2.04) |

*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.

** $m^s2t^6A$ and $k^2C$ were low abundance modifications found close to the detection limit.

**Table S2.** Relative levels of tRNA modifications* in *B. subtilis* PS832 cells cultured in Luria Bertani (LB) rich medium at various growth phases.

| Modification | OD 0.5 | OD 1.0 | OD 1.5 |
|---|---|---|---|
| **U** | 24.7 (3) | 10.1 (6.4) | 11.8 (4.2) |
| **ψ** | 2.2 (0.6) | 1.7 (1.21) | 0.92 (0.52) |
| **$s^4U$** | 0.08 (0.0) | 0.06 (0.029) | 0.03 (0.01) |
| **$mnm^5U$** | 0.02 (0.02) | 0.07 (0.035) | 0.02 (0.01) |
| **$mnm^5s^2U$** | 0.29 (0.07) | 0.47 (0.171) | 0.09 (0.02) |
| **$i^6A$** | 2.0 (0.48) | 0.97 (0.46) | 0.17 (0.07) |
| **$ms^2i^6A$** | 0.88 (0.23) | 1.0 (0.38) | 0.35 (0.23) |
| **$t^6A$** | 0.2 (0.1) | 0.33 (0.17) | 0.24 (0.1) |
| **$m^s2t^6A$** | 0.003 (0.001) | 0.007 (0.003) | 0.003 (0.001) |
| **Q** | 1.2 (0.38) | 1.7 (0.98) | 0.69 (0.2) |
| **oQ** | ND | ND | ND |
| **$cmnm^5s^2U$** | 0.3 (0.04) | 0.48 (0.13) | 0.19 (0.07) |
| **$cmnm^5U$** | 0.04 (0.02) | 0.1 (0.82) | 0.023 (0.015) |
| **$ct^6A$** | 0.57 (0.26) | 0.05 (0.015) | 0.83 (0.37) |
| **$k^2C$** | 0.06 (0.01) | 0.1 (0.03) | 0.05 (0.01) |
| **$mo^5U$** | 0.42 (0.09) | 0.61 (0.07) | 0.37 (0.13) |
| **$m^5U$** | 1.2 (0.09) | 2.6 (0.6) | 0.95 (0.01) |
| **$m^7G$** | 0.02 (0.03) | 0.01 (0.009) | 0.03 (0.02) |
| **Inosine** | 0.18 (0.04) | 0.15 (0.112) | 0.08 (0.05) |
| **$ac^4C$** | 0.45 (0.07) | 1.1 (1.4) | 0.28 (0.08) |
| **$m^6A$** | 0.39 (0.14) | 3.9 (1.9) | 1.5 (0.55) |
| **$m^1A$** | 0.29 (0.05) | 0.34 (0.09) | 0.37 (0.08) |
| **$m^2A$** | 1.8 (0.29) | 2.9 (0.45) | 2.2 (0.75) |
| **$m^1G$** | 5.2 (0.62) | 7.6 (2.5) | 4.8 (2.4) |
| **Cm** | 0.21 (0.06) | 0.09 (.046) | 0.09 (0.04) |
| **Gm** | 1.8 (0.41) | 3.7 (1.2) | 2.3 (0.98) |

*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.

** $m^s2t^6A$ and $k^2C$ were low abundance modifications found close to the detection limit.

**Table S3:** Relative levels of tRNA modifications in *B. subtilis* PS832 grown under varying sulfur sources.

| | **Sulfur source in the growth medium** | |
|---|---|---|
| **Modification** | **3 mM Methionine (SD)** | **2.5 mM $SSO_4^{2-}$ (SD)** |
| **$mnm^5s^2U$** | 0.41 (.007) | 0.45 (.011) |
| **$cmnm^5s^2U$** | 0.23 (.04) | 0.19 (0.03) |
| **$mnm^5U$** | 0.05 (.01) | 0.040 (0.014) |
| **$cmnm^5U$** | 0.15 (.01) | 0.14 (0.01) |
| **$s^4U$** | 0.08 (.007) | 0.07 (-) |
| **oQ** | 0.685 (0.05) | 0.585 (0.08) |
| **Q** | 0.13 (0.01) | 0.135(0.02) |
| **$m^2A$** | 1.75 (0.27) | 1.808(0.05) |
| **$ms^2i^6A$** | 0.445 (0.22) | 0.345 (0.13) |

*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.

