structural similarity. Viewed from the major groove side of the acceptor stem, a phosphate backbone outline of the tRNAs is shown (tan), and tRNA_{Pyl} is shown in purple. In space filling representation, a glutamyl-adenylate (a) and a pyrrolysyl-adenylate (b) highlight the class I and class II active site pockets, respectively. The small substrates are partially obscured by the protein backbone due to the need to show both aaRS families in the same orientation relative to the tRNA.

Figure 3. DhPylRS:tRNA_{Pyl} interface. (a) View showing the recognition of the CCA terminus by the motif-2 loop of DhPylRS. (b) View showing the recognition of the tRNA_{Pyl} acceptor helix by DhPylRS. (c,d) Views showing the recognition of the tRNA_{Pyl} minimal core by the core-binding surface of DhPylRS.

Figure 4. Comparison of PylRS structures: (a) DhPylRS:tRNA_{Pyl} complex, (b) DhPylRS apo, (c) MmPylRS:Pyl-AMP complex (PDB code 2zim) 6, (d) MmPylRS:ATP, Cyc complex (2q7g) 6, (e) MmPylRS:ATP analog complex (2q7e) 6, (f) MmPylRS apo (2e3c) 10. The structures are colored according to B-factor (indicating more [red] or less [blue] structurally dynamic regions), and four regions that show conformational changes in the different structures are labeled in panel a. Only one subunit of the dimer is shown for clarity.

Full Methods

Additional crystallographic details
To crystallize DhPylRS alone, a 0.8 μl aliquot of the protein solution was mixed with 0.8 μl of a crystallization solution and then equilibrated by hanging-drop vapor diffusion against 500 μl reservoir solution. Crystals appeared at 273°K within a few days in 45 mM sodium cacodylate buffer (pH 6.0) containing 45 mM (NH₄)₂SO₄, 18% PEG400, 10 mM Na-acetate (pH 4.6), and 0.2 mM Na-formate. To crystallize the DhPylRS:tRNA_{Pyl} complex, DhPylRS was mixed with tRNA_{Pyl} in a molar ratio of DhPylRS to tRNA_{Pyl} of 2:2.2, at a final protein concentration of 5 mg/ml. The 0.8 μl aliquot of the complex solution was mixed with 0.8 μl of a crystallization solution and was equilibrated by hanging-drop vapor diffusion against a 500 μl reservoir solution.
of 90 mM MES-NaOH buffer (pH6.0) containing 5.4% 2-propanol, 180 mM calcium acetate, 2% ethanol, 10 mM Tris-HCl (pH8.5).

For data collection under cryogenic conditions, the drop solution was slowly equilibrated against the same reservoir solution as the crystallization condition. The obtained crystals were briefly transferred to 1.2× reservoir solution containing 25% (w/v) PEG400, and were flash-cooled in a cryo-stream of nitrogen gas at 100 K. The data sets of the crystals were collected at station BL41XU of SPring-8 (Harima, Japan) and the NW12 Beam Line of PF-AR (Tsukuba, Japan). The data set of the DhPylRS:tRNA\textsuperscript{Pyl} was collected at 100 K in a nitrogen gas cryo-stream. The collected datasets were processed with the program HKL2000 (HKL Research).

The complex crystal structure was solved by molecular replacement, using the free-form DhPylIRS structure as a search model. Molecular replacement was done by\textsuperscript{29}; tRNA models were built using program O\textsuperscript{30}. The solution was refined by CNS\textsuperscript{31}, by rigid-body refinement, energy minimization, B-factor refinement, and simulated annealing methods. In the resultant 2m|Fo| - D|Fc| and m|Fo| - D|Fc| maps, we clearly found tRNA electron densities. Throughout the structural refinement, the WC base pair and the non-crystallographic symmetry restraints (weight=300 kcal mol\textsuperscript{-1} Å\textsuperscript{-2}) were applied for the tRNA molecules. The crystallographic, data-collection and refinement statistics are presented in Supplementary Tables 1 and 2.

**Aminoacylation assays**

These assays were performed as described\textsuperscript{32}. Aminoacylation reactions were carried out at 37°C in 100 mM Hepes-NaOH, pH 7.2, 25 mM MgCl\textsubscript{2}, 60 mM NaCl, 5 mM ATP, 1 mM DTT, 10 mM Cyc\textsuperscript{33} and 1 μM 3'-\textsuperscript{32}P]-labeled tRNA. PylRS enzyme is added in excess at a concentration of 1 μM. In the aminoacylation assay including PylSn, DhPylIRS and DhPylSn were added in equimolar ratio (each 1 μM). The Pyl analogue N-ε-cyclopentyloxy carbonyl-L-lysine (Cyc) is used for aminoacylation assays. Reactions were stopped by removing 2 μl reaction mix, adding it to 3 μl of 2.5 units/ml of nuclease P1 (American Bioanalytical, Natick, MA) in 0.1 M sodium citrate (pH 4.5)
at 25°C for 30 min, thus releasing \([\alpha-^{32}\text{P}]\text{AMP}\) from uncharged tRNA and Cyc\-[\alpha-^{32}\text{P}]\text{AMP}\) from charged tRNA. \([\alpha-^{32}\text{P}]\text{AMP}\) and Cyc\-[\alpha-^{32}\text{P}]\text{AMP}\) were separated by thin layer chromatography (TLC) on PEI cellulose plates (JT Baker, Phillipsburg, NJ) in 0.1 M sodium acetate and 5% acetic acid\(^{34}\). \([\alpha-^{32}\text{P}]\text{AMP}\) and Cyc\-[\alpha-^{32}\text{P}]\text{AMP}\) were exposed to phosphoimager plates (FujiFilms, Valhalla, NY), visualized using a Molecular Dynamics Storm 860 scanner (Amersham, Piscataway, NJ) and quantified using ImagQuant software. The data represent the results of at least three independent experiments.

