Mutually orthogonal pyrrolysyl-tRNA synthetase/tRNA pairs

Supplementary Information

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Methods

Identification of PylRS/^{Pyl}tRNA sequences

We identified protein sequences homologous to the C-terminal region of *Mm*PylRS by protein BLAST[1] using *Mm*PylRSΔ184 as the query sequence and filtering for Expect values below 1×10^{-10}. We next identified protein sequences homologous to *Dh*PylSn by protein BLAST using *Dh*PylSn as the query sequence and filtering for Expect values below 1×10^{-10}. We sorted identified protein sequences which are homologous to the C-terminal region of *Mm*PylRS according to whether we found sequence homology to *Dh*PylSn within the same protein sequence (pylSn-pylSc fusion class), within the same genome in a separate protein sequence (pylSn class), or not within the same genome (ΔpylSn class). We generated protein sequence alignments and percentage identity scores using Clustal Omega[2], discarding duplicate sequences with a percentage identity score above 98%. Protein sequence alignments were visualised using Clustal X[3] and phylogenetic trees generated by phyloT were visualised using ITOL[4].

DNA constructs

PylRS and ^{Pyl}tRNA genes were synthesised by IDT and we inserted the genes into pKW vectors by Gibson cloning to be expressed from constitutively active *glmS* and *lpp* promoters respectively. We appended the gene for *Mm*PylRS with a sequence encoding a C-terminal Ser$(Gly_4Ser)_4$FLAG-tag, while all other PylRS genes were appended with a sequence encoding a C-terminal Ser$(Gly_4Ser)_4His_6$SerGlyStrep-tag II. We used these plasmids together with pBAD GFP(150TAG)His_6 (in which sfGFP containing an amber stop codon at position 150 and a C-terminal His_6 tag is expressed from the arabinose promoter of pBAD; GFP refers to sfGFP throughout). We also created versions of pKW *Ma*PylRS/^{Pyl}$tRNA_{CUA}$ and pKW *G1*PylRS/^{Pyl}$tRNA_{CUA}$ in which the PylRS gene contains no C-terminally appended sequence. *Mm*PylRS-AF, *Ma*PylRS-AF, *Mm*PylRS-MutRS2 and *Ma*PylRS-MutRS1 genes were synthesised by IDT and we inserted the genes into pKW vectors[5] by Gibson cloning.

For library generation of *Ma*^{Pyl}tRNA with randomised variable loop sequences, *Ma*^{Pyl}tRNA genes containing nucleotide positions 41, 42 and 43 replaced with either NNN, NNNN, NNNNN or NNNNNN were synthesised by IDT and we inserted the genes into an empty pKW vector by Gibson cloning. We transformed each library separately into competent *E. coli* DH10B cells to give a library diversity of 1×10^6,

exceeding the theoretical diversity of 4×10^4 required for complete library coverage. The libraries of *Ma*^{Pyl}tRNA plasmids were used together with pBAD *Ma*PylRS GFP(150TAG)His_6 CAT(111TAG) and pBAD *Mm*PylRS GFP(150TAG)His_6 CAT(111TAG). We created these plasmids by transferring the relevant PylRS cassette into the pBAD GFP(150TAG)His_6 vector together with a CAT(111TAG) gene under constitutive expression (chloramphenicol acetyl transferase containing an amber stop codon at position 111).

We created pKW-DUAL plasmids to assess the mutual orthogonality of the PylRS/^{Pyl}tRNA pairs in the same cell and to enable incorporation of distinct ncAAs into a single polypeptide. We created these plasmids by transferring the relevant *Ma*PylRS/*Ma*^{Pyl}tRNA cassette into pKW *Mm*PylRS/*Mm*^{Pyl}tRNA vectors by Gibson cloning. The pRSF vector containing ribo-Q1 rRNA with o-GST-CaM(1TAG, 40AGGA) was previously reported.

Measuring the activity and specificity of PylRS/^{Pyl}tRNA$_{CUA}$ pairs

To measure the activity and specificity of cognate and non-cognate PylRS/^{Pyl}tRNA combinations we transformed pKW PylRS/^{Pyl}tRNA plasmids into competent *E. coli* DH10B cells bearing pBAD GFP(150TAG)His_6. We recovered the transformed cells for 1 h at 37°C in 0.5 ml super optimal broth (SOB) medium. The transformation was used to inoculate 6 ml 2XTY-ST (2XTY medium with 37.5 μg ml^{-1} spectinomycin and 12.5 μg ml^{-1} tetracycline) and incubated overnight (37°C, 16h, 220 rpm). The overnight culture was used to inoculate 190 μl 2XTY-STA (2XTY medium with 37.5 μg ml^{-1} spectinomycin, 12.5 μg ml^{-1} tetracycline and 0.2% arabinose) in 96-well microtitre plate format, supplemented with or without the indicated ncAA (8 mM BocK (**1**), 8 mM BCNK (**2**), 3 mM CbzK (**3**), or 2 mM CypK (**4**)). OD_{600} and GFP fluorescence (λ_{ex} 485 nm, λ_{em} 520 nm) measurements were recorded over a 20 h time course at 10 min intervals using a Tecan Infinite M200 Pro at 37°C, 220 rpm. At the maximum OD_{600} for each sample the GFP fluorescence measurement was normalised by the OD_{600} measurement to give a value for the amber suppression activity in normalised fluorescence units.

