Supplemental Information

Table S1. Primers, plasmids and strains used in experiments

Primers	Sequence
BsyrvO-NcoI5	5’-GCGACCATGGAACGGATTTATTTAG-3’
BsyrvO’-BamHI3	5’-GGATCCGGTCACTCTCTTCGCCG-3’
BsmnmA-NdeI5	5’-GACCATATGGAAAAACGGCCGGAGG-3’
BsmnmA-BamHI3	5’-GCTGGATCCTTTTATACGTACCACAATTTTGTTCCG-3’

Plasmids	Relevant Gene Cloned	Vector	Reference
pDS 16	*yrvO* PCR product using *BsyrvO*-NcoI5 and *BsyrvO*-BamHI3 primers. It places NcoI and BamHI sites at 5’ and 3’ sites flanking *yrvO* coding sequence	pCR2.1 TOPO	This work
pDS 22	1.1 kb NcoI-BamHI *yrvO* fragment ligated into NcoI-BglII sites of pAra13.	pAra13	This work
pDS 31	827bp BamHI-BglII fragment of pDS16 containing *'yrvO* was cloned into BamHI of pMutin4.	pMutin4	This work
pDS 123	*mnmA* PCR product using *BsmnmA*-NdeI5 and *BsmnmA*-BamHI3 primers. It places NdeI and BamHI sites at 5’ and 3’ sites flanking *mnmA* coding sequence	pCR2.1 TOPO	This work
pDS143	1.2 kb NdeI-BamHI fragment of *mnmA* ligated into NdeI-BamHI sites of pET16b	pet16b	This work
pDS 144	*yrvO-mnmA* PCR product using *BsyrvO*-NcoI5 and *BsmnmA*-BamHI3 primers. It places NcoI and BamHI sites at 5’ and 3’ sites flanking *yrvO* and *mnmA* coding sequences respectively	pCR2.1 TOPO	This work
pDS 145	2.3 kb NcoI-BamHI fragment of *yrvO*-mnmA ligated into NcoI-BglII sites of pBad	pBad	This work
pDS151	1.2 kb NcoI-BamHI fragment of mnmA ligated into NcoI-BglII sites of pBad	pBad	This work
pDS 173	pDS 151 containing codon substitution at position 104 of *mnmA* (C104A, TGC to GCC)	pBad	This work
pDS 174	pDS 143 containing codon substitution at position 104 of *mnmA* (C104A, TGC to GCC)	pet16b	This work
pDS 175	pDS 145 containing codon substitution at position 104 of *mnmA* (C104A, TGC to GCC)	pBad	This work
pDS 178	pDS 143 containing codon substitution at position 200 of *mnmA* (C200A, TGC to GCC)	pet16b	This work
pDS 179	pDS 145 containing codon substitution at position 200 of *mnmA* (C200A, TGC to GCC)	pBad	This work

pDS 180	pDS 151 containing codon substitution at position 200 of *mnmA* (C200A, TGC to GCC)	pBad	This work
pDS 194	pDS 143 containing codon substitution at position 51 of *mnmA* (C51A, TGC to GCC)	pet16b	This work
pDS 195	pDS 151 containing codon substitution at position 51 of *mnmA* (C51A, TGC to GCC)	pBad	This work
pDS 196	pDS 143 containing codon substitution at position 66 of *mnmA* (C66A, TGC to GCC)	pet16b	This work
pDS 197	pDS 151 containing codon substitution at position 66 of *mnmA* (C66A, TGC to GCC)	pBad	This work
pDS 198	pDS 143 containing codon substitution at position 304 of *mnmA* (C304A, TGC to GCC)	pet16b	This work
pDS 199	pDS 151 containing codon substitution at position 304 of *mnmA* (C304A, TGC to GCC)	pBad	This work
pDS 201	pDS 143 containing codon substitution at position 325 of *mnmA* (C325A, TGC to GCC)	pet16b	This work
pDS 202	pDS 145 containing codon substitution at position 325 of *mnmA* (C325A, TGC to GCC)	pBad	This work
pDS 203	pDS 151 containing codon substitution at position 325 of *mnmA* (C325A, TGC to GCC)	pBad	This work
pDS 217	pDS 145 containing codon substitution at position 304 of *mnmA* (C304A, TGC to GCC)	pBad	This work
pDS 219	pDS 22 containing codon substitution at position 325 of *yrvO* (C325A, TGC to GCC)	pAra13	This work
pDS 220	pDS 145 containing codon substitution at position 325 of *yrvO* (C325A, TGC to GCC)	pBad	This work
pDS 221	pDS 145 containing codon substitution at position 51 of *mnmA* (C51A, TGC to GCC)	pBad	This work
pDS 222	pDS 145 containing codon substitution at position 66 of *mnmA* (C66A, TGC to GCC)	pBad	This work

