Supporting Information

Non-ribosomal Peptide Extension by a Peptide Amino-acyl tRNA Ligase

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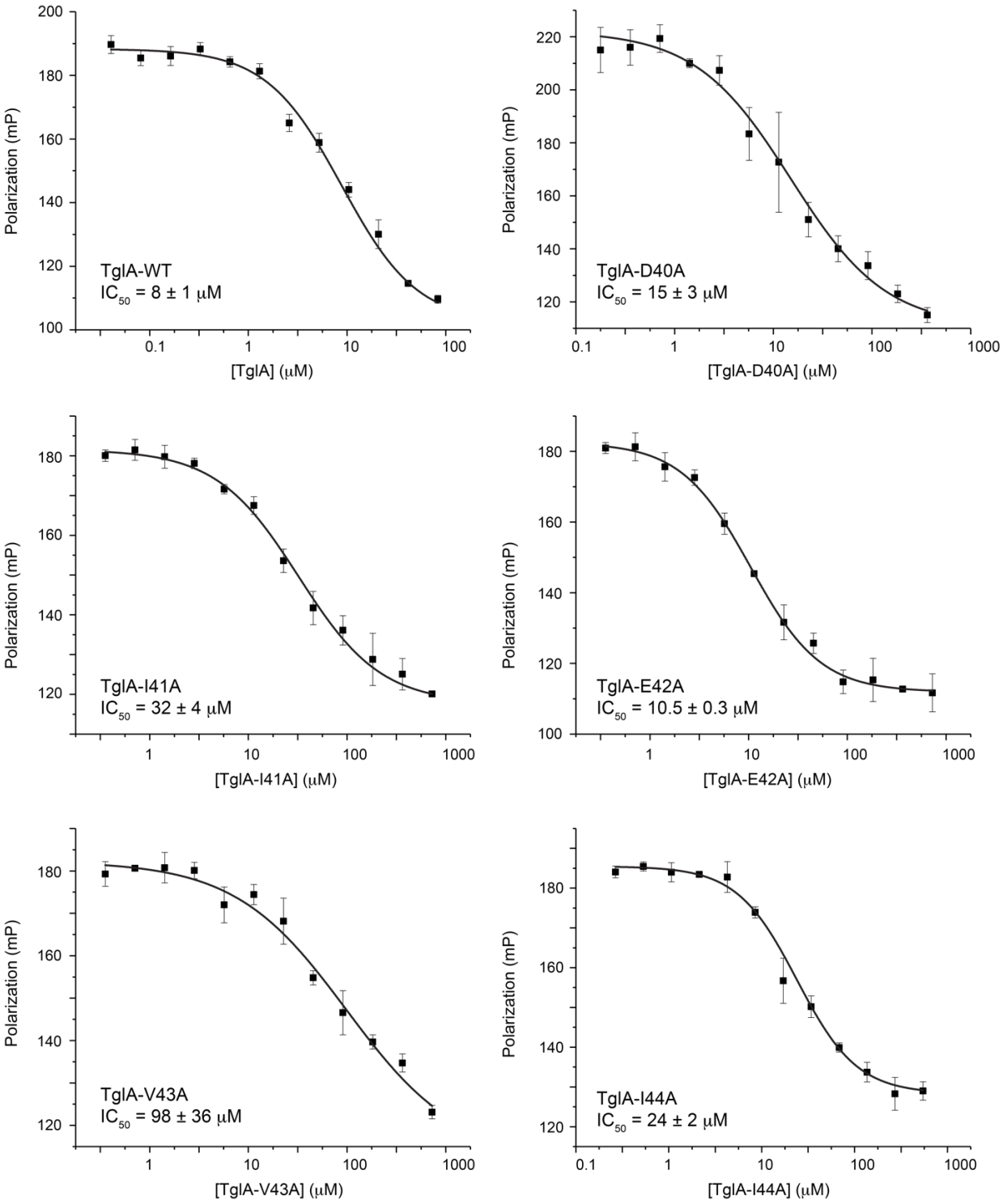
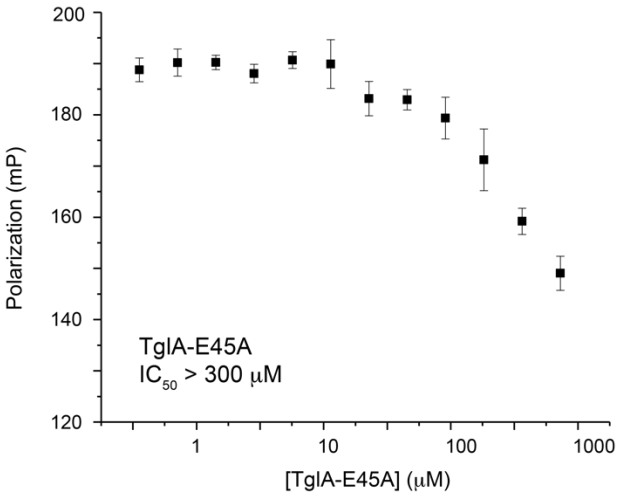
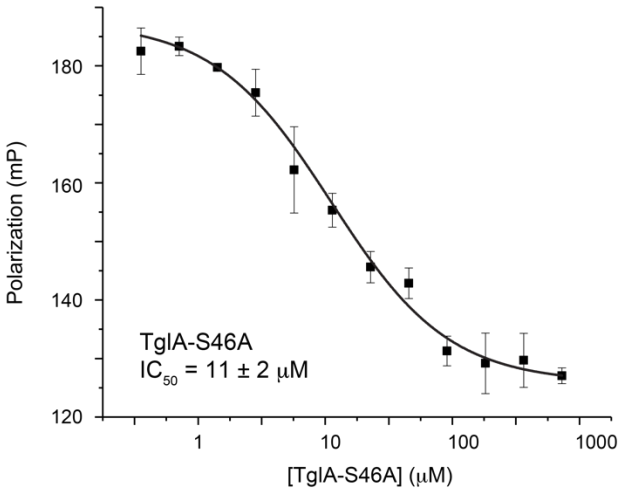
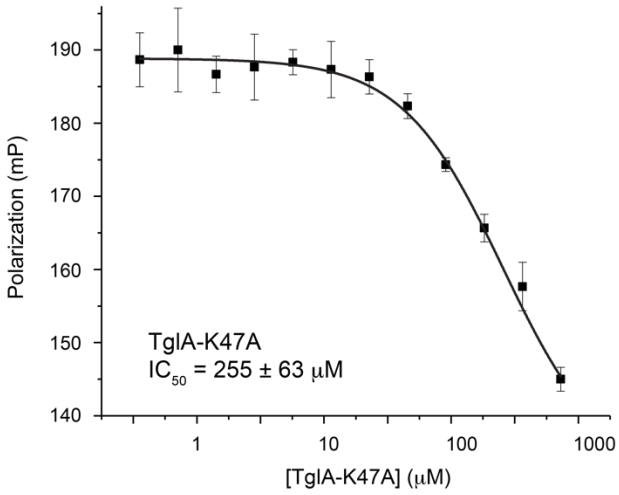
Figure S1. Competitive binding of TglA variants to TglB. Competitive fluorescence polarization traces for binding of wild-type (WT) TglA and TglA variants to TglB. These peptides were used to titrate TglB (3 μM) complexed with fluorescein-labeled peptide consisting of the 20 C-terminal amino acids of TglA (25 nM). Error bars represent standard deviation (s.d.) of three independent replicates. Errors on IC_{50} are the standard error of the mean (s.e.m.) given by regression analysis.