To express GFP incorporating BocK (**1**) for mass spectrometry we transformed pKW PylRS/^{Pyl}tRNA plasmids in which PylRS contains no C-terminally appended sequence into competent *E. coli* DH10B cells bearing pBAD GFP(150TAG)His_6. We recovered the transformed cells for 1 h at 37°C in 0.5 ml SOB medium. The

transformation was used to inoculate 2XTY-STA (2XTY medium with 37.5 μg ml^{-1} spectinomycin, 12.5 μg ml^{-1} tetracycline and 0.2% arabinose) supplemented with 8 mM BocK (**1**) and incubated overnight (37°C, 16 h, 220 rpm). 50 ml culture was pelleted, washed with 800 μl PBS and flash frozen. The cell pellets were resuspended in 1 ml of lysis buffer (1X BugBuster Protein Extraction Reagent supplemented with 1X cOmplete protease inhibitor cocktail, 1 mg ml^{-1} lysozyme and 1 mg ml^{-1} DNase I) and lysed for 1 h at 25°C, 1,400 rpm. The lysate was clarified by centrifugation (21,000 g, 30 min, 4°C) and imidazole added to a final concentration of 10 mM. GFP was purified by its C-terminal His_6 tag using 100 μl Ni-NTA agarose beads and left to bind for 1 h at 4°C. The beads were washed four times with 800 μl PBS supplemented with 10 mM imidazole and eluted in three volumes of 60 μl PBS supplemented with 250 mM imidazole. The three eluting fractions were pooled together to reach a combined volume of 180 μl. The imidazole was removed using 7K MWCO Zeba spin desalting columns and the samples were then concentrated using an Eppendorf Concentrator Plus and analysed by electrospray ionisation mass spectrometry.

To express GFP incorporating CbzK (**2**) or CypK (**3**) we transformed pKW-DUAL PylRS/^{Pyl}tRNA plasmids into competent *E. coli* DH10B cells bearing pBAD GFP(150TAG)His_6. We recovered the transformed cells for 1 h at 37°C in 0.5 ml SOB medium. The transformation was used to inoculate 2XTY-STA (2XTY medium with 37.5 μg ml^{-1} spectinomycin, 12.5 μg ml^{-1} tetracycline and 0.2% arabinose) supplemented with or without the indicated ncAAs (3 mM CbzK (**3**), or 2 mM CypK (**4**)) and incubated overnight (37°C, 16 h, 220 rpm). 50 ml culture was pelleted, washed with 800 μl PBS and flash frozen. The cell pellets were resuspended in 1 ml of lysis buffer (1X BugBuster Protein Extraction Reagent supplemented with 1X cOmplete protease inhibitor cocktail, 1 mg ml^{-1} lysozyme and 1 mg ml^{-1} DNase I) and lysed for 1 h at 25°C, 1,400 rpm. The lysate was clarified by centrifugation (21,000 g, 30 min, 4°C). PylRS was removed by its C-terminal Strep-tag II using 100 μl StrepTactin sepharose beads and left to bind for 1 h at 4°C. Imidazole was added to the recovered supernatant to a final concentration of 10 mM. GFP was purified by its C-terminal His_6 tag using 100 μl Ni-NTA agarose beads and left to bind for 1 h at 4°C. The beads were washed four times with 800 μl PBS supplemented with 10 mM imidazole and eluted in three volumes of 60 μl PBS supplemented with 250 mM imidazole. The three eluting fractions were pooled together to reach a combined volume of 180 μl. Samples were both analysed by electrospray ionisation mass

spectrometry and analysed on 4-12% Bis-Tris SDS-PAGE gels, visualised with InstantBlue Coomassie stain and imaged using a ChemiDoc XRS+.

Discovering orthogonal *Ma*^{Pyl}tRNA variants

To discover *Ma*^{Pyl}tRNA variants orthogonal to *Mm*PylRS, we transformed the *Ma*^{Pyl}tRNA libraries into competent *E. coli* DH10B cells bearing pBAD *Ma*PylRS CAT(111TAG) GFP(150TAG)His_6. We recovered the transformed cells for 1 h at 37°C in 0.5 ml SOB medium supplemented with 8 mM BocK (**1**). The transformation was plated on LB agar containing 37.5 μg ml^{-1} spectinomycin, 12.5 μg ml^{-1} tetracycline and 100 μg ml^{-1} chloramphenicol. The plates were incubated at 37°C for 40 h. After incubation, colonies on the plates were washed off and collected in 2XTY buffer and the plasmids were extracted by DNA midiprep. To remove the pBAD *Ma*PylRS CAT(111TAG) GFP(150TAG)His_6 plasmid, the extracted DNA was digested with both NcoI restriction endonuclease and T5 exonuclease and re-purified using a PCR purification column. The remaining pKW plasmids were transformed into competent *E. coli* DH10B cells bearing pBAD *Mm*PylRS CAT(111TAG) GFP(150TAG)His_6. The transformed cells were recovered for 1 h at 37°C in 0.5 ml SOB medium. The transformation was plated on LB agar containing 37.5 μg ml^{-1} spectinomycin and 12.5 μg ml^{-1} tetracycline. The plates were incubated at 37°C for 20 h. For each library, 376 colonies were picked from the plates using a QPix 420 Colony Picking System and inoculated into 190 μl 2XTY-STA in 96-well microtitre plate format supplemented with 8 mM BocK (**1**). The plates were incubated at 37°C, 220 rpm and OD_{600} and GFP fluorescence (λ_{ex} 485 nm, λ_{em} 520 nm) measurements were recorded after 20 h using a SpectraMax i3. Cells from 96 wells with the lowest GFP/OD_{600} ratios were used to inoculate 2XTY medium with 75 μg ml^{-1} spectinomycin, and the pKW plasmids containing *Ma*^{Pyl}tRNA variants were extracted by DNA miniprep and then sequenced. Each hit corresponding to a distinct *Ma*^{Pyl}tRNA sequence was cloned into a pKW *Ma*PylRS vector and a pKW *Mm*PylRS vector and re-phenotyped with pBAD GFP(150TAG)His_6.