Strain	**Relevant Genotype**	**Reference**
B. subtilis PS832	wild type strain	Corfe et al 1994
B. subtilis DD19	'*yrvO*::pMutin4	This work
B. subtilis J1235	*yqhL::spc ytwF::erm yrkF::erm::cat ybfQΔ::kan*	T.J. Larson laboratory stock
E. coli MG1655	wild type strain	Laboratory stock
E. coli BW25113	wild type strain	Laboratory stock
E. coli CL100	Δ*iscS*	Lauhon and Kambampati, 2000
E. coli JW1119	Δ*mnmA*	Laboratory stock

Table SII Gram-positive species containing MnmA and cysteine desulfurase genes

Gram-positive species containing *mnmA* and cysteine desulfurase within same genomic neighborhood

Species Name	MnmA Locus Tag	Cysteine Desulfurase Locus Tag
Acidothermus cellulolyticus 11B	Acel_0689	Acel_0688
Actinomyces odontolyticus ATCC 17982	ACTODO_01767	ACTODO_01768
Anaerostipes caccae DSM 14662	ANACAC_00390	ANACAC_00392
Anoxybacillus flavithermus WK1	Aflv_0738	Aflv_0737
Arthrobacter aurescens TC1	AAur_2710	AAur_2712
Bacillus amyloliquefaciens plantarum FZB42	RBAM_024610	RBAM_024620
Bacillus anthracis Sterne	BAS4291	BAS4292
Bacillus cereus E33L (ZK)	BCZK4139	BCZK4140
Bacillus halodurans C-125	BH1261	BH1260
Bacillus licheniformis DSM 13 Goettingen	BLi02875	BLi02876
Bacillus subtilis subtilis 168	BSU27500	BSU27510
Bacillus weihenstephanensis KBAB4	BcerKBAB4_4243	BcerKBAB4_4244
Brevibacterium linens BL2	BlinB01001207	BlinB01001208
Caldicellulosiruptor saccharolyticus DSM 8903	Csac_2252	Csac_2254
Carboxydothermus hydrogenoformans Z-2901, DSM 6008	CHY_2197	CHY_2199
Clavibacter michiganensis michiganensis NCPPB 382	CMM_1403	CMM_1402
Clostridium beijerinckii NCIMB 8052	Cbei_1100	Cbei_1098
Clostridium botulinum BoNT/A1 Hall	CLC_1228	CLC_1225
Clostridium cellulolyticum H10	Ccel_1930	Ccel_1932
Clostridium difficile 630 (epidemic type X)	CD1281	CD1279
Clostridium perfringens ATCC 13124	CPF_2037	CPF_2039
Collinsella aerofaciens ATCC 25986	COLAER_01430	COLAER_01431
Corynebacterium diphtheriae bv. Gravis	DIP1074	DIP1072
Corynebacterium glutamicum R	cgR_1317	cgR_1309
Deinococcus geothermalis DSM 11300	Dgeo_0792	Dgeo_0781
Desulfotomaculum reducens MI-1	Dred_0766	Dred_0764
Dorea longicatena DSM 13814	DORLON_02440	DORLON_02442
Enterococcus faecalis V583	EF2070	EF2072
Eubacterium siraeum DSM 15702	EUBSIR_02454	EUBSIR_02456
Exiguobacterium sibiricum 255-15, DSM 17290	Exig_2078	Exig_2079
Finegoldia magna ATCC 29328	FMG_1586	FMG_1587
Frankia alni ACN14a	FRAAL5861	FRAAL5862
Geobacillus kaustophilus HTA426	GK2563	GK2564
Heliobacterium modesticaldum Ice1	HM1_1866	HM1_1864
Janibacter sp. HTCC2649	JNB_19083	JNB_19078
Kineococcus radiotolerans SRS30216	Krad_1308	Krad_1307
Lactobacillus brevis ATCC 367	LVIS_1434	LVIS_1434
Lactobacillus casei ATCC 334	LSEI_1292	LSEI_1289
Lactobacillus johnsonii NCC 533	LJ0986	LJ0984
Leifsonia xyli xyli CTCB07	Lxx14390	Lxx14420

