SUPPLEMENTARY INFORMATION

Specificity determinants for the two tRNA substrates of the cyclodipeptide synthase AlbC from *Streptomyces noursei*

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tRNA^{Phe}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC CCG GAT AGC TCA GTC GGT AGA GCA-3′
	Matrix	5′-CTC GGA ATC GAA CCA AGG ACA CGG GGA TTT TCA ATC CCC TGC TCT ACC GAC TGA GCT A-3′
	Reverse	5′-TG GTG CCC GGA CTC GGA ATC GAA CCA AGG-3′
tRNA^{LeuCAA}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC CGA AGT GGC GAA ATC GGT AGA CGC-3′
	Matrix	5′-GCC GGA CTC GAA CCG GCA CGT ATT TCT ACG GTT GAT TTT GAA TCA ACT GCG TCT ACC GAT TTC GCC A-3′
	Reverse	5′-TG GTG CCG AAG GCC GGA CTC GAA CCG GCA-3′
tRNA^{LeuTAG}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC GGG AGT GGC GAA ATT GGT AGA CGC-3′
	Matrix	5′-GCG AGA CTT GAA CTC GCA CAC CTT GCG GCG CCA GAA CCT AAA TCT GGT GCG TCT ACC AAT TTC GCC A-3′
	Reverse	5′-TG GTG CGG GAG GCG AGA CTT GAA CTC GCA-3′
tRNA^{LeuGAG}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC CGA GGT GGT GGA ATT GGT AGA CAC-3′
	Matrix	5′-AC GGG ACT TGAACC CGT AAG CCC TAT TGG GCA CTA CCA CCT CAA GGT AGC GTG TCT ACC AAT TCC ACC A-3′
	Reverse	5′-T GGT ACC GAG GAC GGG ACT TGA ACC CGT AAG-3′
tRNA^{LeuTAA}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC CCG GAT GGT GGA ATC GGT AGA CAC-3′
	Matrix	5′-GC GGG ACT TGA ACC CGC ACA GCG CGA ACG CCG AGG GAT TTT AAA TCC CTT GTG TCT ACC GAT TCC ACC A-3′
	Reverse	5′-T GGT ACC CGG AGC GGG ACT TGA ACC CGC ACA-3′
tRNA^{LeuCAG}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC GAA GGT GGC GGA ATT GGT AGA CGC-3′
	Matrix	5′-GG GGG ACT TGA ACC CCC ACG TCC GTA AGA ACA CTA ACA CCT GAA GCT AGC GCG TCT ACC AAT TCC GCC A-3′
	Reverse	5′-T GGT GCG AGG GGG GGG ACT TGA ACC CCC ACG-3′
tRNA^{LeuCAG*}	Forward	5′-TT**T AAT ACG ACT CAC TAT A**GC GAA GGT GGC GGA ATT GGT AGA CGC-3′
	Matrix	5′-GG GGG ACT TGA ACC CCC ACG TCC GTA AG**G** ACA CTA ACA CCT GAA GCT AGC GCG TCT ACC AAT TCC GCC A-3′
	Reverse	5′-T GGT GCG AGG GGG GGG ACT TGA ACC CCC ACG-3′
G1C72 tRNA^{LeuTAA}	Forward	5′-**T**TT **AAT ACG ACT CAC TAT A**GC CCG GAT GGT GGA ATC GGT AGA CAC-3′
	Matrix	5′-GC GGG ACT TGA ACC CGC ACA GCG CGA ACG CCG AGG GAT TTT AAA TCC CTT GTG TCT ACC GAT TCC ACC A-3′
	Reverse	5′-T GGT **G**CC CGG AGC GGG ACT TGA ACC CGC ACA-3′
A1U72 tRNA^{LeuTAA}	Forward	5′-TT**T AAT ACG ACT CAC TAT AA**C CCG GAT GGT GGA ATC GGT AGA CAC-3′
	Matrix	5′-GC GGG ACT TGA ACC CGC ACA GCG CGA ACG CCG AGG GAT TTT AAA TCC CTT GTG TCT ACC GAT TCC ACC A-3′
	Reverse	5′-T GGT ACC CGG AGC GGG ACT TGA ACC CGC ACA-3′

Table S1.

Oligonucleotides used for *in vitro* transcription of tRNA^{Phe} and tRNA^{Leu} isoacceptors.