Polarization (mP)
[TglA-E45A] (μM)
TglA-E45A
IC50 > 300 μM


Polarization (mP)
[TglA-S46A] (μM)
TglA-S46A
IC50 = 11 ± 2 μM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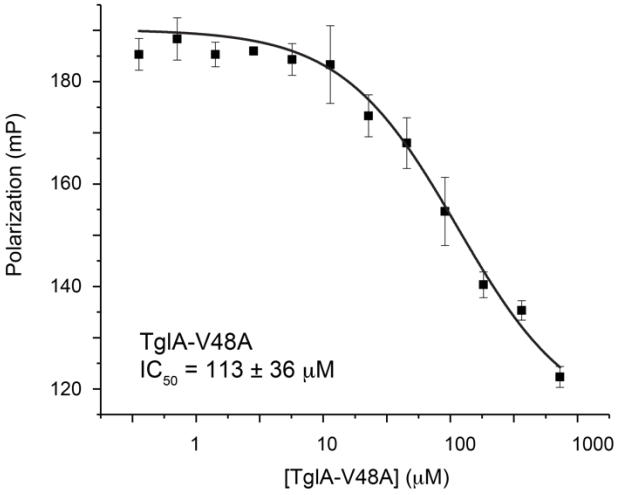
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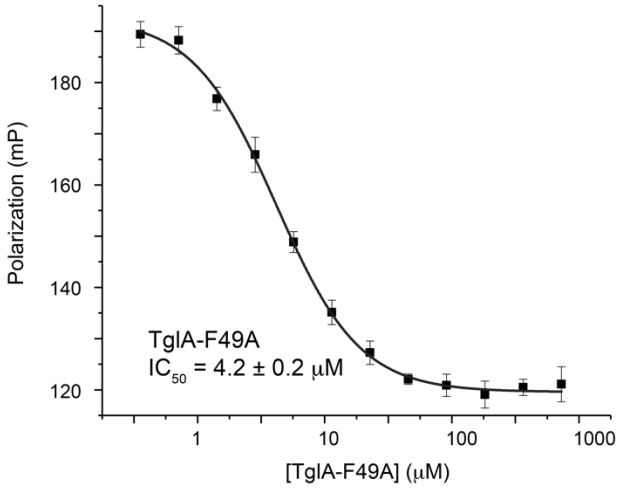
Polarization (mP)
[TglA-F49A] (μM)
TglA-F49A
IC50 = 4.2 ± 0.2 μM

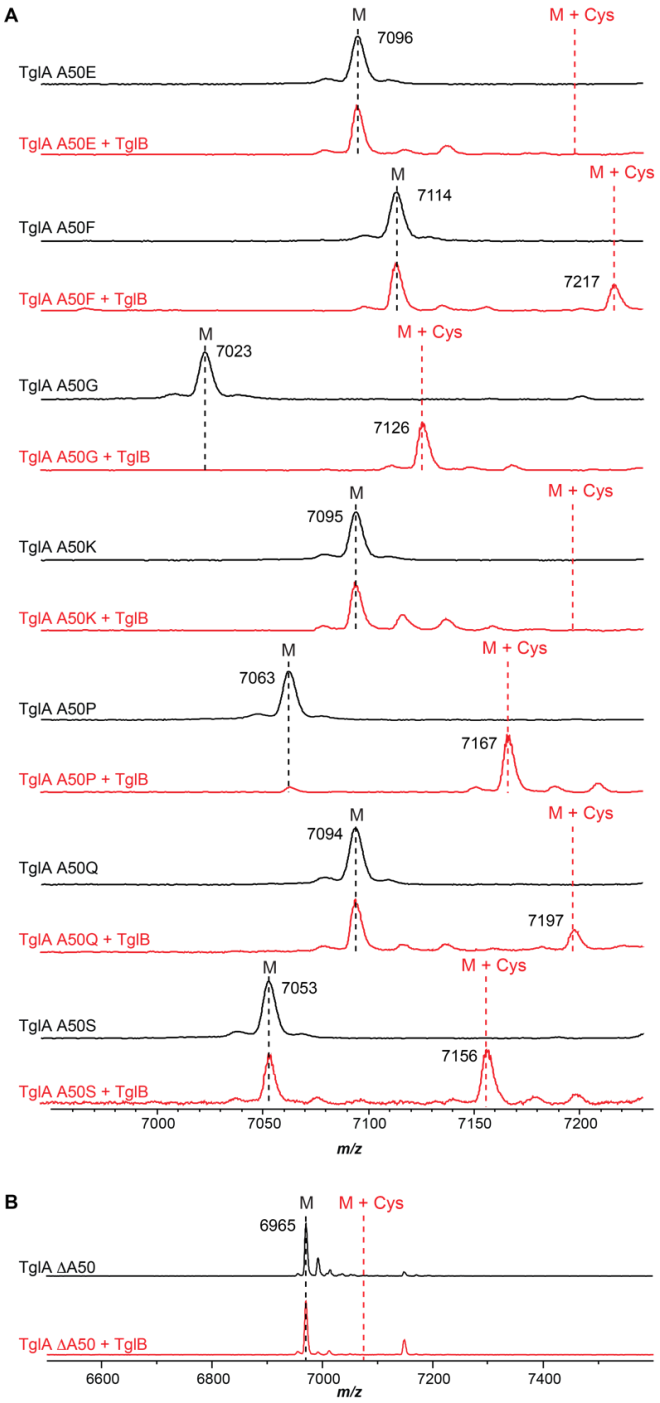

Figure S2. TglB is relatively promiscuous towards a change of the last amino acid of TglA. MALDI-TOF mass spectra of (**A**) TglA-Ala50 variants and their co-expression products with TglB in *E. coli*. (TglA A50E, calc. $[M+H]^+$, 7095. TglA_A50F, calc. $[M+H]^+$, 7114; TglA_A50F_Cys, calc. $[M+H]^+$, 7217. TglA_A50G, calc. $[M+H]^+$, 7023; TglA_A50G_Cys, calc. $[M+H]^+$, 7127. TglA_A50K, calc. $[M+H]^+$, 7095. TglA_A50P, calc. $[M+H]^+$ 7063; TglA_A50P_Cys, calc. $[M+H]^+$, 7167. TglA_A50Q, calc. $[M+H]^+$, 7094; TglA_A50Q_Cys, calc. $[M+H]^+$, 7198. TglA_A50S, calc. $[M+H]^+$, 7053; TglA_A50S_Cys, calc. $[M+H]^+$, 7157). (**B**) TglA ΔA50 variant and its product after co-expression with TglB in *E. coli*. (TglA_ΔA50, calc. $[M+H]^+$, 6966). All m/z values are average masses.