**Complementation of tryptophan auxotroph**

To determine charging by DhPylRS \textit{in vivo}, \textit{E. coli} mutant strain FTP5822\(^{35,36}\) with an amber mutation in position 243 (Gln243 to UAG) of TrpA was co-transformed with pCBS-PylS and pTECH-PylT, respectively. \(\text{PylS}\) was cloned into the \(\text{Nde}\)I and \(\text{Kpn}\)I sites of pCBS (ampicillin marker), and \(\text{pylT}\) was cloned into the pTECH vector (chloramphenicol marker) as described\(^{33}\). In the complementation that included the \(D.\ hafniense\ pylSn\), the \(\text{pylSn}\) gene was fused to the \(\text{lpp}\) promoter by PCR and then cloned into the pCBS-\(\text{pylS}\) vector between \(\text{Sma}\)I and \(\text{Bgl}\)II.

The experiment was performed as described previously\(^{33}\). The PylS and PylT were under the control of constitutive promoters \(\text{trpS}\) and \(\text{lpp}\), respectively. The transformants were grown overnight at 37°C in LB liquid media. Subsequently, cells were washed thrice with M9 minimal medium and streaked onto M9 minimal agar plates supplemented with 19 amino acids (20 \(\mu\text{g/ml}\)) without tryptophan and 10 mM \(N-\varepsilon\)-cyclopentyloxy carbonyl L-lysine. Plates were incubated for 3-5 days at 37°C.

**Bioinformatic analysis**

For comparison of the PylRS:tRNA\textsuperscript{Pyl} interface with other class II aaRS:tRNA complexes, protein structures were downloaded from the Protein Databank\(^{34}\) and structurally aligned based on the protein backbone of the conserved catalytic core domain using Multiseq 2.0 in VMD 1.8.6\(^{37}\). The PDB codes for the aligned structures
are as follows for class I: 1f7v, 1gax, 1gr1 1n78, 1qf6, 1qu3, 1u0b, 1wz2, 2azx, 2ct8, 2cv2, 2dlc, 2dr2, 2re8 ; and class II aaRSs: 1asy, 1asz, 1c0a, 1efw, 1eiy, 1h4s, 1il1, 1il2,1qf6, 1ser, 1wle, 1wyd, 1x54, 1yfs, 2cj9, 2du3, 2du4, 2du7, 2i40, 2iy5, 2jl1, 2odr, 2zim. Multiseq was also used for sequence alignment, alignment editing and calculation of sequence and structure conservation. Protein sequences were downloaded from the National Center for Biotechnology Information and from the Joint Genome Institute Integrated Microbial Genomes with Microbiome Samples database\(^\text{38}\).

Molecular graphics pictures in Figures 1, 3 and Supplementary Figures 4 and 5 were prepared with the program CueMol (http://www.cuemol.org/). Figures 2 and 4 and Supplementary Figures 6 and 7 were prepared with the program VMD 1.8.6\(^\text{39}\).

**Supplemental References for methods**

29. Vagin, A. and Teplyakov, A. MOLREP: an automated program for molecular replacement. *J. Appl. Crystallogr.* 30, 1022-1025 (1997).

30. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* 47 (Pt 2), 110-119 (1991).

31. Brünger, A. T. *et al.* Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 54, 905-921 (1998).

32. Herring, S., Ambrogelly, A., Polycarpo, C. R., and Söll, D. Recognition of pyrrolysine tRNA by the *Desulfitobacterium hafniense* pyrrolysyl-tRNA synthetase. *Nucleic Acids Res.* 35, 1270-1278 (2007).

33. Polycarpo, C. R. *et al.* Pyrrolysine analogues as substrates for pyrrolysyl-tRNA synthetase. *FEBS Lett.* 580, 6695-6700 (2006).

34. Berman, H. M. *et al.* The Protein Data Bank. *Nucleic Acids Res.* 28, 235-242 (2000).

35. Yanofsky, C. and Horn, V. Tryptophan synthetase chain positions affected by mutations near the ends of the genetic map of *trpA* of *Escherichia coli*. *J. Biol. Chem.* 247, 4494-4498 (1972).

36. Murgola, E. J. tRNA, suppression, and the code. *Annu. Rev. Genet.* 19, 57-80 (1985).

37. Roberts, E., Eargle, J., Wright, D., and Luthey-Schulten, Z. MultiSeq: Unifying sequence and structure data for evolutionary analysis. *BMC Bioinformatics* 7, 382 (2006).

38. Markowitz, V. M. *et al.* IMG/M: a data management and analysis system for
metagenomes. *Nucleic Acids Res.* 36, D534-538 (2008).

39. Humphrey, W., Dalke, A., and Schulten, K. VMD: visual molecular dynamics. *J Mol. Graph.* 14, 33-38, 27-38 (1996).