

Direct charging of tRNA_{CUA} with pyrrolysine *in vitro* and *in vivo*

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Pyrrolysine is the 22nd amino acid^{1–3}. An unresolved question has been how this atypical genetically encoded residue is inserted into proteins, because all previously described naturally occurring aminoacyl-tRNA synthetases are specific for one of the 20 universally distributed amino acids. Here we establish that synthetic L-pyrrolysine is attached as a free molecule to tRNA_{CUA} by PylS, an archaeal class II aminoacyl-tRNA synthetase. PylS activates pyrrolysine with ATP and ligates pyrrolysine to tRNA_{CUA} *in vitro* in reactions specific for pyrrolysine. The addition of pyrrolysine to *Escherichia coli* cells expressing *pylT* (encoding tRNA_{CUA}) and *pylS* results in the translation of UAG *in vivo* as a sense codon. This is the first example from nature of direct aminoacylation of a tRNA with a non-canonical amino acid and shows that the genetic code of *E. coli* can be expanded to include UAG-directed pyrrolysine incorporation into proteins.

Most organisms employ UAG as a stop codon, but translation is not terminated at in-frame UAGs in some methyltransferases of methanogenic Archaea^{4,5}. Rather, these codons serve as sense codons⁶ and, as determined by crystal structure analyses, UAG encodes pyrrolysine, that is, lysine with the epsilon nitrogen in amide linkage to (4*R*, 5*R*)-4-substituted-pyrroline-5-carboxylate¹, the 22nd amino acid found to be genetically encoded in nature. A key question is whether the UAG-translating tRNA_{CUA} (ref. 2) is first charged with lysine^{2,7} and then modified to pyrrolysine for incorporation into the growing polypeptide or whether pyrrolysine is attached as the fully synthesized amino acid to tRNA_{CUA} (refs 2, 8). Here we show that the latter possibility is feasible by demonstrating the direct pyrrolysylation of tRNA_{CUA} *in vitro*. This is the first example found in nature of specific aminoacylation of a tRNA with a non-canonical amino acid. The results reported show further that the expression of only two genes, *pylT* and *pylS*, which encode tRNA_{CUA} and pyrrolysyl-tRNA synthetase, can expand the genetic code of *E. coli* to include pyrrolysine. This procedure could potentially be used to immediately expand the genetic code of any species that can incorporate exogenously added pyrrolysine.

The 4-substituent of pyrrolysine could not be initially assigned, but recent mass spectrometry with MtmB peptide fragments has provided accurate mass measurements indicating that the substituent is a methyl group (K.B.G.-C., J. A. Soares, L. Zhang, R. L. Pitsch, N. M. Kleinholz, R. Benjamin Jones, J. J. Wolff, J. Amster and J.K., unpublished observations). Crystallographic studies have also indicated that the most likely substituent at the 4-position of the pyrrolysine ring is a methyl group, and this form of L-pyrrolysine has been synthesized⁹. Recombinant PylS-His₆ was purified by Ni affinity chromatography from lysates of *Escherichia coli* expressing the recombinant *Methanosarcina barkeri* *pylS* gene modified so as to add a carboxy-terminal hexahistidine tag to the gene product

(see Supplementary Fig. S1). The tRNA pool extracted from *Methanosarcina acetivorans* or tRNA_{CUA} transcribed *in vitro* was used in charging experiments. Charged and uncharged tRNA species were separated by electrophoresis in a denaturing acid-urea polyacrylamide gel^{10,11} and tRNA_{CUA} was specifically detected by northern blotting with an oligonucleotide probe. The oligonucleotide complementary to tRNA_{CUA} could hybridize to a tRNA in the pool of tRNAs isolated from wild-type *M. acetivorans* but not to the tRNA pool from a *pylT* deletion mutant of *M. acetivorans* (A.M., A. Patel, J. Soares, R.L. and J.A.K., unpublished observations).

Both tRNA_{CUA} and aminoacyl-tRNA_{CUA} were detectable in the isolated cellular tRNA pool (Fig. 1). Alkaline hydrolysis deacylated the cellular charged species, but subsequent incubation with pyrrolysine, ATP and PylS-His₆ resulted in maximal conversion of 50% of deacylated tRNA_{CUA} to a species that migrated with the same electrophoretic mobility as the aminoacyl-tRNA_{CUA} present in the extracted cellular tRNA pool. The aminoacyl-tRNA_{CUA} synthesized *in vitro* was also sensitive to mild alkaline hydrolysis (Supplementary Fig. S2). This charged species was not formed by PylS-His₆ in the presence of a mixture of the 20 canonical amino acids each at 50 μM, or only 50 μM lysine, but was formed after the further addition of synthetic pyrrolysine (Fig. 1). PylS-His₆ conversion of tRNA_{CUA} to the charged species was therefore dependent on pyrrolysine, even in the presence of other amino acids. To determine whether pyrrolysine itself was present in the cytoplasm, we prepared a cell extract of *M. acetivorans* and separated the low-molecular-mass metabolite pool from macromolecules by ultrafiltration. The small molecule pool contained a PylS-His₆ substrate for aminoacylation of tRNA_{CUA} (Fig. 1). We also demonstrated that PylS-His₆ does not aminoacylate tRNA^{lys} in the *M. acetivorans* tRNA pool with either pyrrolysine or lysine (Supplementary Fig. S3). tRNA_{CUA} transcribed *in vitro* was also aminoacylated with synthetic pyrrolysine by PylS-His₆ (Supplementary Fig. S4) in an ATP-dependent reaction. We observed that PylS-His₆ aminoacylated with pyrrolysine a maximum of 43% of *in-vitro*-transcribed tRNA_{CUA} during the course of our experiments.