Listeria monocytogenes sv. 1/2a EGD-e	lmo1512	lmo1513
Listeria welshimeri sv. 6b, SLCC5334	lwe1525	lwe1526
Mycobacterium abscessus CIP 104536	MAB_3356c	MAB_3357c
Mycobacterium bovis AF2122/97	Mb3050c	Mb3051c
Mycobacterium leprae TN	ML1707	ML1708
Mycobacterium smegmatis MC2 155	MSMEG_2358	MSMEG_2357
Mycobacterium tuberculosis H37Ra	MRA_3055	MRA_3056
Nocardia farcinica IFM 10152	nfa42670	nfa42680
Nocardioides sp. JS614	Noca_3445	Noca_3446
Pediococcus pentosaceus ATCC 25745	PEPE_1172	PEPE_1174
Pelotomaculum thermopropionicum SI	PTH_1056	PTH_1054
Propionibacterium acnes KPA171202	PPA1117	PPA1116
Renibacterium salmoninarum ATCC 33209	RSal33209_1673	RSal33209_1674
Rhodococcus jostii RHA1	RHA1_ro06467	RHA1_ro06466
Rubrobacter xylanophilus DSM 9941	Rxyl_1354	Rxyl_1353
Ruminococcus gnavus ATCC 29149	RUMGNA_01754	RUMGNA_01752
Salinispora arenicola CNS-205	Sare_1107	Sare_1106
Staphylococcus aureus RF122	SAB1492c	SAB1493c
Staphylococcus epidermidis ATCC 12228	SE1304	SE1305
Streptococcus pneumoniae sv. 14 CGSP14	SPCG_0121	SPCG_1185
Streptomyces avermitilis MA-4680	SAV2753	SAV2756
Thermoanaerobacter pseudethanolicus 39E, ATCC 33223	Teth39_0281	Teth39_0280
Thermobifida fusca YX	Tfu_0597	Tfu_0595
Tropheryma whipplei TW08/27	TW353	TW352
marine actinobacterium PHSC20C1	A20C1_05071	A20C1_05076

Gram-positive species containing *mnmA* and cysteine desulfurase genes at two separate locations in the genome

Species Name	**MnmA Locus Tag**	**Cysteine Desulfurase Locus Tag**
Acholeplasma laidlawii PG-8A	ACL_0559	ACL_1215
Alkaliphilus oremlandii OhILAs	Clos_0891	Clos_1670
Anaerofustis stercorihominis DSM 17244	ANASTE_01135	ANASTE_02089
Candidatus Desulforudis audaxviator MP104C	Daud_2035	Daud_0907
Candidatus Phytoplasma onion yellows OY-M	PAM125	PAM468
Deinococcus radiodurans ATCC BAA-816	DR1759	DR0215
Eubacterium dolichum DSM 3991	EUBDOL_01338	EUBDOL_01200
Lactobacillus acidophilus NCFM	LBA0822	LBA1177
Lactococcus lactis cremoris MG1363	llmg_1725	llmg_2048
Mesoplasma florum L1	Mfl412	Mfl248
Paenibacillus larvae larvae BRL-230010	Plarl_010100015834	Plarl_010100022313
Streptococcus agalactiae sv. V/V 2603V/R	SAG2144	SAG1098
Streptococcus pyogenes sv. M1 GAS SF370	SPy2188	SPy1122
Streptococcus thermophilus LMD-9	STER_1979	STER_1426
Syntrophomonas wolfei Goettingen, DSM 2245B	Swol_0464	Swol_1912

Table SIII. pmol of s^2U formed in *in vitro* s^2U reactions

Reaction Components	**pmol of s^2U***	
	- DTT	+ DTT
Cysteine	0.2 (±0.2)	0.1 (±0.03)
Cysteine + YrvO + MnmA	26.0 (±0.76)	43.1 (±1.75)
Sulfide + MnmA	22.5 (±1.31)	19.9 (±2.3)

[*] Standard deviations were obtained from at least three independent experiments

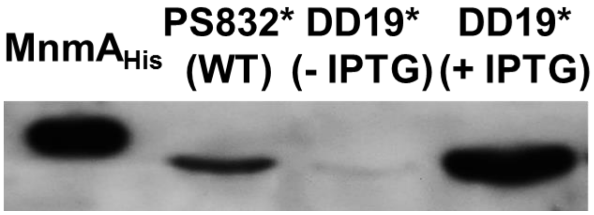


Figure S1: MnmA expression levels in protein crude extracts (40 ug aliquots) visualized by western analysis using an antibody against *B. subtilis* MnmA. **A.** MnmA expression in *B. subtilis* PS832 wild type strain and in the *mnmA* conditional knockout DD19 strain, in absence of the IPTG inducer (-IPTG), or in the presence of 1 mM IPTG (+IPTG). Overexposure of the same western blot displayed in **Figure 2A** allowed detection of trace amounts of MnmA expression in the *mnmA* conditional knockout DD19 strain, in absence of the IPTG inducer (-IPTG).