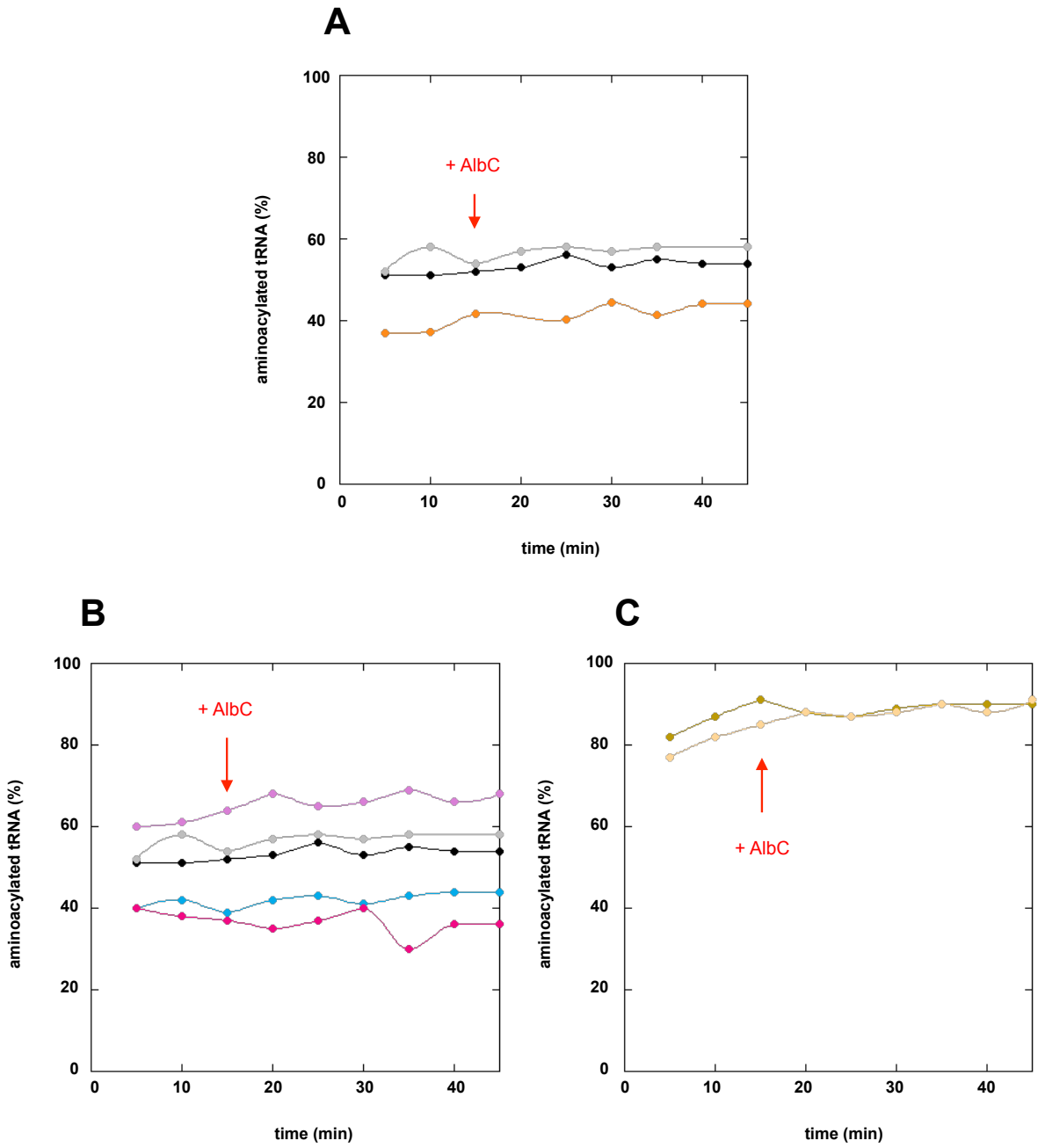


Figure S1.

Control of the stability of aa-tRNA concentrations throughout the CDPS enzymatic assays. The concentrations of aminoacylated tRNAs were determined as described in 'Materials and Methods'. All assays contained 5 µM in both $tRNA^{Phe}$ and $tRNA^{Leu}$, both PheRS and LeuRS (1 µM) and 50 µM Phe and Leu (either Phe/^{14}C-Leu (for $tRNA^{Leu}$ control) or ^{14}C-Phe/Leu (for $tRNA^{Phe}$ control)). The reactions were initiated by the addition of PheRS and LeuRS. AlbC (50 nM) was added after 15 min. (**A**) Acylation of wild-type tRNAs: $tRNA^{LeuCAG}$ (black), $tRNA^{LeuTAA}$ (gray), and $tRNA^{Phe}$ obtained by TIV (orange). (**B**) Acylation of $tRNA^{LeuCAG}$ (black), $tRNA^{LeuTAA}$ (gray), G^{1}-C^{72}-RNA^{LeuTAA} (blue), A^{1}-U^{72} $tRNA^{LeuTAA}$ (purple) and C^{1}-G^{72} $tRNA^{LeuCAG}$ (magenta). (**C**) Acylation of wild-type $tRNA^{Phe}$ purified from *E. coli* (brown) and C^{1}-G^{72} $tRNA^{Phe}$ (sand).