Incorporating distinct ncAAs

To express GST-CaM proteins incorporating two distinct ncAAs we co-transformed competent *E. coli* DH10B cells with pKW-DUAL *Mm*PylRS/*Mm*^{Pyl}$tRNA_{UCCU}$ *Ma*PylRS-MutRS1/*Ma*^{Pyl}$tRNA(6)_{CUA}$ and pRSF ribo-Q1 o-GST-CaM(1TAG,

40AGGA). We recovered the transformed cells for 1 h at 37°C in 0.5 ml SOB medium. The transformation was used to inoculate 6 ml LB-KS (LB medium with 25 μg ml^{-1} kanamycin and 37.5 μg ml^{-1} spectinomycin) and incubated overnight (37°C, 16 h, 220 rpm). The overnight culture was diluted to OD_{600} 0.1 in LB-KS and incubated at 37°C, 220 rpm. At OD_{600} 0.5, IPTG was added to a final concentration of 1 mM together with or without the indicated ncAAs (3 mM CbzK (**3**) or 2 mM CypK (**4**)). After 4 h incubation at 37°C, 220 rpm, 50 ml culture was pelleted, washed with 800 μl PBS and flash frozen. The cell pellets were resuspended in 1 ml of lysis buffer (1X BugBuster Protein Extraction Reagent supplemented with 1X cOmplete protease inhibitor cocktail, 1 mg ml^{-1} lysozyme and 1 mg ml^{-1} DNase I) and lysed for 1 h at 25°C, 1,400 rpm. The lysate was clarified by centrifugation (21,000 g, 30 min, 4°C). GST-containing proteins from the lysate supernatant were left to bind to 70 μl glutathione sepharose beads for 1 h at 4°C. The beads were washed four times with 800 μl PBS before eluting in 180 μl 1X NuPAGE LDS sample buffer supplemented with 100 mM DTT at 95°C for 5 min. Samples were analysed on 4-12% Bis-Tris SDS-PAGE gels, visualised with InstantBlue Coomassie stain and imaged using a ChemiDoc XRS+.

To express GFPHis$_6$Strep proteins incorporating two distinct ncAAs we transformed competent *E. coli* DH10B cells with pKW-DUAL *Mm*PylRS/*Mm*^{Pyl}tRNA$_{UCCU}$ *Ma*PylRS-MutRS1/*Ma*^{Pyl}tRNA(6)$_{CUA}$ and pRSF ribo-Q1 and po-GFP(40AGGA, 150TAG)His$_6$Strep. We recovered the transformed cells for 1 h at 37°C in 0.5 ml SOB medium. The transformation was used to inoculate 20 ml 2XTY-KST (2XTY medium with 20 μg ml^{-1} kanamycin, 30 μg ml^{-1} spectinomycin and 10 μg ml^{-1} tetracycline) and incubated overnight (37°C, 16 h, 220 rpm). The overnight culture was diluted 1:50 in LB-KST and IPTG was added to a final concentration of 1 mM together with or without the indicated ncAAs (3 mM CbzK (**3**) or 2 mM CypK (**4**)). After 16 h incubation at 37°C, 220 rpm, 50 ml culture was lysed and used to purify GFPHis$_6$Strep as described above but instead using 70 μl StrepTactin sepharose beads and eluting in three volumes of 60 μl PBS supplemented with 2.50 mM desthiobiotin. The three eluted fractions were pooled together into a combined volume of 180 μl and analysed by electrospray ionisation mass spectrometry.

Electrospray ionization mass spectrometry

Mass spectra for protein samples were acquired using an Agilent 1200 LC-MS with a 6130 Quadrupole spectrometer. The solvent system used for liquid chromatography was 0.2% formic acid in water as buffer A and 0.2% formic acid in acetonitrile as buffer B. Samples were injected into a Phenomenex Jupiter C4 column (150 x 2 mm, 5 μm) and subsequently into the mass spectrometer. Spectra were acquired in the positive mode and analyzed using OpenLAB CDS Chemstation software. The de-convolution program provided in the software was used to obtain the mass spectra. The theoretical molecular weights of proteins with ncAAs was calculated by first computing the theoretical molecular weight of wild-type protein using an online tool (http://web.expasy.org/protparam/) and then manually correcting for the theoretical molecular weight of ncAAs.