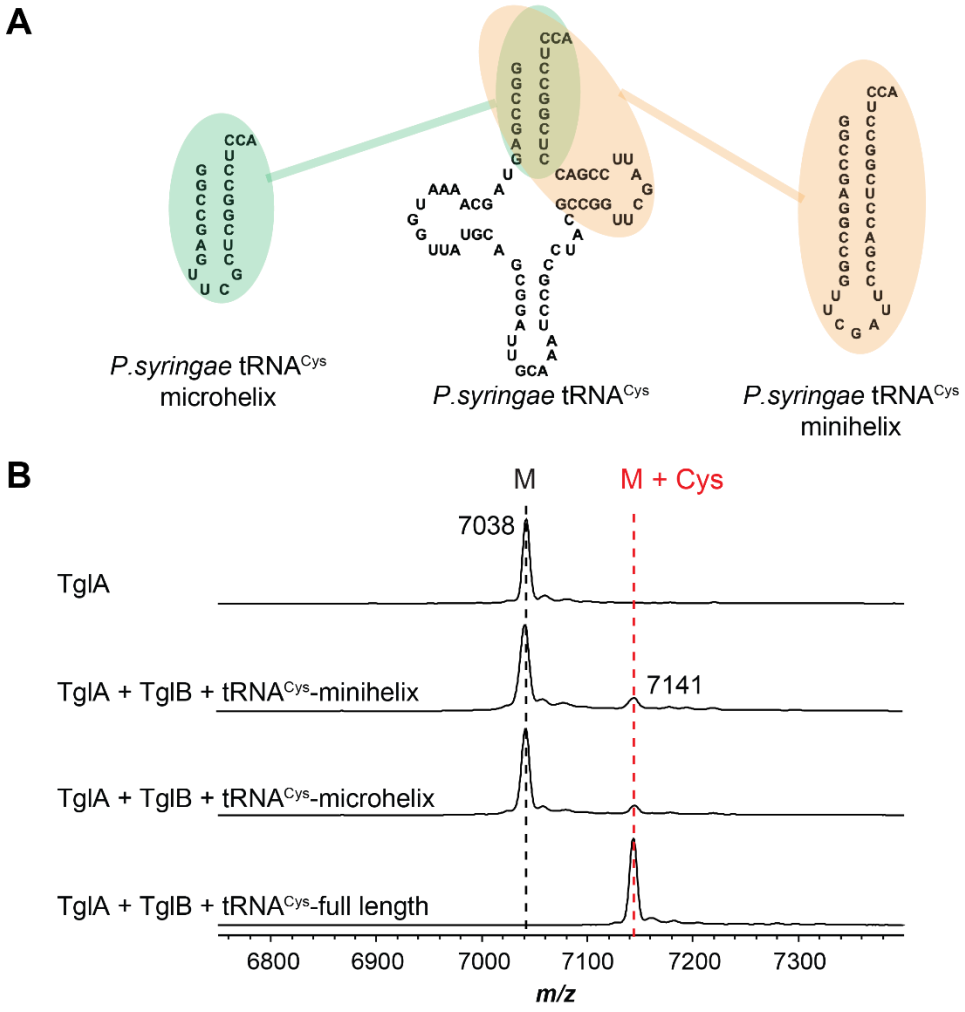

Figure S3. *P. syringae* tRNA^{Cys} mini- or micro-helix is sufficient to catalyze the in vitro cysteinylation reaction. (**A**) Secondary structure of full length *P. syringae* tRNA^{Cys}, and its mini- or micro-helix. (**B**) MALDI-TOF-MS analysis of in vitro cysteinylation reactions with *P. syringae* tRNA^{Cys} mini- or micro-helix under standard assay conditions. (TglA, calc. $[M+H]^+$, 7037; TglA_Cys, calc. $[M+H]^+$, 7141).

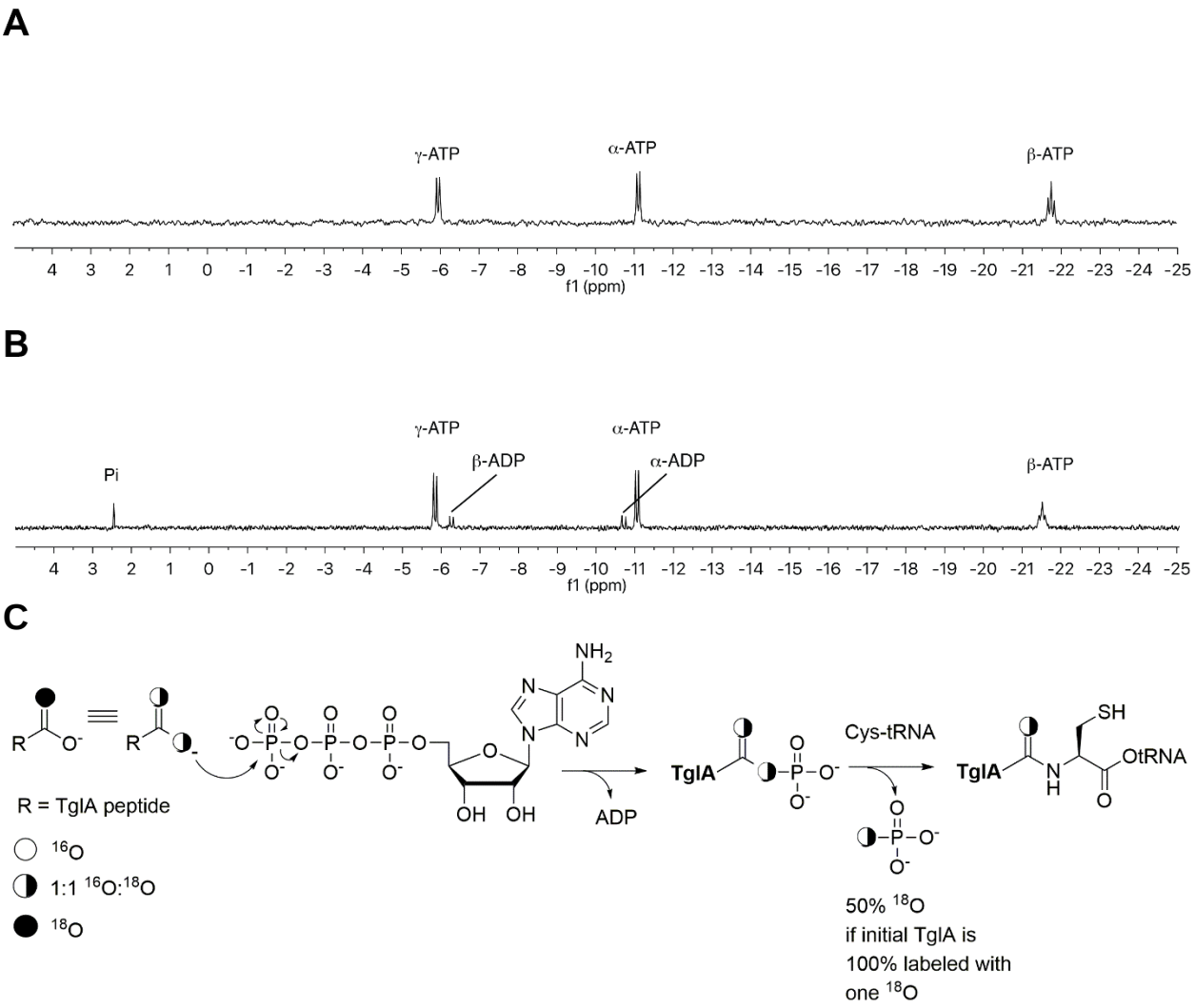

Figure S4. **TglB hydrolyzes ATP in the absence of substrate TglA.** ^{31}P NMR analysis of 500 µM ATP incubated in HEPES assay buffer (100 mM HEPES pH 7.5; 5 mM $MgCl_2$, 100 mM NaCl) at 30 ºC for 30 min in the absence (**A**) and presence of 50 µM TglB (**B**). (**C**) If TglA was 100% labeled with one ^{18}O at the C-terminal carboxylate, then upon phosphorylation 50% of the label would be in the carbonyl group and 50% in the bridging oxygen of the phosphate ester. Upon attack by the Cys-tRNA, this would lead to 50% of labeled phosphate. Using the same reasoning, when 85% of TglA is labeled with one ^{18}O, then the phosphorylated peptide will be 15% unlabeled, 42.5% labeled in the carbonyl oxygen, and 42.5% in the bridging oxygen, and hence after attack by Cys-tRNA ~42% of the released phosphate would be ^{18}O labeled, as mentioned in the main text. 42.5% ^{18}O and 57.5% ^{16}O corresponds to a calculated ratio of 1:1.35. The observed ratio was 1:2 ^{18}O:^{16}O. However, a significant amount of ^{16}O arises from hydrolysis of ADP to phosphate and AMP as shown by NMR analysis (Figure 3). The fraction of the total phosphate that was derived from ADP hydrolysis was estimated by taking the difference in integration of the phosphate peak and either of the two ADP peaks (it was harder to integrate the AMP peak which was overlapping with other peaks). This amount of phosphate produced from ADP hydrolysis was subtracted from the total amount of ^{16}O-phosphate observed to arrive at the amount of ^{16}O-phosphate produced from ATP. This correction leads to a ratio of ^{18}O:^{16}O of 1:1.4.