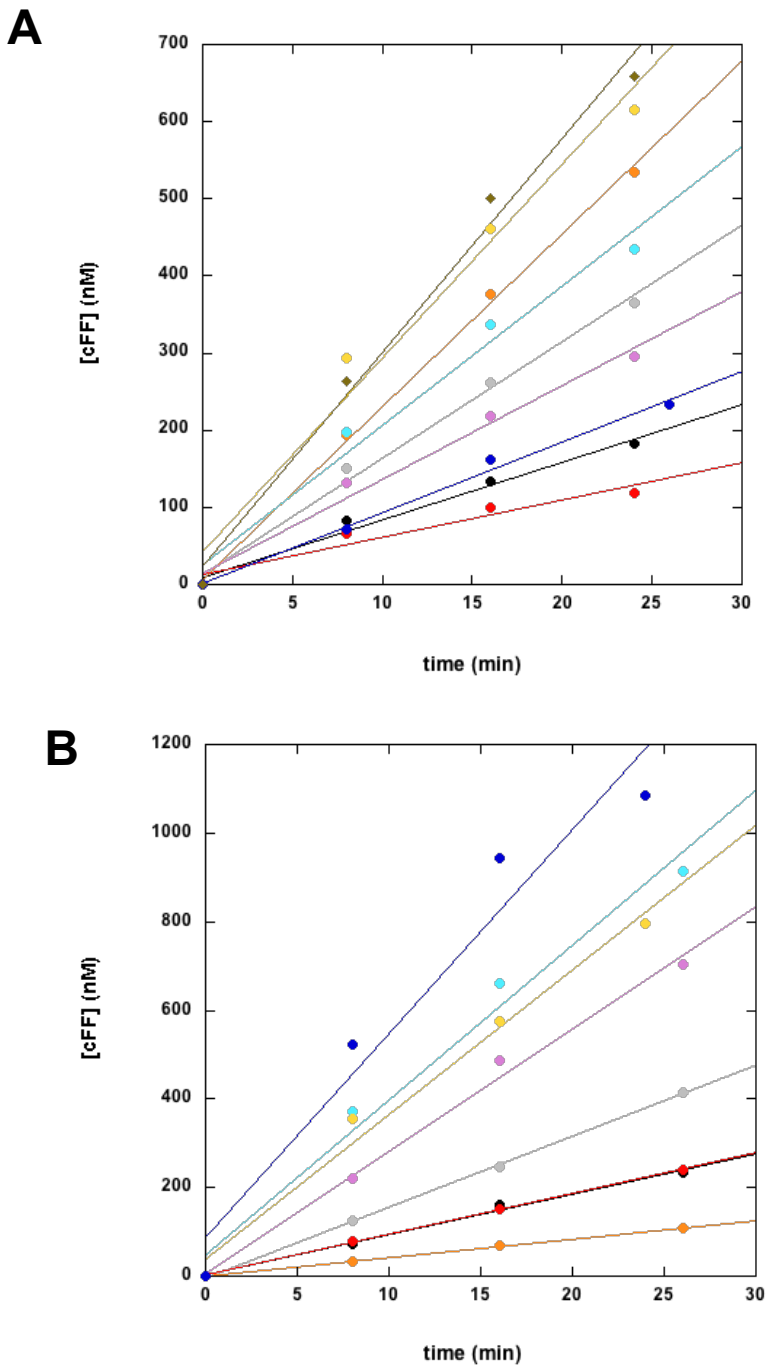


Figure S2.