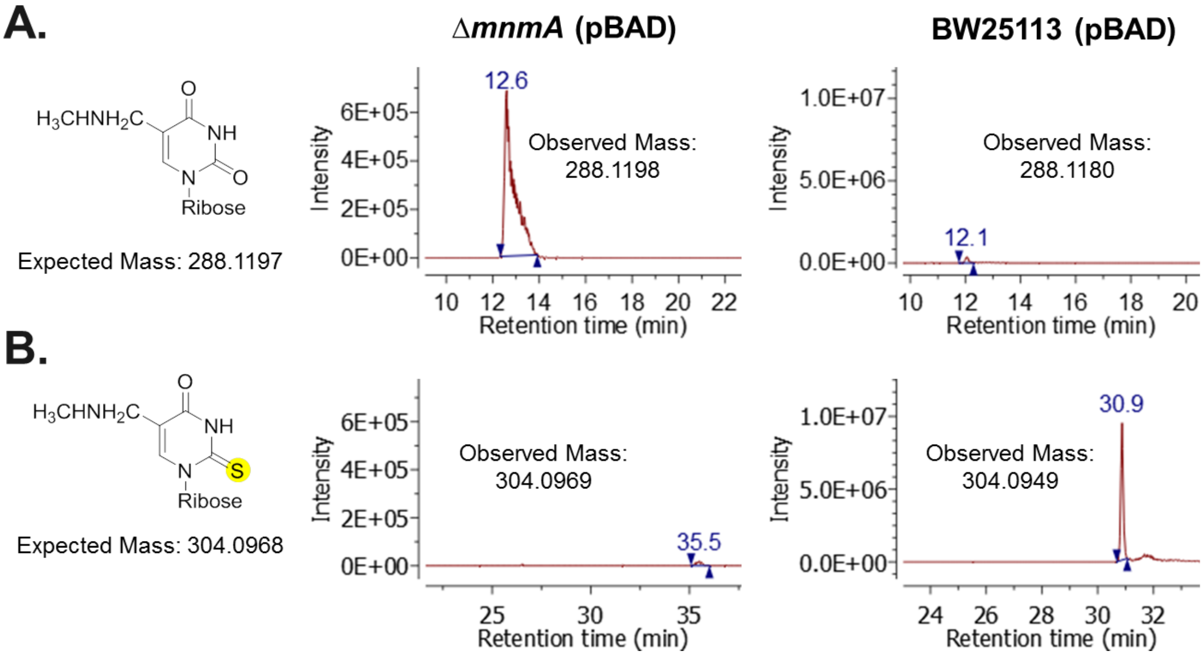
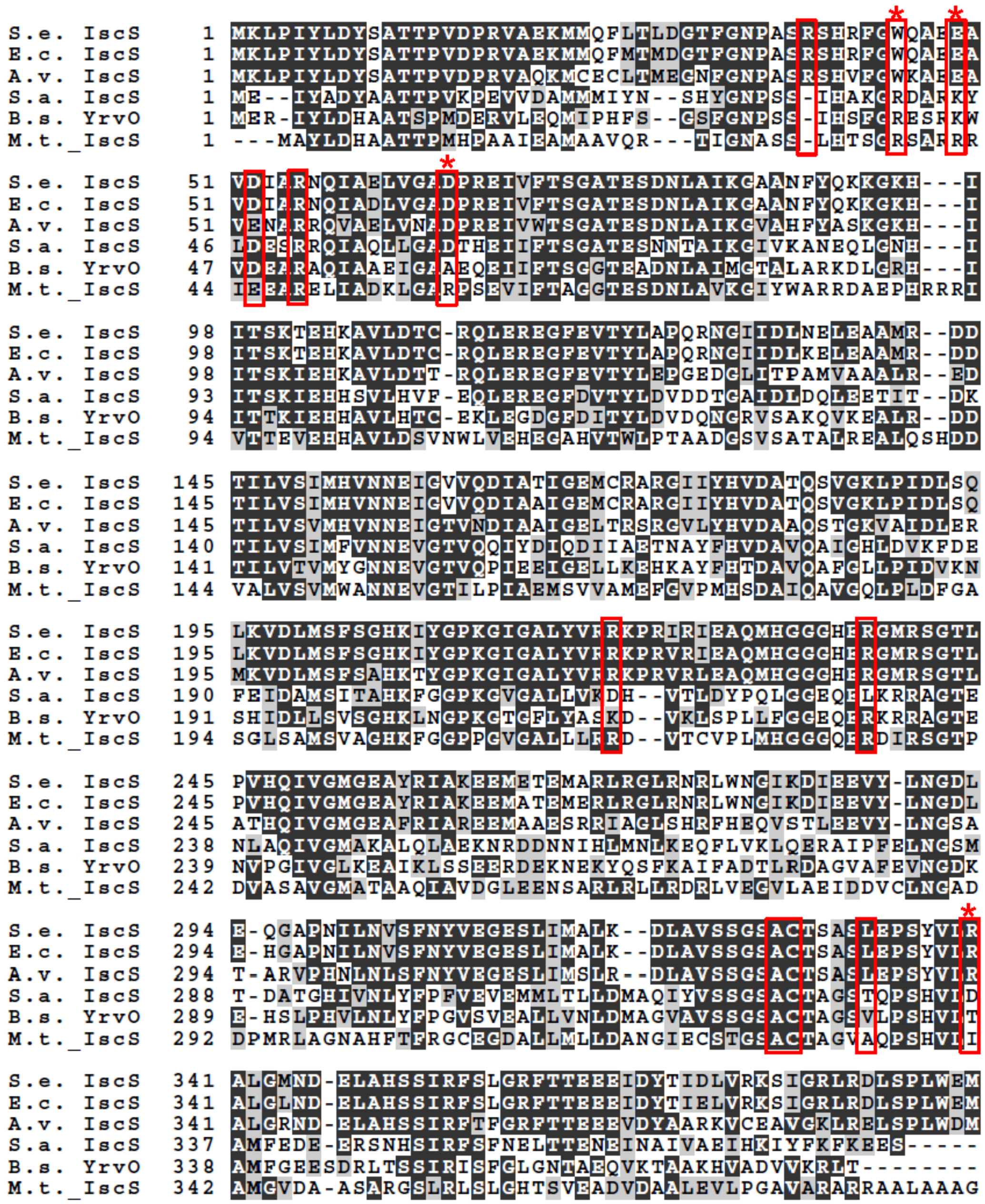