References

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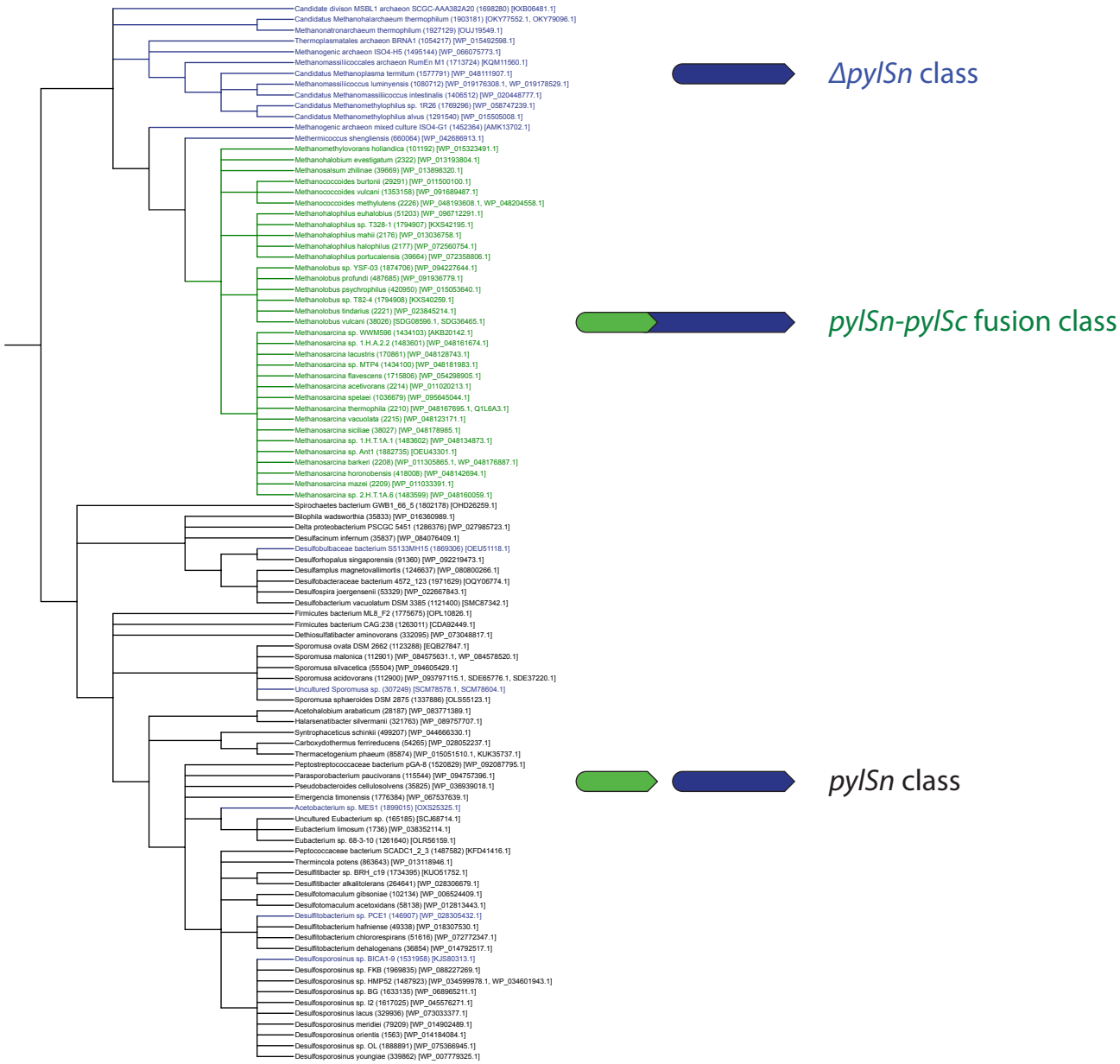
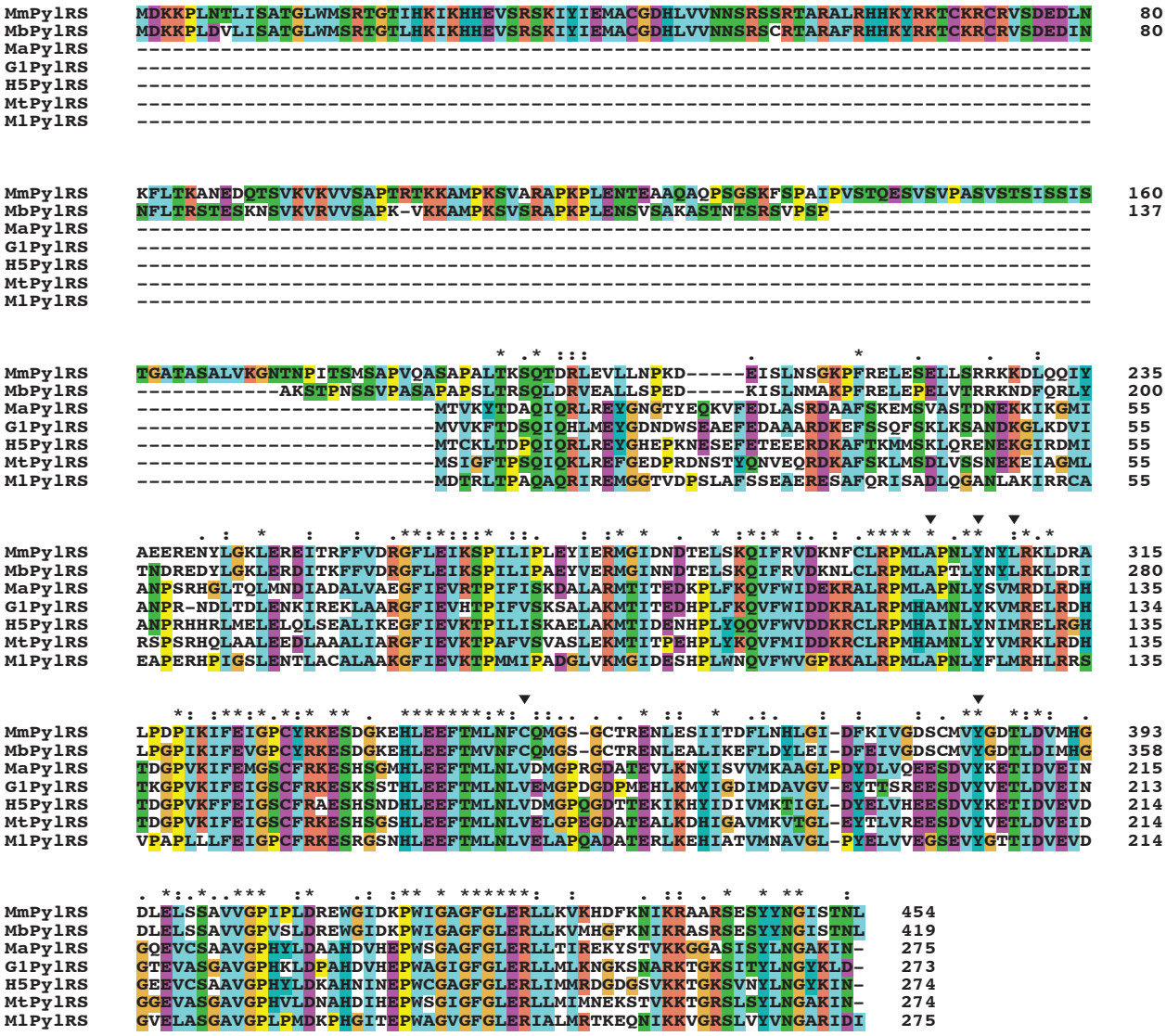
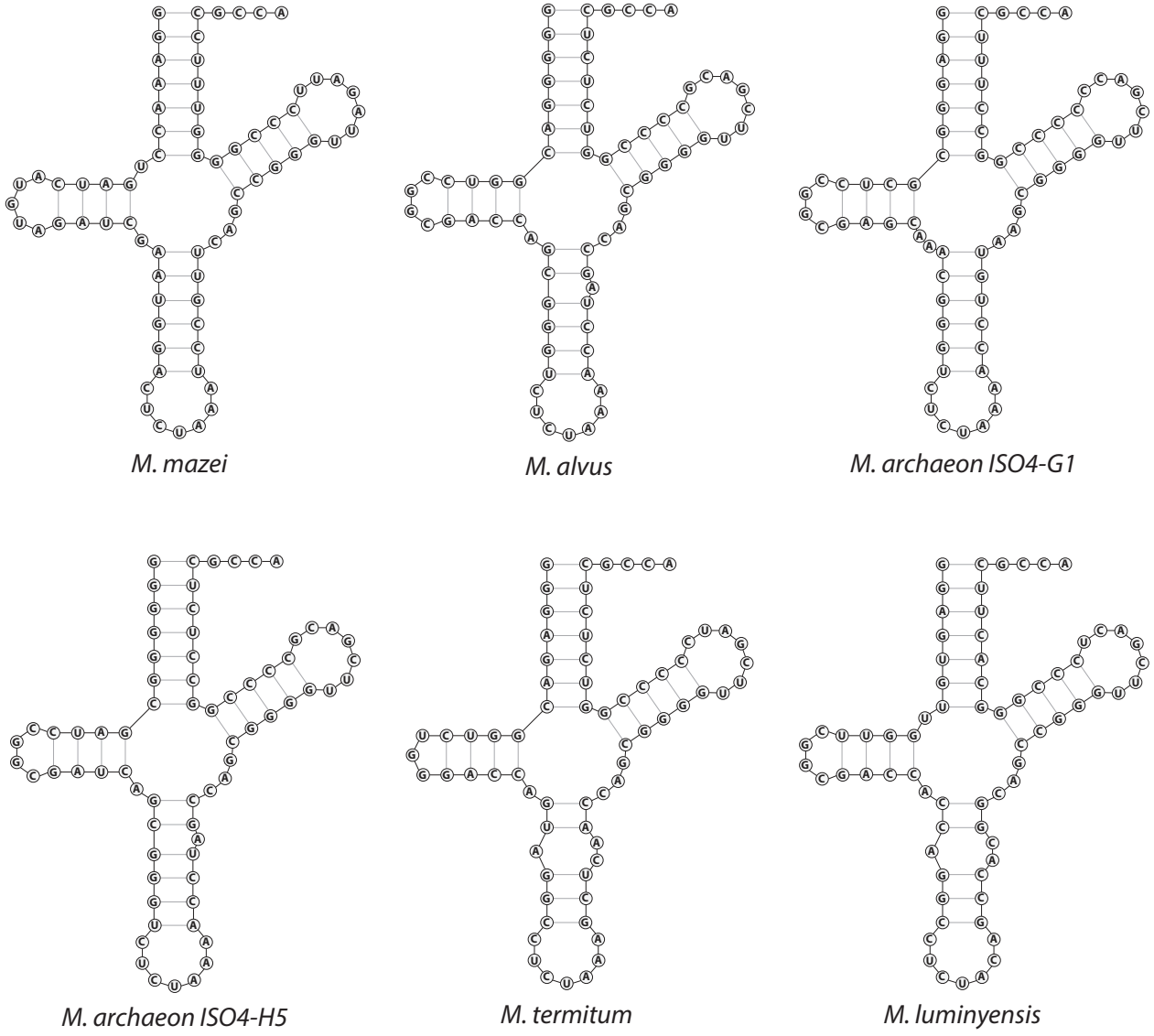