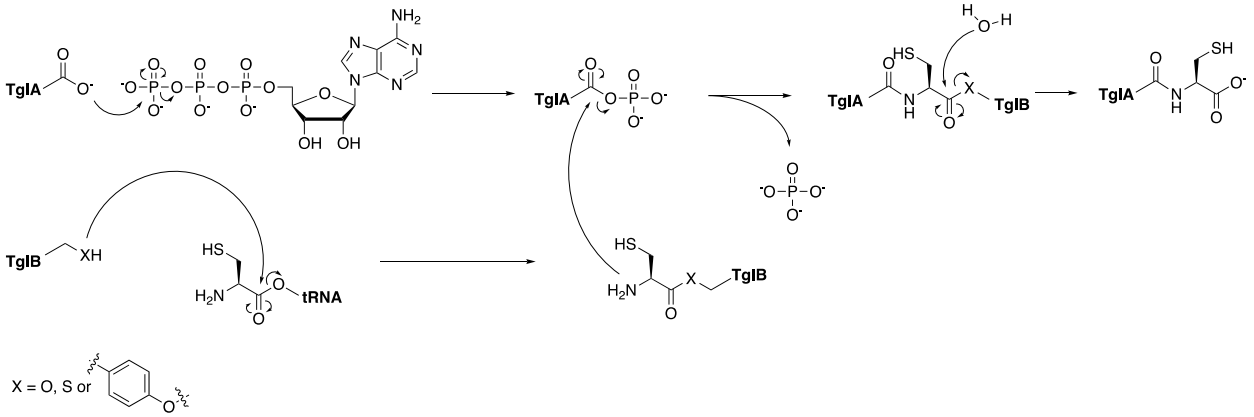

Figure S5. **Potential ping-pong mechanism involving the formation of an aminoacyl-enzyme intermediate.** The amino acylated tRNA first transfers the amino acid onto a residue of TglB. Then, the amino group of the tethered amino acid attacks the activated TglA peptide. Finally, hydrolysis of the protein-peptide complex releases the product.

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WP_102649326.1      ASGL---VEHLLTVLP-----GDFHRAAFAL-------AADVLGAGIAERIRLGHTILKR
WP_110653318.1      LGQF---SEDLLCVYS-----TPLHRLGFAL-------AGSAVAKHDHRRIWLGRTLYKR
WP_048368865.1      LGQF---SEDLLCVYP-----TPLHRIGFTL-------ADSAVAKHEYRRIWLGRTLYKR
TglB                AGQF---SEDLVCVYP-----TPMHRLGFAL-------AGSVVAKNDRRRIGLGKTLYKR
WP_117166554.1      AGEF---AEDLLCVYS-----TPMHRLGFAL-------AGSAVARGDRRRIWLGKTLYKR
WP_112718210.1      LGYF---NEDLLCVHN-----TQLHQLLFDL-------AGDIIPRHECRRILFNRSIYKR
WP_095098125.1      DGAF---HEDLLCVSG-----TSLHQLLFRL-------AGDVLPRHEPRRIRVNRTLYKR
CUW02180.1          DGAF---HEDLLCVNS-----TALHQLLFQL-------AGDMLPRHEPRRIRVNRTLYKR
                           :  .               . :       . .         : ..     *

WP_010898205.1      AKWHISKQDVWSKKHTGSSIELFYEAYRFHLKESLPKEGFAKLPGQPKPVYVNFTSYFLI
BAB05753.1          EHWVFEPTDWGEVHASLDGFQQMACLQEIKVKYQLPQVGYLRFSQELKPVWFDFNNPFCV
WP_102649326.1      RTWTLQASALPIAERVGESMPAFLAWRRWADALGLPRHIFVKADTEPKPVFVDFDCPLSL
WP_110653318.1      ASWLFARDRLPEPKGSIDELEHTLQWRAWAGAHGLPRYAFVKIDTEPKPLFLDFDNPLSF
WP_048368865.1      ASWLFTREQLPEPKGTIEELEYTMQWRAWAVSQGLPRYAFIKIDTEPKPLFLDFDNPLSL
TglB                ASWWFSPEQLPCSEFSVDKLDDVLAWRAWAVEHGLPRYVFAKIDIEPKPIFIDFDNPLSL
WP_117166554.1      ASWWFTQEQFPCPEFSVDKLDDTLAWRAWAQEHGLPRYVFAKIDIEPKPIFIDFDNPLSF
WP_112718210.1      RTWSFESSDWPVS--SKDELGLFVQWRLWKDKYQMPRWIFVKCDSEPKPFFVDFDNPLSL
WP_095098125.1      RTWAFNAGGWPEA--VSDEFMAFIQWRDWQQRQALPRWVFIKCDSEPKPLFIDFDNPLSL
CUW02180.1          RTWAFNTGGWPEA--VSDEFMAFIQWQEWQQRQALPRWVFIKCDSEPKPLFIDFDNPLSL
                      * :            . :              :*.  : .   : **.:.:*   : .