As a prerequisite of tRNA aminoacylation, an aminoacyl-adenylate and pyrophosphate are formed from the amino acid and ATP by an aminoacyl-tRNA synthetase¹². This reversible activation reaction can be assayed by the isotopic exchange of ³²P-pyrophosphate into ATP dependent on the addition of the cognate amino acid for the aminoacyl-tRNA synthetase in question¹³. PylS-His₆ catalyses a pyrophosphate-ATP isotopic exchange reaction on the addition of synthetic pyrrolysine (Fig. 2). This reaction is not dependent on the addition of cellular tRNA. Exchange activity independent of tRNA is typical of a class II aminoacyl-tRNA synthetase¹⁴. The apparent *K_m* values for pyrrolysine and ATP were 53 μM and 2 μM, respectively. The apparent *V_{max}* was 120 nmol min⁻¹ per mg PylS, giving a *k_{cat}* of 6 min⁻¹ for the

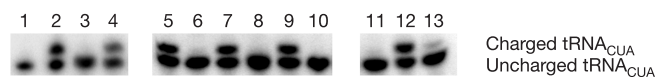


Figure 1 Aminoacylation of tRNA_{CUA} in cellular tRNA pools monitored by acid-urea gel electrophoresis and northern blotting to detect tRNA_{CUA}. Lane 1 contains the alkali-deacylated cellular tRNA pool with only uncharged tRNA_{CUA}, lane 2 contains cellular tRNA as isolated and shows charged (upper band) and uncharged (lower band) tRNA_{CUA}. Aminoacylation of tRNA_{CUA} in the deacylated cellular tRNA pool by PylS was assayed as described in Methods except that the amino-acid substrate (50 μM) was varied. Each lane represents reactions with the following: 3, no amino acid; 4, pyrrolysine; 5, pyrrolysine; 6, lysine; 7, pyrrolysine plus lysine; 8, a mixture of the 20 canonical amino acids; 9, pyrrolysine plus a mixture of the 20 canonical amino acids; 10, pyrrolysine but lacking PylS-His₆; 11, no amino acid; 12, pyrrolysine; 13, the low-molecular-mass fraction from *M. acetivorans* cell extract. Sample groups 1–4 and 5–13 are from two different experiments. Samples 5–13 are from different parts of the same gel.

exchange reaction. Incubation for as long as 30 min resulted in no detectable isotopic exchange into ATP above background in the presence of a mixture of the canonical 20 amino acids each at 100 μ M, or in the presence of 1 mM lysine.

In contrast with the inability of PylS-His₆ to synthesize lysyl-tRNA_{CUA}, we previously observed this activity with amino-terminally His-tagged PylS (His₆-PylS) as assayed by acid precipitation of tRNA ligated to radioactive lysine². However, no lysyl-tRNA synthetase activity was detectable with His₆-PylS by using the gel-shift aminoacylation assay, in agreement with a recent report⁷. In contrast, His₆-PylS does have pyrrolysyl-tRNA synthetase activity as demonstrated by the gel-shift aminoacylation assay (Supplementary Fig. S5). To determine whether PylS lacking either an N-terminal or C-terminal tag sequence acts as a lysyl- or pyrrolysyl-tRNA synthetase, we undertook the following experiments to test whether PylS allows the translation of UAG codons *in vivo* in *E. coli*, and whether this would be dependent on the presence of pyrrolysine. As a reporter of UAG translation as a sense codon, we introduced the *mtmB1* gene into *E. coli* BL21 (DE3). The *mtmB1* gene encodes the methylamine methyltransferase MtmB (refs 5, 15), in which pyrrolysine was identified¹. *E. coli* BL21 (DE3) expresses recombinant *mtmB1* with only trace amounts of the UAG read-through product, and instead primarily produces a truncated MtmB protein terminating at codon 202, the internal UAG that encodes pyrrolysine in the 452-codon *mtmB1* reading frame⁶. Plasmids were constructed bearing combinations of *mtmB1*, *pylS* and/or *pylT* under the control of the T7 promoter, and transformed into *E. coli*. Expression of these genes was induced in cells growing in the presence and the absence of exogenous 1 mM pyrrolysine. Total cellular proteins from each strain were then separated by SDS-polyacrylamide-gel electrophoresis and *mtmB1* gene products were detected by subsequent immunoblotting with polyclonal antibody specific for purified *M. barkeri* MtmB (ref. 6). All strains produced the amber-truncated product of *mtmB1* as a 23-kDa protein; however, the strain expressing *pylT*, *pylS* and *mtmB1* further expressed large amounts of 50-kDa MtmB, showing UAG read-