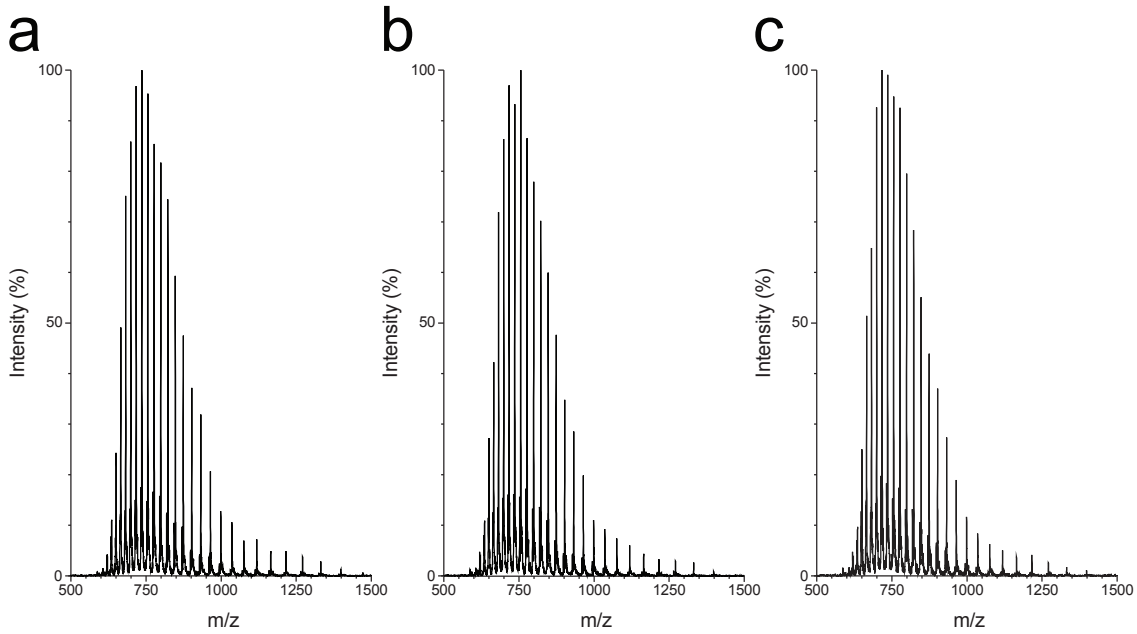
Supplementary Figure 1. Cladogram derived from the phylogenetic analysis of identified PylRS-containing species using phyloT, visualised using iTOL. PylRS sequences are classified and coloured according to the presence of a domain homologous to *D. hafniense* PylSn either within the same gene (*pylSn-pylSc* fusion class, green), present within a separate gene (*pylSn* class, black), or absent entirely (*ΔpylSn* class, blue). Labels are of the form: Species name (Taxonomy ID) [Protein sequence accession number(s)].