WP_010898205.1      EMLEGLWPDGENCTFSEMLPNLSELWLSKEDQKTHTAEIRLSY---FVERS---------
BAB05753.1          DLFFNMAKKAEWFTFSRMDPGVEDLFL-KDDRGHYCCELRTFA---YRQEITSKDMLMG-
WP_102649326.1      DALTSVSARAQTLGISEMLPAPHQVWL-ADERGAFCAEFRTSIAGMGDDEGAQSHSPQQH
WP_110653318.1      DGISNALKNAGHVKFSEMRPCPDELWL-EEARGRFCCEIRTTF---STCEALAT------
WP_048368865.1      DGIINALKSAGHVKFSEMRPGPDELWL-EDARGRFCCEIRTTF---SINKALAT------
TglB                DGVSNSMKKAGHVKFSEMCPAPDQLWL-EETRGHFCCEIRTTF---RDNGVTRDE-----
WP_117166554.1      DGVSNSMKKATHVKFSEMCPSPDELWL-EEARGRFCCEIRTTF---TSDGVVPDEQ----
WP_112718210.1      DALVSALKKAKVIRVSEMLPDPNALWF-ADKRGKFCAEIRTSMIISDNNKPVSSEQYE--
WP_095098125.1      DALATALKKARVIQVSEMLPTPDELWF-NDARGRVCCEIRTTF---SPIKQETTENAE--
CUW02180.1          DTLATALKKARVIHVSEMLPTPDELWL-NDTRGRVCCEIRTTF---SPIKQEASENAE--
                    : .      .    .* * *    :::  : .    .*:*
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WP_010898205.1      -----
BAB05753.1          -----
WP_102649326.1      EEALP
WP_110653318.1      -----
WP_048368865.1      -----
TglB                -----
WP_117166554.1      -----
WP_112718210.1      -----
WP_095098125.1      -----
CUW02180.1          -----
```

Figure S6. Sequence alignment of TglB homologs. The protein sequence for TglB was used to identify homologs via BLAST search. Homologs with E-values < 1E-39 were aligned by sequence with MUSCLE (MUltiple Sequence Comparison by Log- Expectation),[1] and highly conserved residues were targeted for alanine replacement. Shown in red are residues replaced with alanine for enzymatic activity assessment in this study.

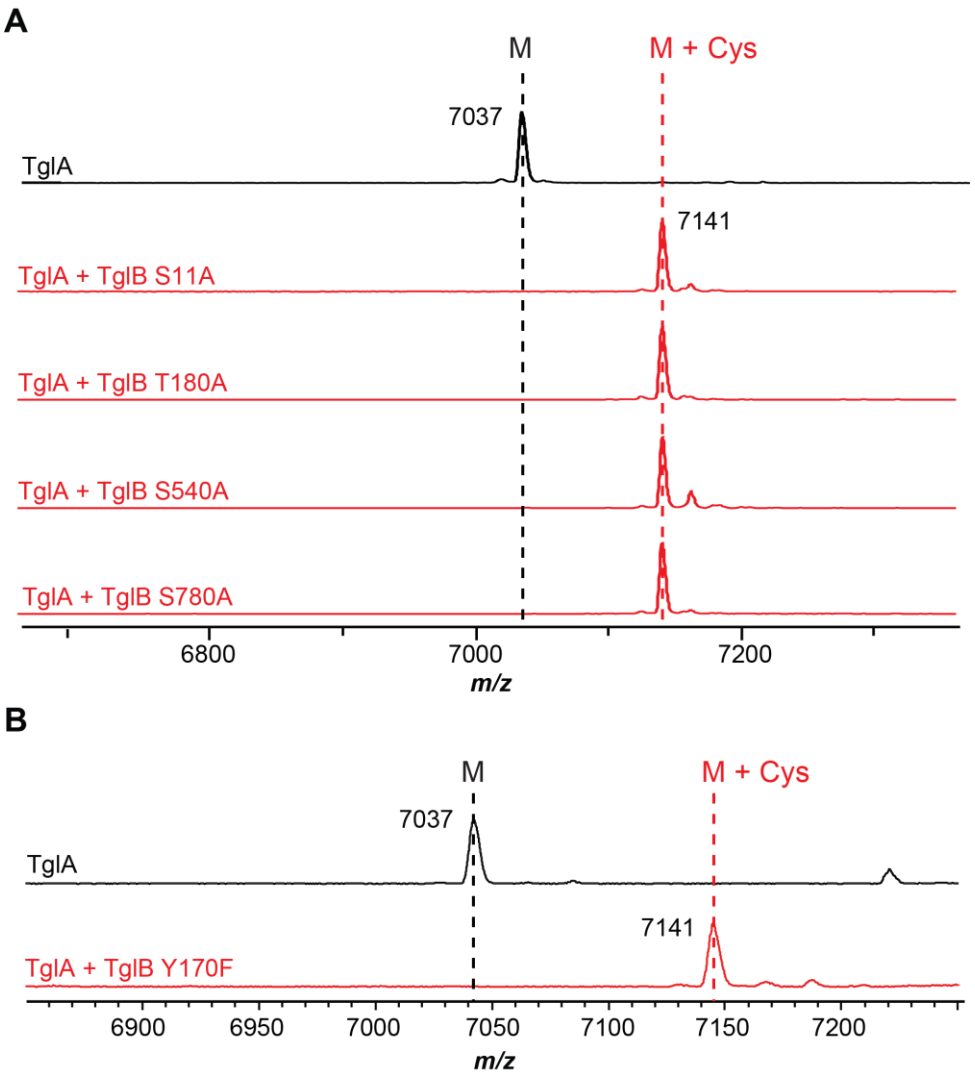

Figure S7. Mutagenesis of selective Ser, Thr and Tyr residues of TglB does not support the ping-pong mechanism in Figure S5. MALDI-TOF-MS spectra of TglA co-expressed in *E. coli* with TglB variants in which selective (**A**) Ser or Thr residues were replaced with Ala, (**B**) a Tyr residue was replaced with a Phe. All experiments were done under the standard TglB assay conditions.

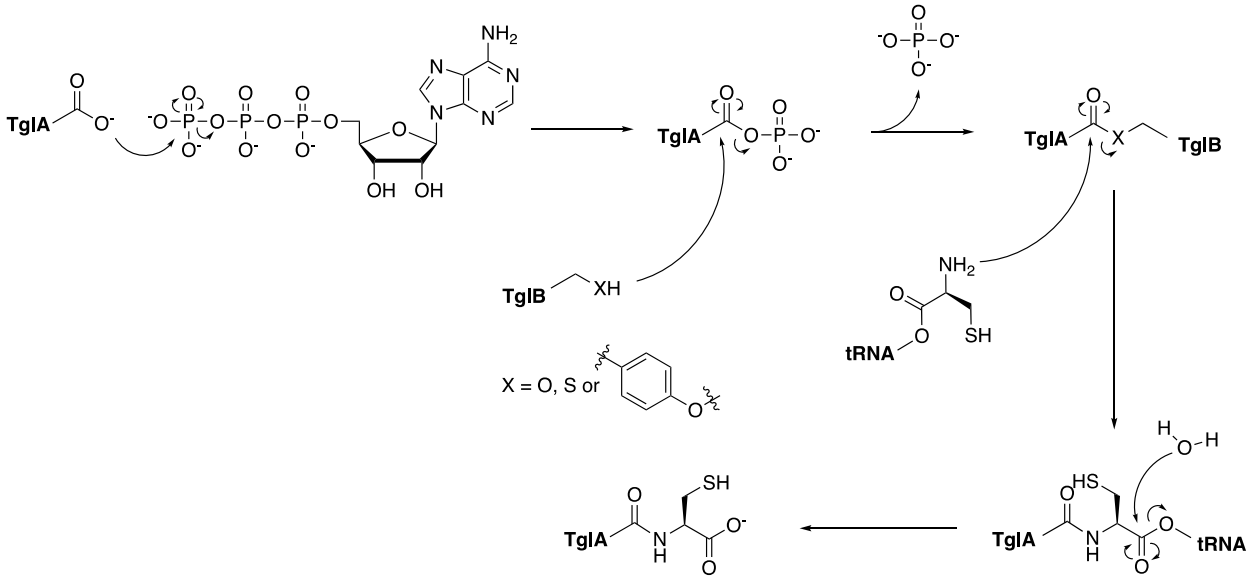

Figure S8. **A potential mechanism in which TglA is covalently linked to a hydroxyl group on TglB.** In this mechanism, the C-terminal carboxylate is first activated by ATP through phosphorylation. Then, a hydroxyl group from a side chain of a residue on TglB attacks the activated carboxylate to load the TglA peptide onto the protein via an ester that in turn can be attacked by the amino group of the cysteinyl-tRNA. Subsequent hydrolysis of the tRNA would produce the final product.