**Table S4**: Intracellular levels of cysteine in *B. subtilis* cells cultured under different sulfur concentrations.

| **Sulfate (mM) in the medium** | **Intracellular cysteine (mM)** |
|---|---|
| 0.1 | 0.35 (±0.07) |
| 1 | 0.39 (±0.08) |
| 50 | 0.36 (±0.03) |

**Table S5**: Sequences of Oligonucleotides Used in this Study*.

| **Primers** | **Sequence** |
|---|---|
| *Northern blot* 5'-Cy5 *labeled primers* | |
| *Bs* $tRNA^{Lys}$ | 5'-GCGACCATGGAACGGATTTATTTAG-3' |
| *Bs* $tRNA^{Glu}$ | 5'-GGATCCGGTCACTCTCTTCGCCG-3' |
| *Bs* $tRNA^{Gln}$ | 5'-GACCATATGGAAAAACGGCCGGAGG-3' |
| *Bs* $tRNA^{Thr}$ | 5'-GCTGGATCCTTTTATACGTACCACAATTTTGTTCCG-3' |
| *qPCR primers* | |
| *Bs* MnmA 5' | 5'-GGATATCATGAAGGGCGAAGAG-3' |
| *Bs* MnmA 3' | 5'-GTCATGCGCACTGTTACTTTATG -3' |
| *Bs* YrvO 5' | 5'-ATGGGTGGATGAAGCAAGAG -3' |
| *Bs* YrvO 3' | 5'-TCCCATGATCGCCAAGTTATC-3' |
| *Bs* CymR 5' | 5'-CCTGTTGAAGTGCTGGAAGA -3' |
| *Bs* CymR 3' | 5'-AGCTGGCAAGATCCTCTAATG-3' |
| *Bs* IDH 5' | 5'-GCTCCCTGCTGAAACATTAGA-3' |
| *Bs* IDH 3' | 5'-CTGAGCGCTACGTTCAAAGA -3' |
| *Bs* SufS 5' | 5'-GCTGTCATTGAAACACTGGATAAG-3' |
| *Bs* SufS 3' | 5'-TCACGCGCTCCTTCATAAC-3' |
| *Bs* NifZ 5' | 5'-CACATGCTTGAGGAACAGGATA-3' |
| *Bs* NifZ 3' | 5'-CTCTCCTTTGCCCATCTCTAAG -3' |
| *Bs* NifS 5' | 5'-CGAACAAGAAGCTCTGCAATTTA-3' |
| *Bs* NifS 3' | 5'-CCCAAAGCACTGTAAACGTATG-3' |

*Oligonucleotides were obtained from Genewiz

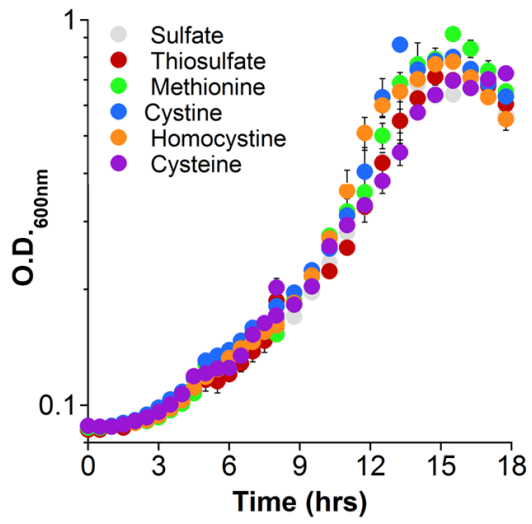


**Figure S1** – Growth profile of *B. subtilis* PS832 cultured in the presence of various sulfur sources. Spizizen's minimal media contains as the sole sulfur source 1 mM $(NH_4)_2SO_4$, thiosulfate, methionine, cystine, cysteine, or homocysteine. Growth was monitored through optical density (600 nm) while shaking at 300 RPM, 37ºC. When not visible, error bars are smaller than the symbols.

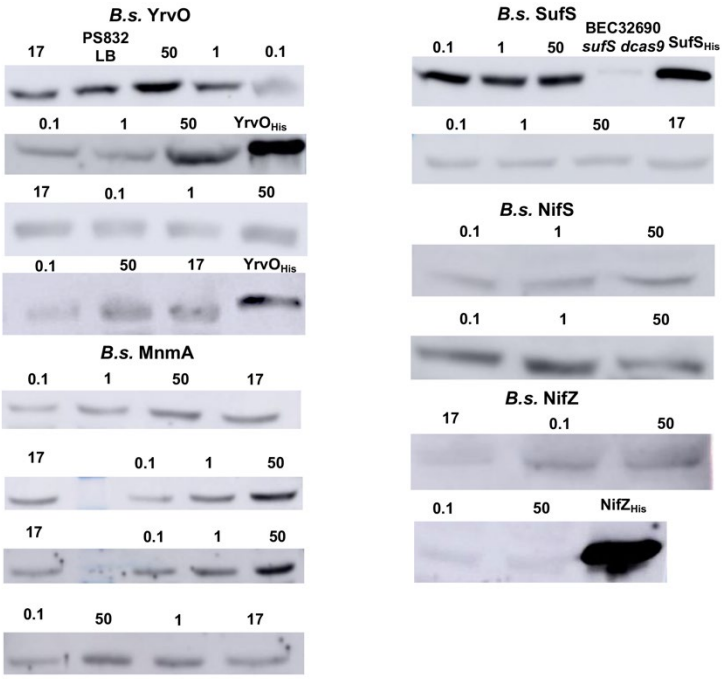


**Figure S2**- Western blots monitoring the expression levels of *B. subtilis* YrvO, MnmA, SufS, NifS, and NifZ in 50 µg of soluble crude extracts prepared from *B.s.* PS832 cells cultured to OD 0.5 in Spizizen's minimal medium containing 0.1, 1, 17, and 50 mM $(NH_4)_2SO4$.