Figure S2: LC/MS analysis for quantification of thionucleoside levels in *E. coli* BW25113 wild type and Δ*mnmA* strains expressing the empty pBAD expression vector. The Extracted Ion Chromatograms of the masses associated with **A.** mnm^{5}U (m/z 288.1197) and **B.** mnm^{5}s^{2}U (m/z 304.0968) are depicted.



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S.e. IscS      1 MKLPIYLDYSATTPVDPRVAEKMMQFLTLDGTFGNPASRSHRFGWQAEEA
E.c. IscS      1 MKLPIYLDYSATTPVDPRVAEKMMQFMTMDGTFGNPASRSHRFGWQAEEA
A.v. IscS      1 MKLPIYLDYSATTPVDPRVAQKMCECLTMEGNFGNPASRSHVFGWKAEEA
S.a. IscS      1 ME--IYADYAATTPVKPEVVDAMMMIYN--SHYGNPSS-IHAKGRDARKY
B.s. YrvO      1 MER-IYLDHAATSPMDERVLEQMIPHFS--GSFGNPSS-IHSFGRESRKW
M.t._IscS      1 ---MAYLDHAATTPMHPAAIEAMAAVQR---TIGNASS-LHTSGRSARRR

S.e. IscS     51 VDIARNQIAELVGADPREIVFTSGATESDNLAIKGAANFYQKKGKH---I
E.c. IscS     51 VDIARNQIADLVGADPREIVFTSGATESDNLAIKGAANFYQKKGKH---I
A.v. IscS     51 VENARRQVAELVNADPREIVWTSGATESDNLAIKGVAHFYASKGKH---I
S.a. IscS     46 LDESRRQIAQLLGADTHEIIFTSGATESNNTAIKGIVKANEQLGNH---I
B.s. YrvO     47 VDEARAQIAAEIGAAEQEIIFTSGGTEADNLAIMGTALARKDLGRH---I
M.t._IscS     44 IEEARELIADKLGARPSEVIFTAGGTESDNLAVKGIYWARRDAEPHRRRI

S.e. IscS     98 ITSKTEHKAVLDTC-RQLEREGFEVTYLAPQRNGIIDLNELEAAMR--DD
E.c. IscS     98 ITSKTEHKAVLDTC-RQLEREGFEVTYLAPQRNGIIDLKELEAAMR--DD
A.v. IscS     98 ITSKIEHKAVLDTT-RQLEREGFEVTYLEPGEDGLITPAMVAAALR--ED
S.a. IscS     93 ITSKIEHHSVLHVF-EQLEREGFDVTYLDVDDTGAIDLDQLEETIT--DK
B.s. YrvO     94 ITTKIEHHAVLHTC-EKLEGDGFDITYLDVDQNGRVSAKQVKEALR--DD
M.t._IscS     94 VTTEVEHHAVLDSVNWLVEHEGAHVTWLPTAADGSVSATALREALQSHDD