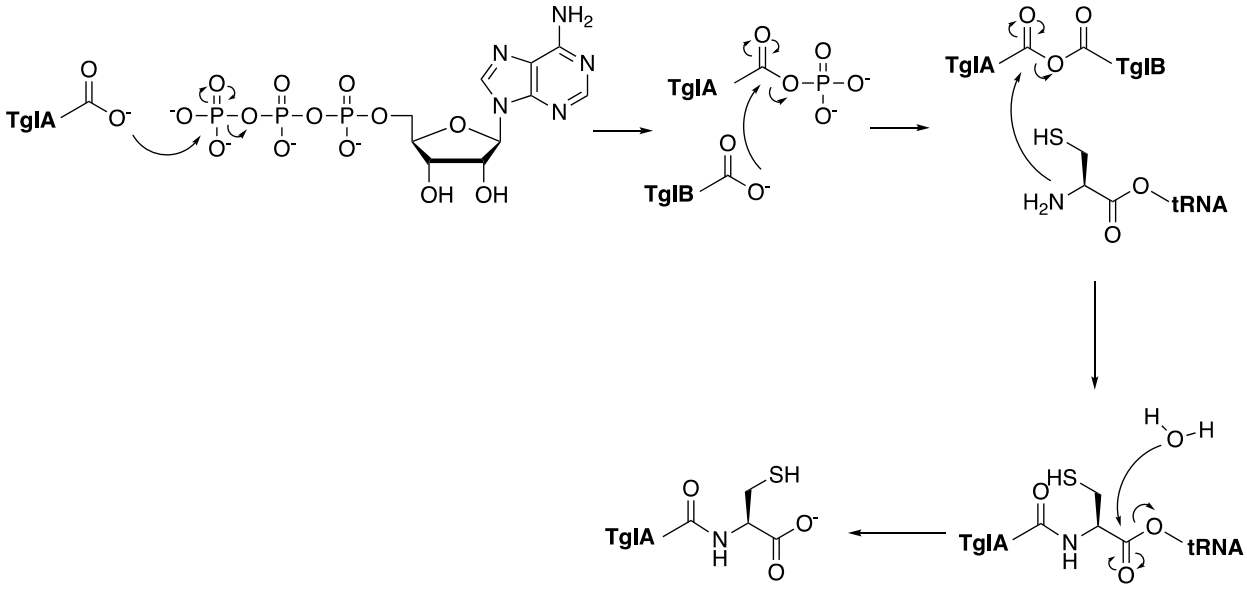

Figure S9. **A potential mechanism in which the C-terminus of TglA is first activated by ATP, followed by attack from a carboxylate residue on TglB to form an anhydride.** Attempts to trap such an intermediate with hydroxylamine or sodium borohydride were unsuccessful.

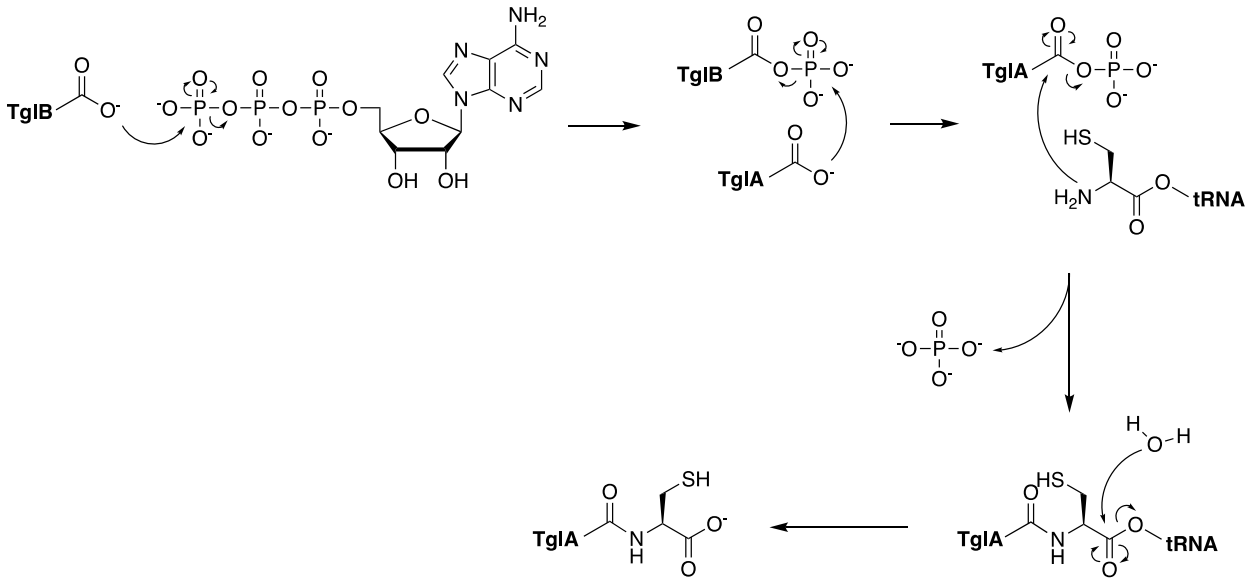

Figure S10. A potential mechanism with a covalent acyl-phosphate intermediate. In this mechanism, a phosphate group is first transferred from ATP onto a carboxylate containing residue on TglB, before further reaction with the TglA peptide.

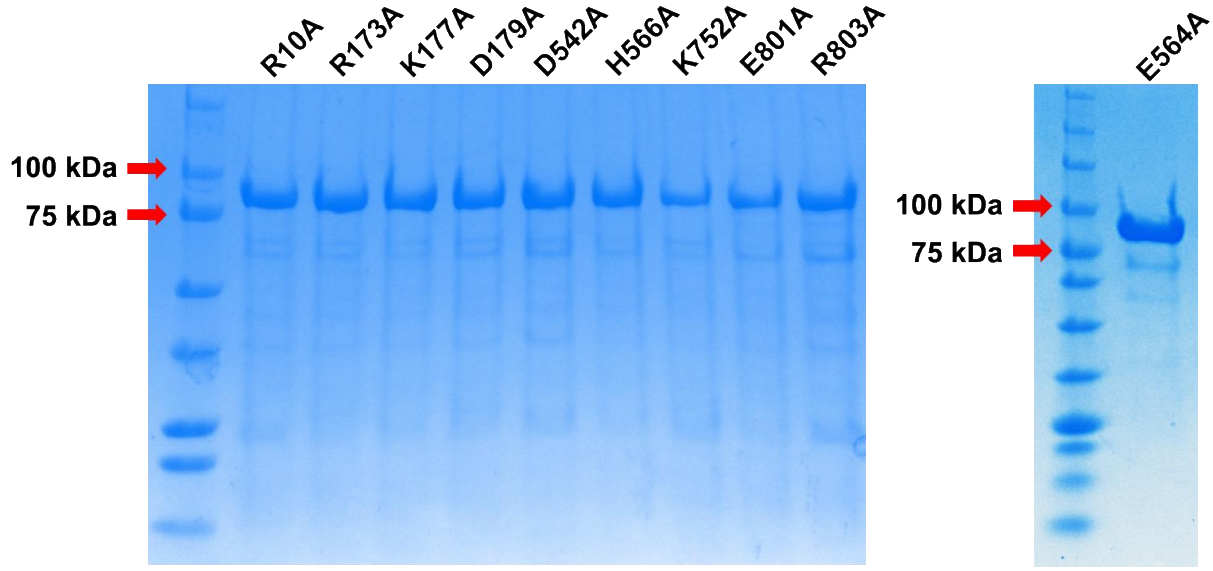

Figure S11. SDS-PAGE analysis of TglB variants with diminished activity. All proteins were expressed as N-terminal His_6-tag fusions.

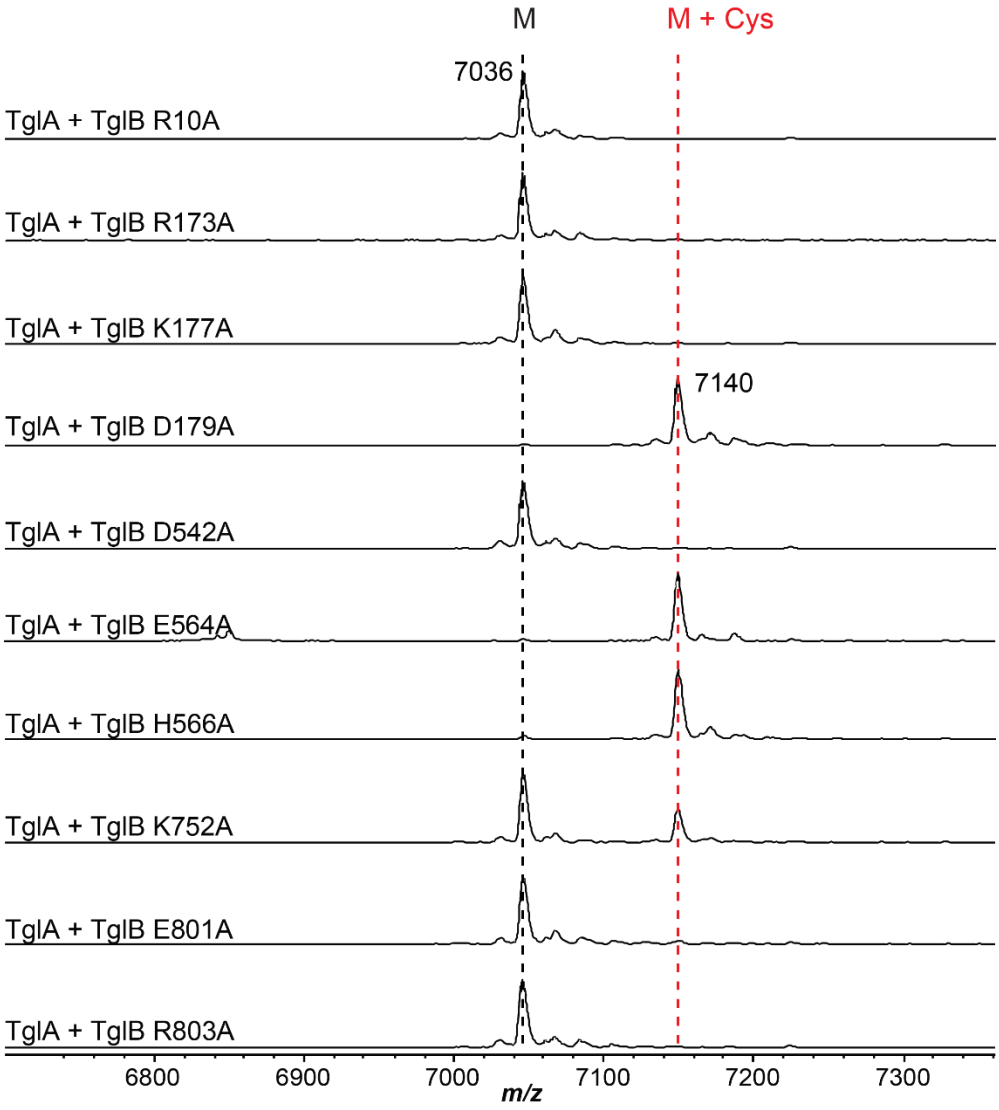

Figure S12. MALDI-TOF-MS spectra of in vitro cysteinylation of TglA with TglB variants. The in vitro activity of each TglB variant mirrors the activity observed when co-expressed with TglA in *E. coli* (Table S1). All experiments were done under the standard TglB assay conditions.

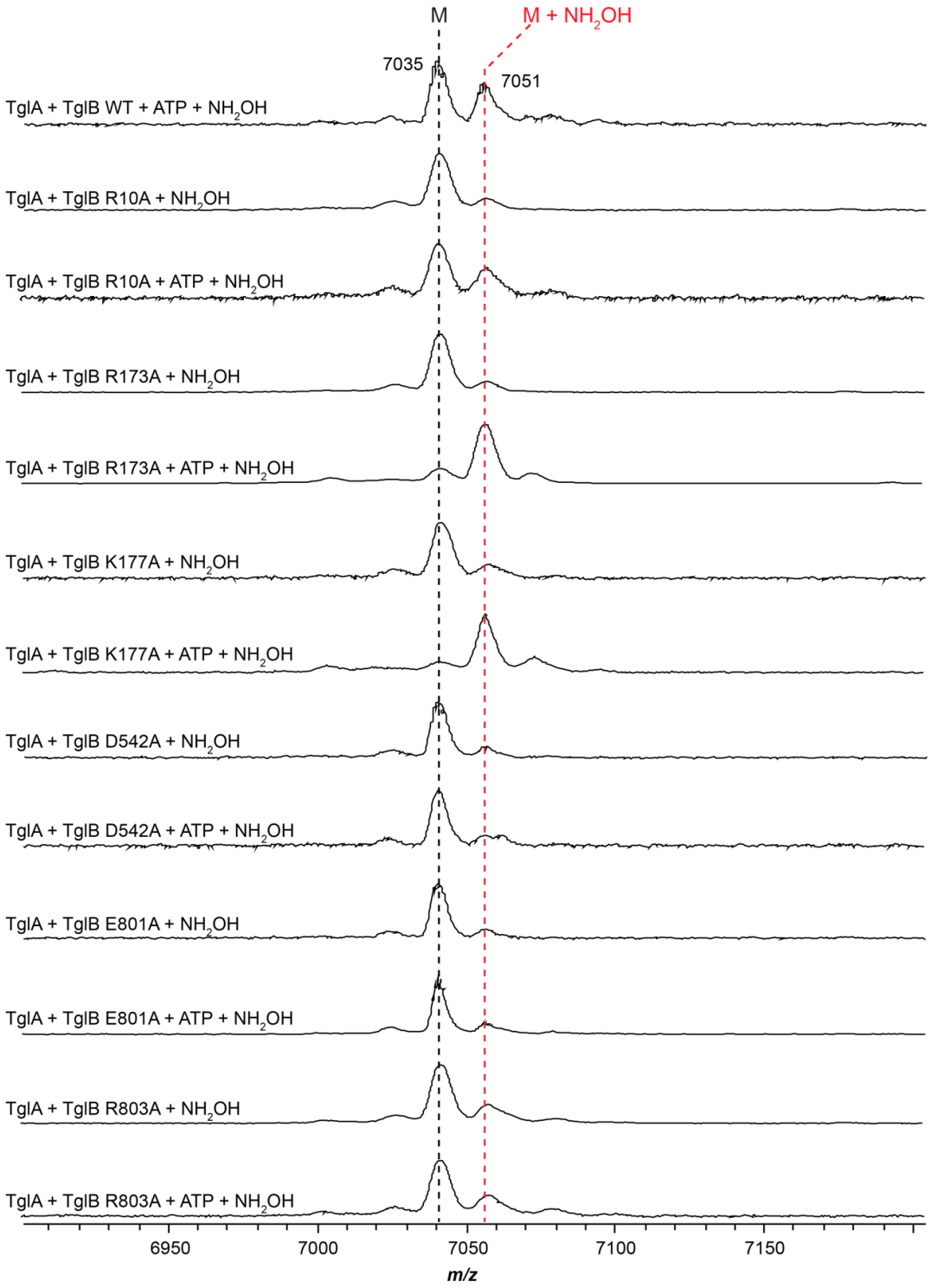

Figure S13. MALDI-TOF-MS spectra of NH_2OH quenching assays with TglB mutants that do not conjugate Cys to TglA. (TglA, calc. $[M+H]^+$, 7037; TglA_NHOH, calc. $[M+H]^+$, 7052). Assays were performed by reacting 50 µM TglB and 50 µM TglA in the presence or absence of ATP (5 mM) for 5 min at 30 ºC. Then assays were quenched with NH_2OH to a final concentration of 1 M and incubated at 30 ºC for another 20 min.

Table S1. Activity of alanine-substituted TglB proteins. Sixteen positions of TglB were targeted for alanine replacement by site-directed mutagenesis using the alignment of Figure S6. These proteins were assayed for enzymatic activity by co-expressing with TglA and analyzed using MALDI-TOF-MS. +++ indicates enzyme activity roughly equal to wild-type (full conversion to product); ++ indicates modestly reduced enzyme activity (>50% conversion to product); + indicates severely reduced enzyme activity (low but detectable product formation); - indicates no detectable enzyme activity (no observed product formation).