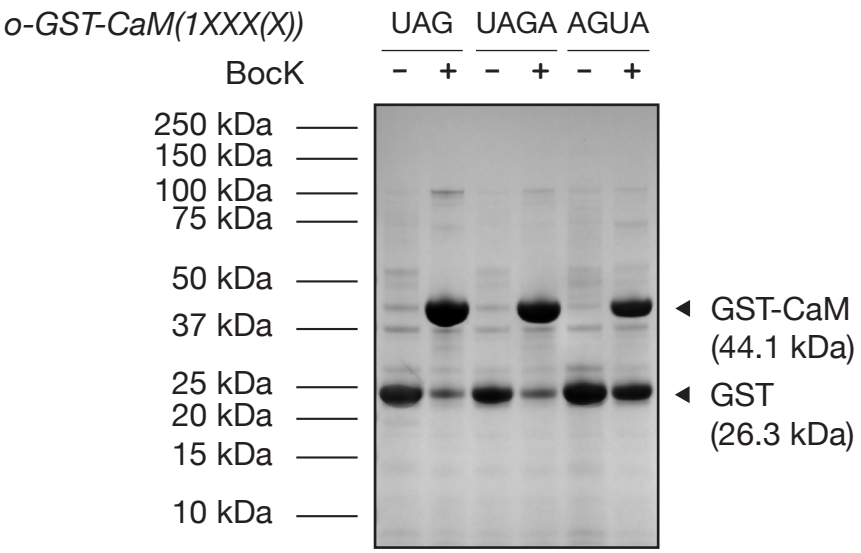
Supplementary Figure 2. Alignment of the indicated PylRS sequences using Clustal Omega, visualised using Clustal X. Active site residues referenced in the main text are indicated by triangles.



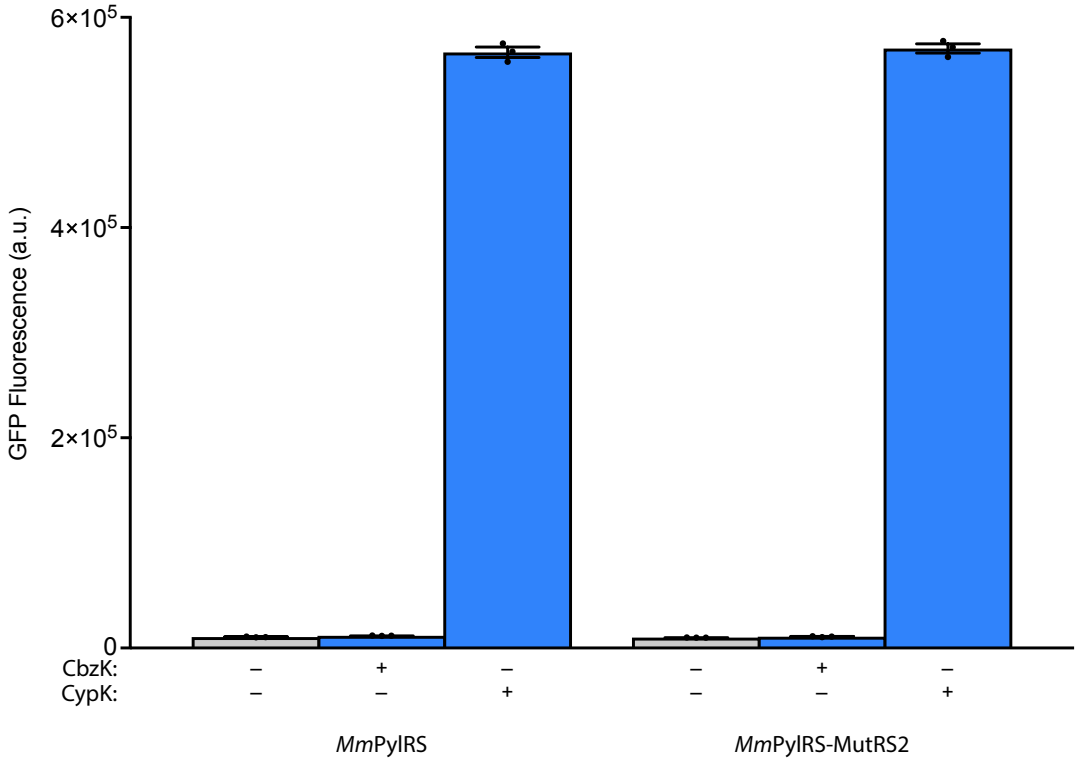
Supplementary Figure 3. ^{Pyl}tRNA sequences from the indicated genomes, and their predicted secondary structures.