through dependent on the presence of pyrrolysine. The pool of amino acids in *E. coli* did not support the synthesis of an aminoacyl-tRNA_{CUA} that could efficiently translate the UAG codon in *mtmB1*. The requirement for pyrrolysine could not be replaced by 1 mM lysine or a mixture of the 20 canonical amino acids each at 1 mM. Translation of the *mtmB1* UAG codon dependent on pyrrolysine was further dependent on the expression of *pylS* and *pylT*, as demonstrated by strains transformed with expression vector containing only *pylT* (Fig. 3) or only *pylS* (Supplementary Fig. S6).

To confirm that synthetic pyrrolysine is incorporated at the UAG-encoded position of *mtmB1* by *E. coli* transformed with *pylT* and *pylS*, the insoluble recombinant full-length MtmB protein was partly purified by differential centrifugation and solubilization with urea. After SDS gel electrophoresis, full-length recombinant MtmB was subjected to in-gel digestion with chymotrypsin. An *m/z* 791.5²⁺ ion was identified, which corresponds to the predicted *m/z* 791.4²⁺ of the MtmB fragment AGRPGM_{ox}GVXGPETSL (residues 194–208), where X is the UAG-encoded residue with the predicted mass of synthetic pyrrolysine. Collision-induced dissociation mass spectrometry confirmed the sequence, and the mass of the UAG-encoded residue was ascertained as 237.2 Da (Supplementary Fig. S7, Supplementary Table S1). The predicted molecular mass of the synthetic pyrrolysyl residue is 237.16 Da, thereby confirming that expression of *pylT* and *pylS* is sufficient to expand the genetic code of *E. coli* to include exogenous pyrrolysine. The amino acid might enter the cell because its amide bond allows recognition by a broad-spectrum peptide transporter such as DppA (ref. 16). The synthesis of full-length MtmB further indicates that the *E. coli* translation factor EF-Tu binds pyrrolysyl-tRNA_{CUA} within a thermodynamic range allowing incorporation into protein during translation¹⁷.

The current data indicate that pyrrolysine is encoded in DNA using the general mechanism employed for the common set of 20 amino acids. Direct charging of pyrrolysine onto tRNA contrasts with selenocysteine, a genetically encoded non-canonical amino acid synthesized only on tRNA^{18,19}. Several systems have been recently developed to expand and manipulate the genetic code^{20–23} to generate recombinant proteins containing unnatural amino

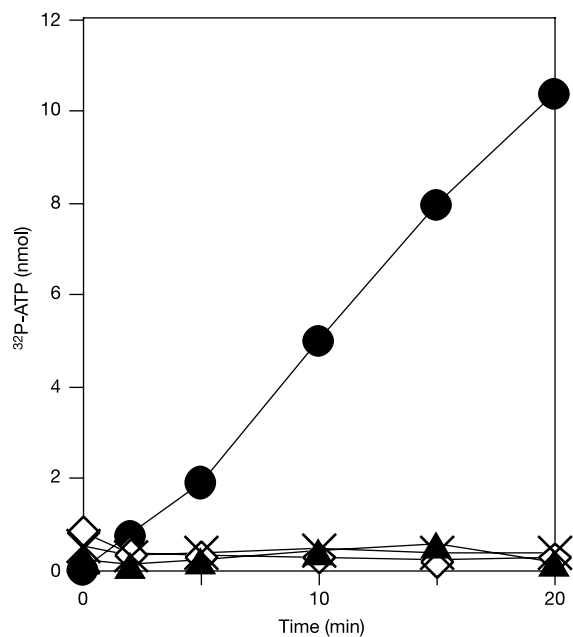


Figure 2 Dependence on pyrrolysine of the ³²P_i-ATP exchange reaction mediated by PylS-His₆. Aliquots were removed at the time points shown for estimation of the amount of ³²P-ATP formed. In the figure are illustrated results from averaged duplicate reactions that were either complete (filled circles) or lacked PylS-His₆ (open diamonds), ATP (filled triangles) or synthetic pyrrolysine (crosses).