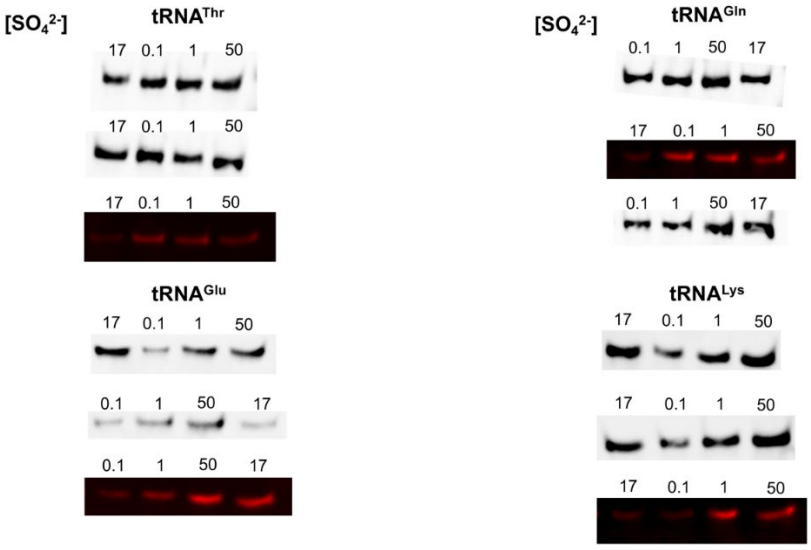


**Figure S3**- Northern blots monitoring the levels of *B. subtilis* $tRNA^{Glu,Gln,Lys,Thr}$ in 5 µg of extracted total RNA isolated from *B.s.* PS832 cells cultured to OD 0.5 in Spizizen's minimal medium containing 0.1, 1, 17, and 50 mM $(NH_4)_2SO4$.

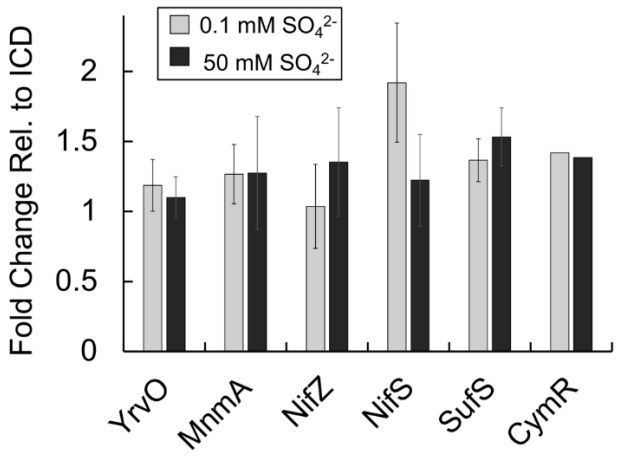


**Figure S4: Transcript abundance in *B. subtilis* total RNA under varying $NH_4SO_4$ concentrations.** Measurement of various transcript levels by RT-qPCR in total RNA isolated from *B. subtilis* cells grown in Spizizen's minimal media supplemented with low (0.1 mM) and high (50 mM) $NH_4SO_4$. The experimentally determined *C*t values were normalized both to the abundance of each corresponding transcript level under standard WT [$NH_4SO_4$] (17 mM), and then normalized against the values of bacterial reference gene *B. subtilis icd* (isocitrate dehydrogenase). Values are reported as fold change in expression relative to ICD. The reported averages and standard deviations were calculated based on data obtained from at least three independent experiments.

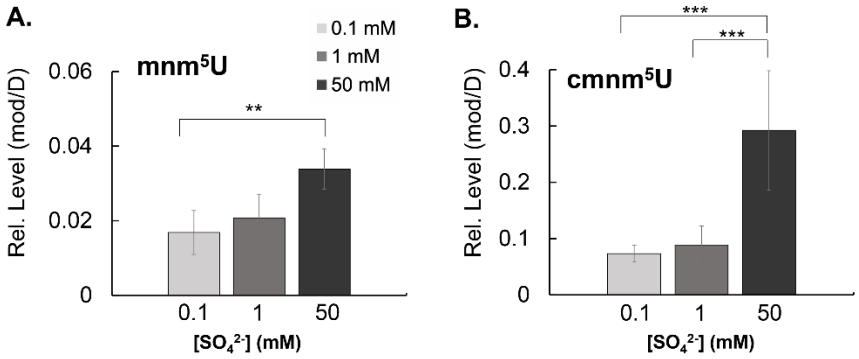


**Figure S5.** Relative levels of partially modified mnm$^5$U (A), cmnm$^5$U (B). tRNA modifications from *B. subtilis* cells cultured under different sulfur concentrations. *B. subtilis* PS832 was cultured to OD 0.5 in Spizizen minimal medium containing different concentrations of $(NH_4)_2SO4$. tRNA 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. A paired t-test compared samples at 0.1,1, and 50 mM $(NH_4)_2SO_4$ (** $p \leq 0.01$; *** $p \leq 0.001$); Values without an asterisk are considered statistically insignificant.