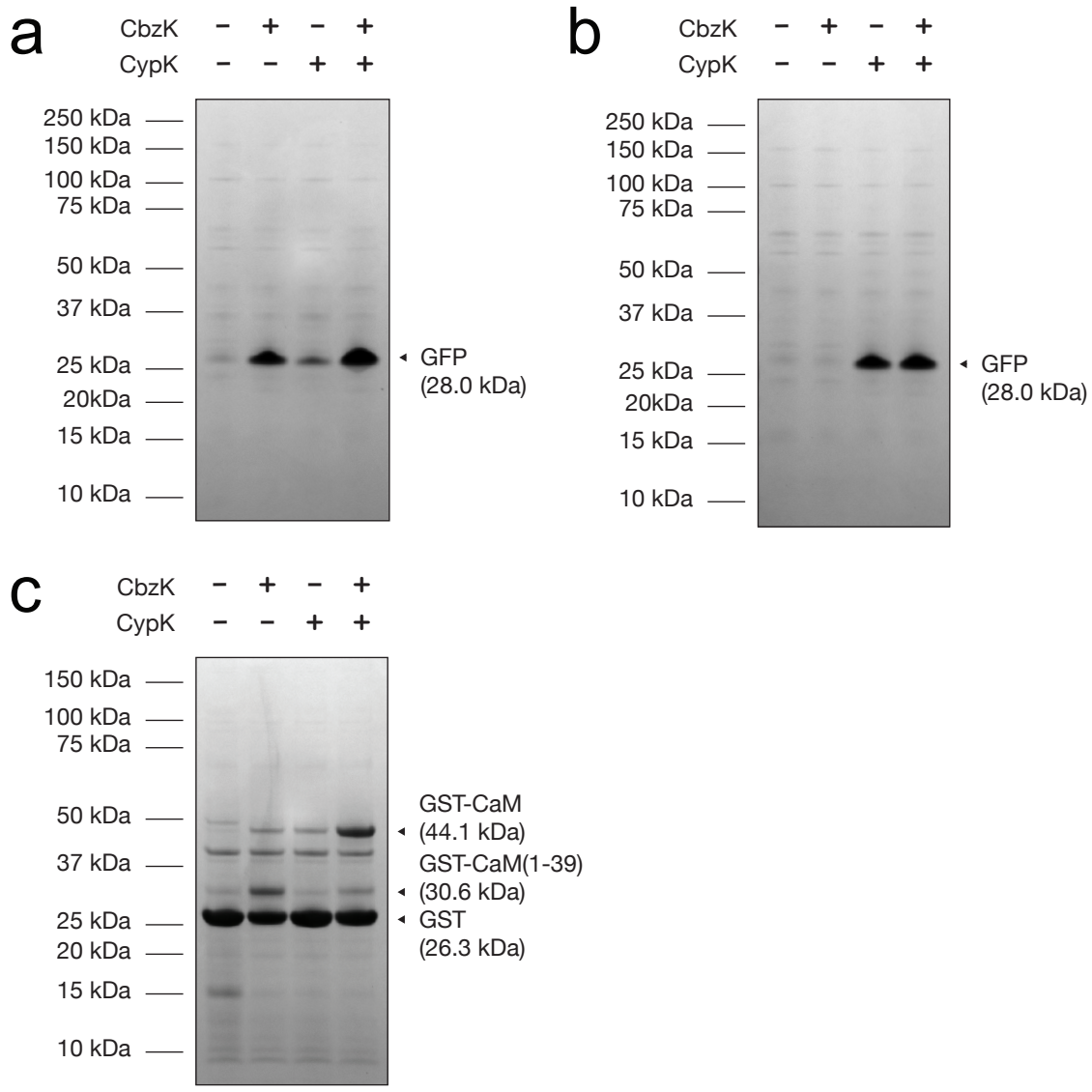
Supplementary Figure 4. Raw ESI-MS spectra for the data shown in **Fig. 2 c,d,e** are shown in panels **a**,**b**,**c** respectively. ESI-MS experiments in (**a**-**c**) were each performed once.



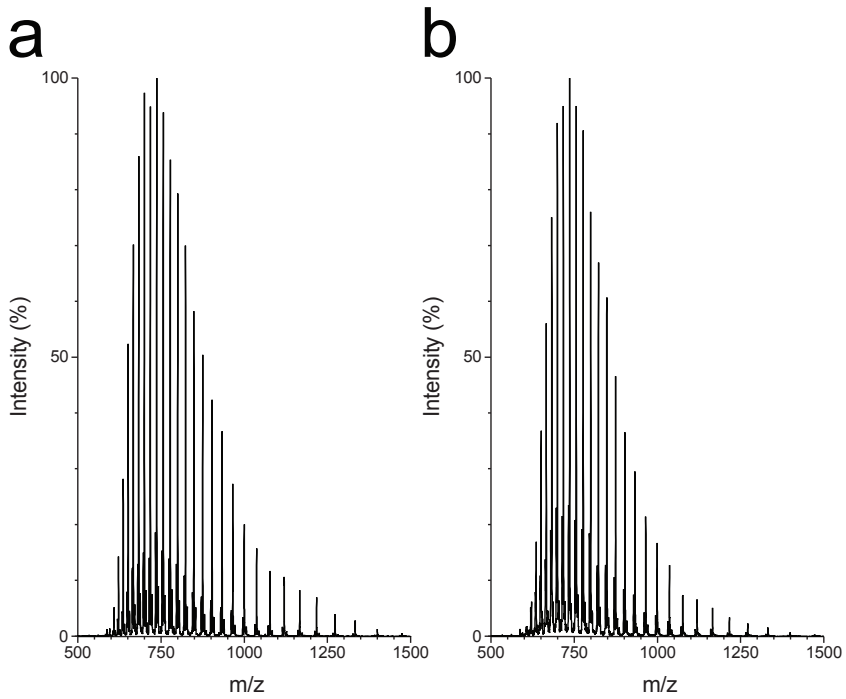
Supplementary Figure 5. *Ma*PylRS/*Ma*^{Pyl}tRNA decoding with altered anticodons. *o-GST-CaM(1UAG)*, *o-GST-CaM(1UAGA)* and *o-GST-CaM(1AGUA)* genes were decoded on ribo-Q by *Ma*PylRS/*Ma*^{Pyl}tRNA$_{CUA}$, *Ma*PylRS/*Ma*^{Pyl}tRNA$_{UCUA}$, *Ma*PylRS/*Ma*^{Pyl}tRNA$_{UACU}$ respectively. Two independent experiments were performed with similar results.