S.e. IscS    145 TILVSIMHVNNEIGVVQDIATIGEMCRARGIIYHVDATQSVGKLPIDLSQ
E.c. IscS    145 TILVSIMHVNNEIGVVQDIAAIGEMCRARGIIYHVDATQSVGKLPIDLSQ
A.v. IscS    145 TILVSVMHVNNEIGTVNDIAAIGELTRSRGVLYHVDAAQSTGKVAIDLER
S.a. IscS    140 TILVSIMFVNNEVGTVQQIYDIQDIIAETNAYFHVDAVQAIGHLDVKFDE
B.s. YrvO    141 TILVTVMYGNNEVGTVQPIEEIGELLKEHKAYFHTDAVQAFGLLPIDVKN
M.t._IscS    144 VALVSVMWANNEVGTILPIAEMSVVAMEFGVPMHSDAIQAVGQLPLDFGA

S.e. IscS    195 LKVDLMSFSGHKIYGPKGIGALYVRRKPRIRIEAQMHGGGHERGMRSGTL
E.c. IscS    195 LKVDLMSFSGHKIYGPKGIGALYVRRKPRVRIEAQMHGGGHERGMRSGTL
A.v. IscS    195 MKVDLMSFSAHKTYGPKGIGALYVRRKPRVRLEAQMHGGGHERGMRSGTL
S.a. IscS    190 FEIDAMSITAHKFGGPKGVGALLVKDH--VTLDYPQLGGEQELKRRAGTE
B.s. YrvO    191 SHIDLLSVSGHKLNGPKGTGFLYASKD--VKLSPLLFGGEQERKRRAGTE
M.t._IscS    194 SGLSAMSVAGHKFGGPPGVGALLLRRD--VTCVPLMHGGGQERDIRSGTP

S.e. IscS    245 PVHQIVGMGEAYRIAKEEMETEMARLRGLRNRLWNGIKDIEEVY-LNGDL
E.c. IscS    245 PVHQIVGMGEAYRIAKEEMATEMERLRGLRNRLWNGIKDIEEVY-LNGDL
A.v. IscS    245 ATHQIVGMGEAFRIAREEMAAESRRIAGLSHRFHEQVSTLEEVY-LNGSA
S.a. IscS    238 NLAQIVGMAKALQLAEKNRDDNNIHLMNLKEQFLVKLQERAIPFELNGSM
B.s. YrvO    239 NVPGIVGLKEAIKLSSEERDEKNEKYQSFKAIFADTLRDAGVAFEVNGDK
M.t._IscS    242 DVASAVGMATAAQIAVDGLEENSARLRLLRDRLVEGVLAEIDDVCLNGAD

S.e. IscS    294 E-QGAPNILNVSFNYVEGESLIMALK--DLAVSSGSACTSASLEPSYVLR
E.c. IscS    294 E-HGAPNILNVSFNYVEGESLIMALK--DLAVSSGSACTSASLEPSYVLR
A.v. IscS    294 T-ARVPHNLNLSFNYVEGESLIMSLR--DLAVSSGSACTSASLEPSYVLR
S.a. IscS    288 T-DATGHIVNLYFPFVEVEMMLTLLDMAQIYVSSGSACTAGSTQPSHVLD
B.s. YrvO    289 E-HSLPHVLNLYFPGVSVEALLVNLDMAGVAVSSGSACTAGSVLPSHVLT
M.t._IscS    292 DPMRLAGNAHFTFRGCEGDALLMLLDANGIECSTGSACTAGVAQPSHVLI

S.e. IscS    341 ALGMND-ELAHSSIRFSLGRFTTEEEIDYTIDLVRKSIGRLRDLSPLWEM
E.c. IscS    341 ALGLND-ELAHSSIRFSLGRFTTEEEIDYTIELVRKSIGRLRDLSPLWEM
A.v. IscS    341 ALGRND-ELAHSSIRFTFGRFTTEEEVDYAARKVCEAVGKLRELSPLWDM
S.a. IscS    337 AMFEDE-ERSNHSIRFSFNELTTENEINAIVAEIHKIYFKFKEES-----
B.s. YrvO    338 AMFGEESDRLTSSIRISFGLGNTAEQVKTAAKHVADVVKRLT--------
M.t._IscS    342 AMGVDA-ASARGSLRLSLGHTSVEADVDAALEVLPGAVARARRAALAAAG
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Figure S3: Amino acid sequence alignment of IscS sequences from Gram-negative *Salmonella enterica* (S.e.), *Escherichia coli* (E.c.), *Azotobacter vinelandii* (A.v.) containing the *iscS* within the ISC operon. The alignment also includes Gram-positive *Staphylococcus aureus* (S.a.), *Bacillus subtilis* (B.s.) and *Mycobacterium tuberculosis* (M.t.) cysteine desulfurase sequences whose coding sequence is located immediately upstream of *mnmA*. Multiple alignment of sequences was accomplished with ClustalW. Conserved and identical residues are colored in dark gray, while similar residues are shaded in light gray. The residues boxed in red with asterisks are those which are necessary for TusA binding and/or s^2U synthesis and are not conserved within *B. subtilis* YrvO.