TglB Protein	Relative Product	TglB Protein	Relative Product
Wild-type	+++	D404A	+++
R10A	-	D542A	-
K161A	+++	E564A	++
Y170F	+++	H566A	++
R173A	-	R704A	+++
K177A	-	W707A	+++
N178A	+++	K746A	+++
D179A	++	K752A	+
R397A	+++	E801A	-
E403A	+++	R803A	-

Table S2. Oligonucleotides used in this study. All sequences are provided 5′ to 3′. Lowercase m indicates 2′ *O*-methylation of the following residue; methylation suppresses random addition of bases at the end of the RNA by T7 RNA polymerase.

Primer	Oligonucleotide Sequence
TglA (-Ala)_F	GCAAGGTCTTTTGAAGCTTGCGGCCGCATAATG
TglA (-Ala)_R	CGCAAGCTTCAAAAGACCTTGCTCTCGATGACTTCAATGTC
P. syringae $tRNA^{Cys}$ F	AATTCCTGCAGTAATACGACTCACTATAGGCCGAGTAGCAAAATGGTTATGCAGC
P. syringae $tRNA^{Cys}$ R	mUmGGAGGCCGAGGTCGGAATCGAACCGGCGTAGGCGGATTTGCAATCCGCTGCATAACC
TglB_Seq_623	CTGGATCAGAGAACG
TglB_Seq_1549	GGCTGATTGTCGAGC
TglA_ 20mer_F	GAGAACCTGTACTTCCAATCCGCGTTGTTTGAAGAGTTTGACC
TglA R	GGATTGGAAGTACAGGTTCTCCGGATCCTGGCTGTG
TglB_S11A_F	CTGGCTTCGCGCTACCGGGTTTGCGGTGC
TglB_S11A_R	CAAACCCGGTAGCGCGAAGCCAGAAATAGTGTG
TglB_T180A_F	CAAAAAATGATGCATCGAGTTTTTTCGGCCC
TglB_T180A_R	GAAAAAACTCGATGCATCATTTTTTGAACAGAATCGCTG
TglB_S540A_F	CACTTTCACGCTCCGGATTTCCTGATTTCCAG
TglB_S540A_R	GAAATCCGGAGCGTGAAAGTGGCTGCCAAATAC
TglB_S780A_F	GTCAAGTTTGCTGAGATGTGTCCTGCTCC
TglB_S780A_R	CACATCTCAGCAAACTTGACGTGCCCC
TglB_R10A_F	CTATTTCTGGCTTGCATCGACCGGGTTTGCG
TglB_R10A_R	CGGTCGATGCAAGCCAGAAATAGTGTGAGCTTTCC
TglB_K161A_F	CAGCCGCAAGGCTCAGAAACTGCGCCTGG
TglB_K161A_R	CAGTTTCTGAGCCTTGCGGCTGTCAGTTC
TglB_Y170F_F	CCTGGAGCTTCGCACAGCGATTCTGTTC
TglB_Y170F_R	GAATCGCTGTGCGAAGCTCCAGGCCAGG
TglB_R173A_F	CTACGCACAGGCATTCTGTTCAAAAAATGATACGTCGAG
TglB_R173A_R	GAACAGAATGCCTGTGCGTAGCTCCAG
TglB_K177A_F	GATTCTGTTCAGCTAATGATACGTCGAGTTTTTTCGG
TglB_K177A_R	CGTATCATTAGCTGAACAGAATCGCTGTGC
TglB_D179A_F	GTTCAAAAAATGCTACGTCGAGTTTTTTCGGC
TglB_D179A_R	CTCGACGTAGCATTTTTTGAACAGAATCGCTGTG
TglB_R397A_F	CATGTATGTCGGTGCATACCCGGTCTACGAGGATTG
TglB_R397A_R	CGGGTATGCACCGACATACATGGCGC
TglB_E403A_F	GGTCTACGCAGATTGTTCGCGCAATATCGATATC
TglB_E403A_R	GCGAACAATCTGCGTAGACCGGGTAGCG
TglB_D404A_F	GGTCTACGAGGCATGTTCGCGCAATATCGATATCAG
TglB_D404A_R	GCGAACATGCCTCGTAGACCGGGTAG
TglB_D542A_F	CACTCTCCGGCATTCCTGATTTCCAGTACCTCG

TglB_D542A_R	GAAATCAGGAATGCCGGAGAGTGAAAGTGGC
TglB_H566A_F	GAGAGGTCGCTCCGGGCGTGCACAC