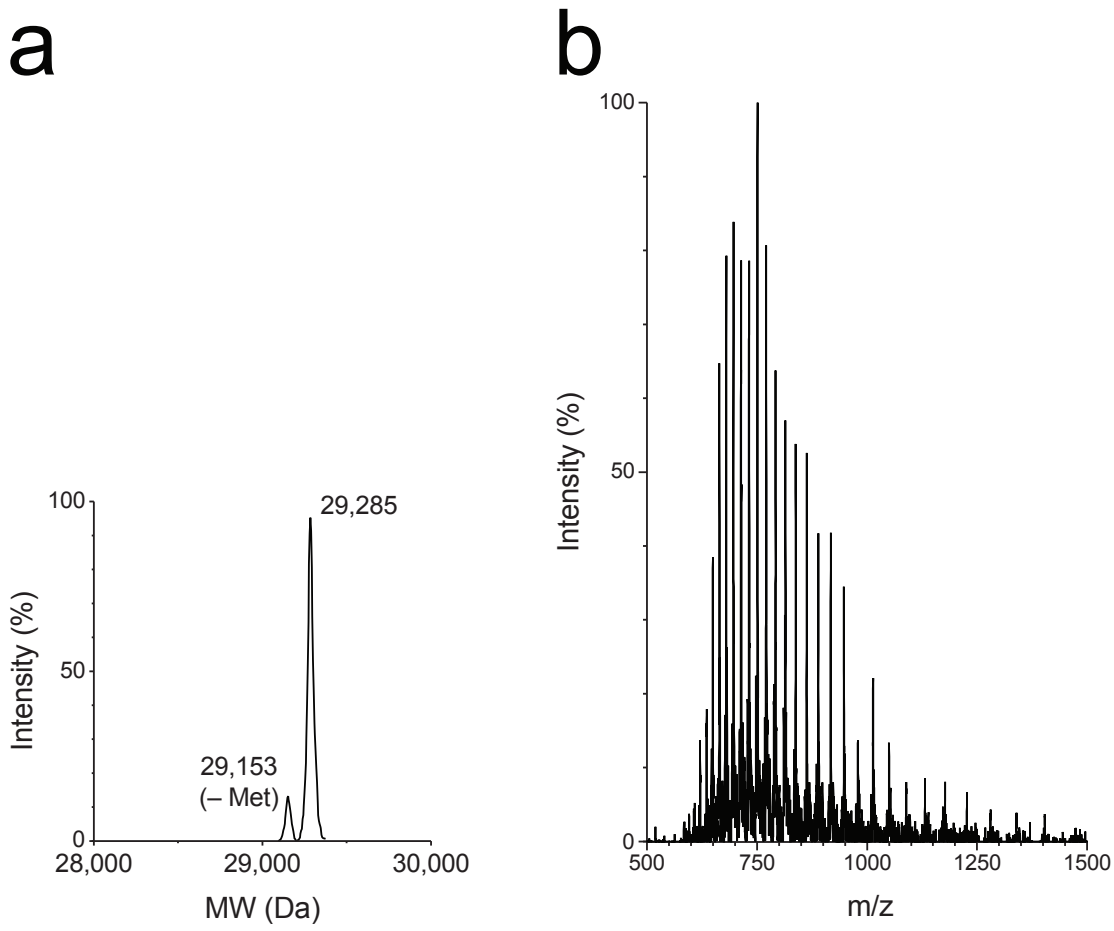
Supplementary Figure 6. Engineering the active site of *Mm*PylRS for selective ncAA incorporation. *In vivo* amber suppression activity assay using *E. coli* DH10B bearing pBAD GFP(150TAG)His_6 and the corresponding pKW PylRS/$^{Pyl}tRNA_{CUA}$ plasmid in the presence and absence of CbzK (**3**) and CypK (**4**) demonstrates the selective incorporation of CypK by *Mm*PylRS-MutRS2 is comparable to that with *Mm*PylRS. Each data point shows the mean of three technical replicates that form one biological replicate; the error bars show the mean and SEM of three independent biological replicates.



Supplementary Figure 7. Encoding distinct ncAAs using mutually orthogonal PylRS/^{Pyl}tRNA pairs. Full gels for **Fig. 6 a, c, e**. are shown in panels **a**, **b** and **c** respectively. Densitometry quantification of the four lanes in panel **a** gives the ratio of relative band intensities as 1 : 17 : 6 : 22. For each panel, two independent experiments were performed with similar results.



Supplementary Figure 8. Raw ESI-MS spectra for the data shown in **Fig 6b, d** are shown in panels **a** and **b** respectively. ESI-MS experiments in panels **a** and **b** were each performed once.



Supplementary Figure 9. GFPHis$_6$Strep purified from *E. coli* DH10B bearing pBAD GFP(40AGGA,150TAG)His$_6$Strep and pKW-DUAL *Mm*PylRS/^{Pyl}tRNA$_{UCCU}$ *Ma*PylRS-MutRS1/^{Pyl}tRNA(6)$_{CUA}$ in the presence of both CbzK (**3**) and CypK (**4**) leads to selective incorporation of distinct ncAAs at distinct codons. Electrospray ionisation mass spectrometry shows a single peak corresponding to CbzK and CypK incorporation (predicted mass 29,285 Da, observed mass 29,285 Da). Raw ESI-MS spectrum before de-convolution is shown in panel **b**. ESI-MS experiments in panels **a** and **b** were each performed once.