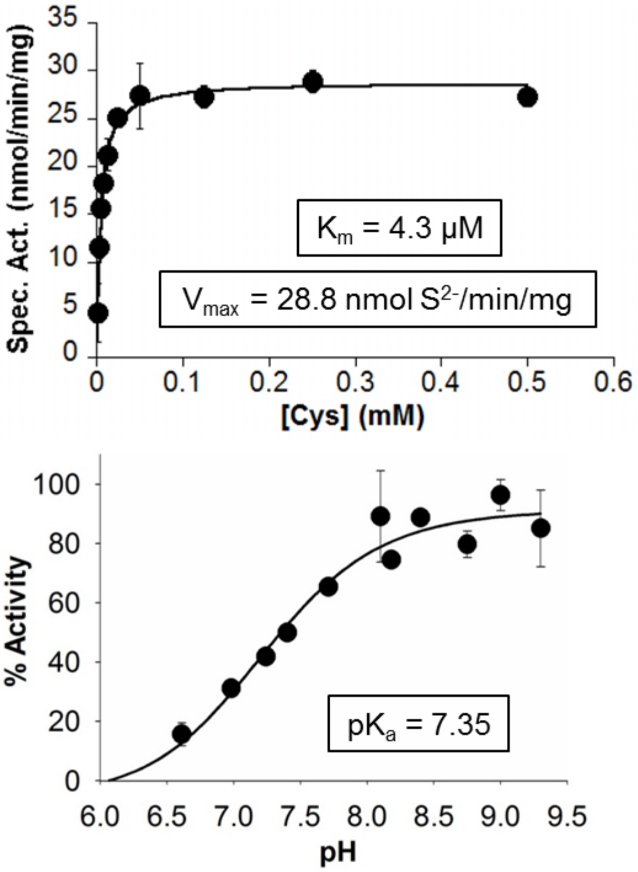

Figure S4: Activity profile of cysteine desulfurase, YrvO. **A.** Substrate saturation curve of YrvO activity was quantified by release of S^{2-}. Assays containing a fixed concentration of YrvO (0.05 mg) and various cysteine concentrations were performed in the presence of 2 mM DTT. Kinetic constants K_m = 4.3± 0.4 μM and V_{max} = 28.8 ± 0.5 nmol S^{2-}/min/mg were determined using the Michaelis-Menten equation. **B**. The pH profile of YrvO displays dependence of YrvO cysteine desulfurase activity on pH. Assays measuring release of S^{2-} were conducted with constant concentrations of YrvO (0.05 mg), L-cysteine (0.5 mM) and DTT (2mM),and a pKa of 7.35 was calculated using the Henderson-Hasselbalch equation. When not visible, error bars are smaller than the symbols.

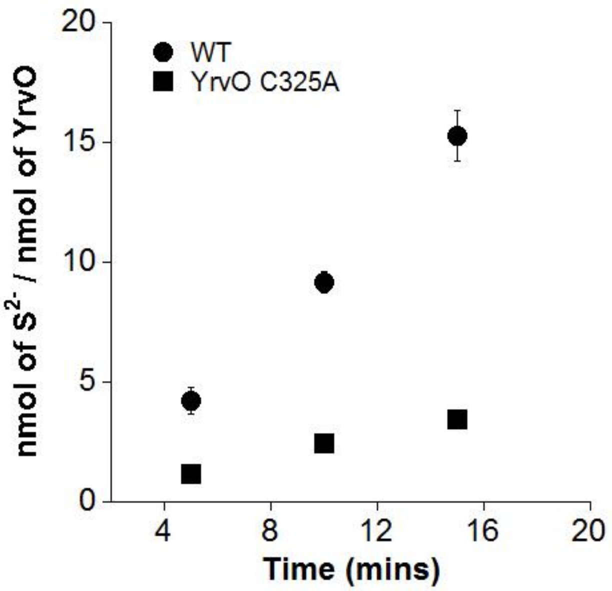

Figure S5: Activity comparison between YrvO WT and C325A mutant. YrvO WT (0.025 mg) or C325A (0.05 mg) were incubated with 0.5 mM L-cysteine and 2 mM DTT, and the activity of each enzyme was quantified by the production of sulfide as mentioned in the materials and methods. When not visible, error bars are smaller than the symbols.