Time-course synthesis of cFF from various concentrations of Phe-tRNA^{Phe} either obtained by *in vitro* transcription (A) or purified from *E. coli* (B). Enzymatic measurements were performed as described in 'Materials and Methods' with 50 nM AlbC. The points reported belong to three independent sets of experiments. (**A**) Kinetics of the formation of cFF were determined for the following concentrations of [Phe-tRNA^{Phe}]: set 1, 0.139 µM (black), 0.278 µM (gray), and 0.385 µM (orange); set 2, 0.094 µM (red), 0.188 µM (violet), 0.282 µM (light blue), and 0.376 µM (yellow); set 3, 0.24 µM (dark blue) and 0.47 µM (brown). (**B**) Kinetics of the formation of cFF were determined for the following concentrations of [Phe-tRNA^{Phe}]: set 1, 0.24 µM (black) and 0.4 µM (gray); set 2, 0.14 µM (orange), 0.256 µM (red), 0.513 µM (violet), and 0.77 µM (light blue); set 3, 0.785 µM (yellow) and 0.985 µM (dark blue).

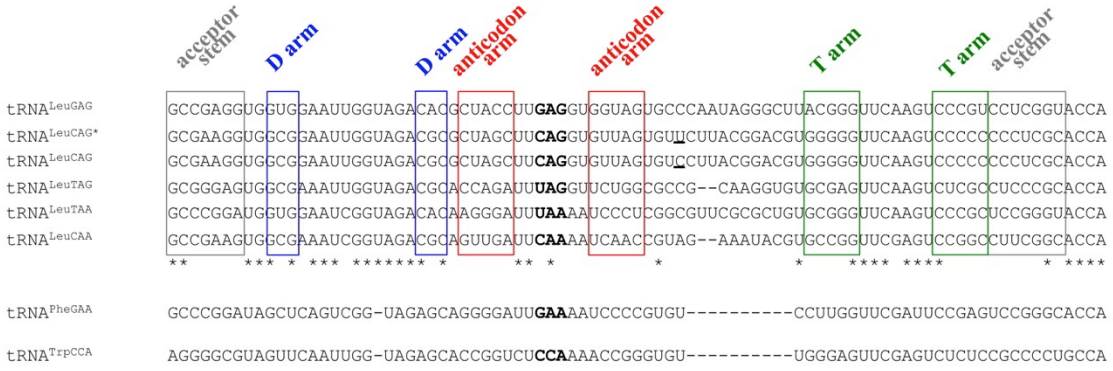
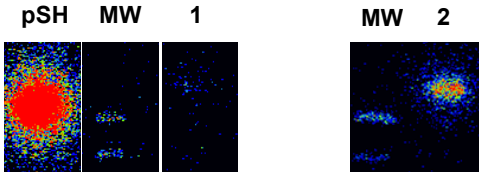


Figure S3

Sequences of the different tRNAs of *Escherichia coli* (K12) mentioned in this work. $tRNA^{LeuCAG}$ and $tRNA^{LeuCAG*}$ differ by a single nucleotide, underlined in black. Base pairing belonging to acceptor stem are boxed in gray, the D arm is boxed in blue, the anticodon arm is boxed in red, the T arm is boxed in green, and the anticodon is shown in bold black.



pSH: pSHaeC06 + Leu-tRNA^{LeuCAG}
1: AlbC + C^{1}-G^{72} Leu-tRNA^{LeuTAA}
2: AlbC + Phe-tRNA^{Phe}

Figure S4

Covalent labelling of AlbC by [^{14}C]Leu transferred from [^{14}C]G^{1}-C^{72} Leu-tRNA^{LeuTAA}. pSHaeC06 (pSH) was used as positive control for the formation of leucyl-enzyme and S37A was used as a negative control in this experiment (not shown). The formation of the phenylalanyl-enzyme for AlbC is reported for comparison. Enzymes were incubated with labelled aa-tRNA, as described in 'Materials and Methods', separated on SDS–PAGE, then transferred onto a PVDF membrane that was analysed with a radioimager.