TglB_H566A_R	CGCCCGGAGCGACCTCTCCCAGAATGATTGAGTAGTCG
TglB_R704A_F	CCTTGTACAAAGCTGCCTCGTGGTGGTTCAG
TglB_R704A_R	CGAGGCAGCTTTGTACAAGGTCTTGCCCAAG
TglB_W707A_F	GCCTCGGCATGGTTCAGTCCAGAGCAAC
TglB_W707A_R	GACTGAACCATGCCGAGGCGCGTTTGTAC
TglB_K746A_F	GTTCGCCGCTATCGATATCGAACCCAAGCC
TglB_K746A_R	CGATATCGATAGCGGCGAACACATAACGGG
TglB_K752A_F	CGAACCCGCTCCGATTTTCATTGATTTCGATAACCC
TglB_K752A_R	CAATGAAAATCGGAGCGGGTTCGATATCGATCTTGGC
TglB_E801A_F	CATTTCTGTTGTGCTATACGTACAACCTTTAGGGACAATGG
TglB_E801A_R	GTTGTACGTATAGCACAACAGAAATGCCCGC
TglB_R803A_F	CTGTTGTGAAATAGCAACAACCTTTAGGGACAATGGAG
TglB_R803A_R	GGTTGTTGCTATTTCACAACAGAAATGCCCG
TglA_ A50F_F	CAAGGTCTTTTTCTGAAGCTTGCGGCCG
TglA_ A50F_R	CAAGCTTCAGAAAAAGACCTTGCTCTCGATGACTTC
TglA_ A50K_F	CAAGGTCTTTAAATGAAGCTTGCGGCCG
TglA_ A50K_R	CAAGCTTCATTTAAAGACCTTGCTCTCGATGACTTC
TglA_ A50E_F	CAAGGTCTTTGAATGAAGCTTGCGGCCG
TglA_ A50E_R	CAAGCTTCATTCAAAGACCTTGCTCTCGATGACTTC
TglA_ A50Q_F	CAAGGTCTTTCAGTGAAGCTTGCGGCCG
TglA_ A50Q_R	CAAGCTTCACTGAAAGACCTTGCTCTCGATGACTTC
TglA_ A50P_F	CAAGGTCTTTCCATGAAGCTTGCGGCCG
TglA_ A50P_R	CAAGCTTCATGGAAAGACCTTGCTCTCGATGACTTC
TglA_ A50S_F	CAAGGTCTTTTCTTGAAGCTTGCGGCCG
TglA_ A50S_R	CAAGCTTCAAGAAAAGACCTTGCTCTCGATGACTTC
pTXB1-TglA_F	GGTCTTTGCCTGCATCACGGGAGATG
pTXB1-TglA_R	GTTGGGTTGTCCCATATGTATATCTCCTTCTTAAAGTTAAAC
TglA_D40A_F	GACCTGGATGCTATTGAAGTCATCGAGAGCAAG
TglA_D40A_R	GACTTCAATAGCATCCAGGTCAAACTCTTCAAAC
TglA_I41A_F	CTGGATGACGCAGAAGTCATCGAGAGCAAGG
TglA_I41A_R	GATGACTTCTGCGTCATCCAGGTCAAACTCTTC
TglA_E42A_F	GATGACATTGCTGTCATCGAGAGCAAGGTC
TglA_E42A_R	CTCTCGATGACAGCAATGTCATCCAGGTCAAAC
TglA_V43A_F	GATGACATTGAAGCTATCGAGAGCAAGGTCTTTG
TglA_V43A_R	GCTCTCGATAGCTTCAATGTCATCCAGGTCAAAC
TglA_I44A_F	CATTGAAGTCGCTGAGAGCAAGGTCTTTGCCTG

TglA_I44A_R	CTTGCTCTCAGCGACTTCAATGTCATCCAGGTC
TglA_E45A_F	GAAGTCATCGCAAGCAAGGTCTTTGCCTGAAG
TglA_E45A_R	GACCTTGCTTGCGATGACTTCAATGTCATCCAGG
TglA_S46A_F	CATCGAGGCAAAGGTCTTTGCCTGAAGC
TglA_S46A_R	CAAAGACCTTTGCCTCGATGACTTCAATGTCATCC
TglA_K47A_F	CATCGAGAGCGCAGTCTTTGCCTGAAGCTTGC
TglA_K47A_R	GCAAAGACTGCGCTCTCGATGACTTCAATGTCATC
TglA_V48A_F	GAGAGCAAGGCATTTGCCTGAAGCTTGCG
TglA_V48A_R	CTTCAGGCAAATGCCTTGCTCTCGATGACTTC
TglA_F49A_F	GAGCAAGGTCGCTGCCTGAAGCTTGCGGC
TglA_F49A_R	GCTTCAGGCAGCGACCTTGCTCTCGATGACTTCAATGTCATC

(1) Madeira, F.; Park, Y. M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A. R. N.; Potter, S. C.; Finn, R. D.; Lopez, R., *Nucleic Acids Res* **2019,** *47* (W1), W636.