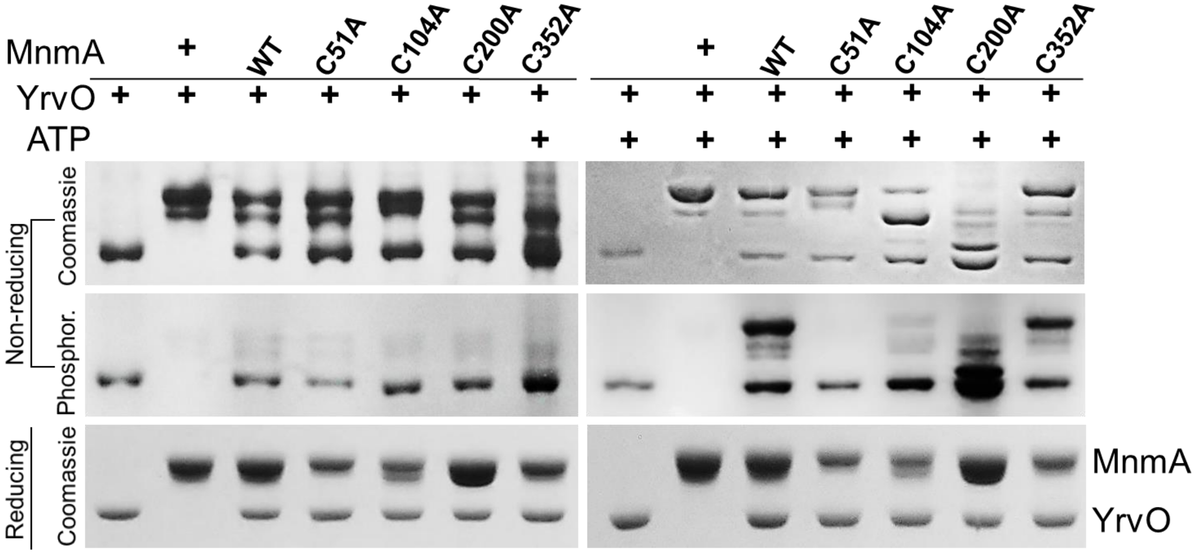

Figure S6: Sulfur transfer from YrvO to MnmA *in vitro*. YrvO (9 µg) and/or MnmA wild type or individual Cys to Ala variants (18 µg) were incubated with ^{35}S-L-cysteine (10 µCi), L-cysteine (200 µM), and $MgCl_2$ (5 mM), in the absence (left), or presence (right) of ATP (1 mM). Reactions were quenched with N-ethylmaleimide (0.4 mM) and subjected to SDS-PAGE analysis. The top panel displays the Coomassie stained gel and the middle panel shows the occurrence of radiolabeling, as imaged using a phosphorimager, both of which were conducted under non-reducing conditions. The bottom panel demonstrates that under reducing conditions, MnmA migrates in a single conformation, establishing that the multiple bands seen in non-reducing gels are all different conformations of the same protein. The phosphorimage associated with the bottom reducing gel showed complete bleaching, indicating reduction of the covalently modified radiolabel (data not shown).

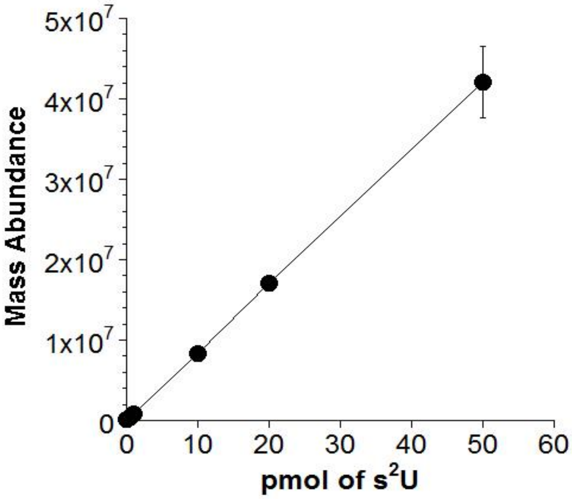

Figure S7: Standard curve generated for determination of pmol of s^{2}U formed during *in vitro* assays. 2-thiouridine was suspended in Optima water and methanol and formic acid were added to the sample to final concentrations of 2% and 0.1% respectively. Next, 10 µL injection volumes containing 0, 0.5, 1, 10, 20 or 50 pmol of s^{2}U were subjected to the same LCMS conditions used for *in vitro* samples. Standard deviations were obtained from at least three independent experiments, and where not visible, error bars are smaller than the symbols.