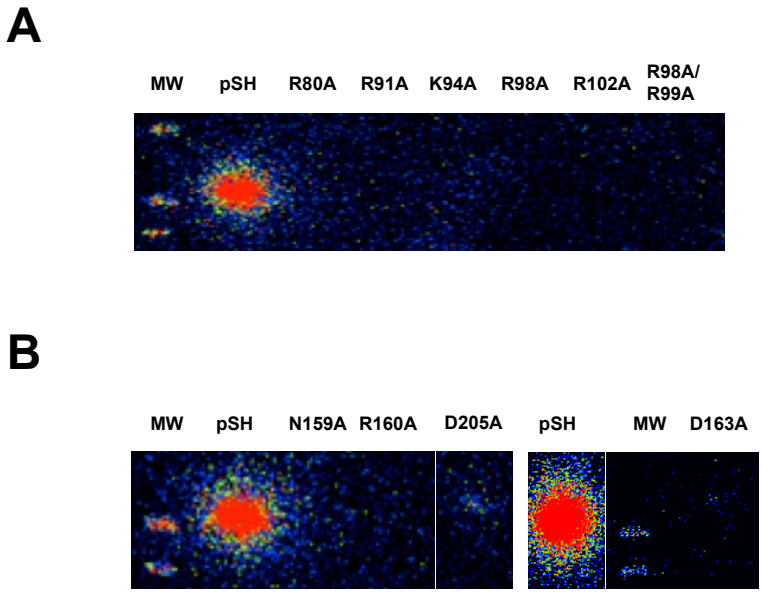


Figure S5

Covalent labelling of AlbC variants by [^{14}C]Leu transferred from [^{14}C]Leu-tRNA^{LeuCAG}. pSHaeC06 (pSH) was used as a positive control for the formation of leucyl-enzyme and S37A was used as a negative control in this experiment (not shown). Enzymes were incubated with labelled aa-tRNA, as described in 'Materials and Methods', separated on SDS–PAGE, then transferred onto a PVDF membrane that was analysed with a radioimager. (**A**) Variants of the basic patch. (**B**) Variants of the loop and variants of residues neighboring the active site. Proteins were analysed on different gels. pSH was loaded onto each gel as a standard.

1. *Nematostella vectensis* NEMVEscaffold_13 genomic scaffold,
Sequence ID: ref|NW_001834401.1|, 1838668 to 1838597

GACACCGTGGCGCAATGGTAGCGCGTCTGACT**CCA**GATCAGAAGGTTGCGTGTTCAAATCACGTCGGGGTCACCA

2. *Nematostella vectensis* NEMVEscaffold_13 genomic scaffold,
Sequence ID: ref|NW_001834401.1|, 1838521 to 1838450

GACTACGTCGCGCAATGGTAGCGCTTCTCACT**CCA**GATCAGAAGGTTGCGTTTCAAATCACGTCGTGGTCACCA

3. *Nematostella vectensis* NEMVEscaffold_2 genomic scaffold,
Sequence ID: ref|NW_001834412.1|, 2087614 to 2087685

GACTCCGTGGCGCAATGGTAGCGCGTCTGACT**CCA**GATCAGAAGGTTGCGTGTTCAAGTCACGTCGGGGTCACCA

4. *Nematostella vectensis* NEMVEscaffold_29 genomic scaffold,
Sequence ID: ref|NW_001834385.1|1459663 to 1459734

GACTCCGTGGCGCAATGGTAGCGCGTCTGACT**CCA**GATCAGAAGGTTGCGTGTTCAAGTCACGTCGGGGTCACCA

5. *Nematostella vectensis* NEMVEscaffold_34 genomic scaffold,
Sequence ID: ref|NW_001834380.1|1112145 to 1112216

TGATCAGTGGGACAATGGTAGCGATTCTCACT**CCA**GATCAGAAGGTTGCGTTTCAAATCACGTCGGGGTCACCA

tRNA^{Trp} (*Escherichia coli*)
AGGGGCGUAGUUCAAUUGGUAGAGCACCGGUCU**CCA**AAACCGGGUGUUGGGAGUUCGAGUCUCUCCGCCCCUGCCA

tRNA^{Trp} (*Drosophila_melanogaster*, chr2R.trna44-TrpCCA)
GACTCCGTGGCGCAACGGTAGCGCGTCTGACT**CCA**GATCAGAAGGTTGCGTGTTCAAATCACGTCGGGGTCACCA

Figure S6

Putative genomic tRNA^{Trp} sequences of *Nematostella vectensis*. Base pairing belonging to the acceptor stem is highlighted in yellow, the D arm is highlighted in blue, the anticodon arm is highlighted in red, the T arm is highlighted in green, and the anticodon is shown in bold black. TRNA^{Trp} of *Escherichia coli* and *Drosophila melanogaster* are shown for comparison.

tRNA^{Trp} sequences were identified from blastn search in the genome available for *Nematostella vectensis* starting from a tRNA^{Trp} sequence of *Danio rerio* (Zebrafish) (GGACCTTTTGCGCAATGGT.A.GCGCATCTGACTCCAGATCAGAAGGtTGTGTGTTCAAATCACGT CAGGTTCA Danio_rerio_chr7.trna144-TrpCCA). The sequences obtained were highly homologous with the sequences of tRNA^{Trp} from *Drosophila melanogaster* (http://gtrnadb.ucsc.edu/).