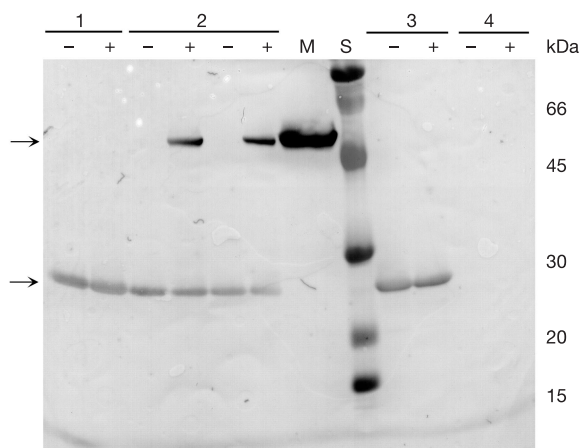


Figure 3 Anti-MtmB immunoblot of cell extracts of *E. coli* strains testing the suppression of the UAG codon in *mtmB1*. The samples loaded were from strains transformed with the following: 1, pEC02, bearing *mtmB1* and *pylT*; 2, pEC03, bearing *mtmB1*, *pylT* and *pylS*; 3, pEC01, bearing *mtmB1*; 4, pET-Duet, the vector from which the pEC plasmids are derived. Recombinant gene expression was induced in the presence (+) and absence (-) of 1 mM exogenous synthetic pyrrolysine. Also present on the immunoblot is purified 50-kDa (full-length) MtmB (M) and dye-coloured protein standards (S) for molecular mass determination; molecular masses are indicated at the right. The arrows at the left point to full-length MtmB (upper arrow) and truncated MtmB (lower arrow) made, respectively, by UAG readthrough or UAG termination during *mtmB1* expression.

acids. By adding *pylS* and *pylT* genes, it should now be possible to generate proteins with the 22nd amino acid incorporated at UAG-targeted sites in any species that can incorporate added pyrrolysine, thereby adding a unique natural amino acid with electrophilic properties. We are now focusing on the pyrrolysine biosynthetic pathway, which offers the possibility of also adding genes that will generate pyrrolysine internally in recombinant organisms. □

Methods

Recombinant proteins

The *M. barkeri* MS *pylS* gene was amplified by polymerase chain reaction (PCR) from isolated genomic DNA²⁴ and cloned into pET 22b (Novagen, Madison, Wisconsin) to create ppylSH6, which produced PylS with a hexahistidine tag at the C terminus (PylS-His₆) in *E. coli* BL21 (DE3) (Stratagene, La Jolla, California). PylS-His₆ was isolated from cell extracts in 20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole pH 7.4, using a Ni-activated trap chelating HP column (Amersham Biosciences Corp., Piscataway, New Jersey). PylS-His₆ eluted at 240 mM imidazole during the application of 10–500 mM imidazole in the same buffer to the column. His₆-PylS (ref. 2) with a hexahistidine N-terminal tag was used as a partly pure fraction from a nickel-affinity column. Control experiments indicated no pyrrolysyl-tRNA synthetase activity in untransformed *E. coli*.

The *lysS* gene was PCR amplified from *M. barkeri* MS genomic DNA for the recombinant expression of *lysS* with an N-terminal hexahistidine sequence (His₆-LysS) that eluted at about 130 mM imidazole from the nickel-affinity column.

PylS substrates

L-Pyrrolysine was synthesized and characterized with the use of ¹³C and ¹H NMR⁹. TLC analysis revealed no other amino acids. The pyrrolysine used in charging experiments was further analysed by electrospray mass spectrometry and revealed two predominant peaks with *m/z* 256.16 (M + H) and 278.14 (M + Na), where M is L-pyrrolysine. The cellular tRNA pool was isolated²⁵ from *M. acetivorans* C2A (OD₆₀₀ 0.6–0.7) growing on trimethylamine at 37 °C in DSM 304 medium²⁶ because this species is easily lysed. Agarose-gel electrophoresis indicated that 30% of the ethidium bromide staining material in the preparation was tRNA. *M. barkeri* Fusaro tRNA_{CUA} transcribed *in vitro* was produced with the DNA template described previously² and the T7-MEGAShortscript transcription kit (Ambion Inc., Austin, Texas).

The low-molecular-mass cell fraction used in aminoacylation reactions was the supernatant of French-pressed trimethylamine-grown *M. acetivorans* (27 g in 30 ml 50 mM MOPS pH 7.0) filtered with a 3-kDa Amicon Centricon apparatus (Millipore, Billerica, Massachusetts), and evaporated to dryness before resuspension in 2 ml doubly distilled water.

Aminoacylation and pyrophosphate exchange assays

The assay for aminoacylation of tRNA_{CUA} (in a volume of 25 μl) contained 0.8–1.7 μM purified PylS-His₆, 50 mM KCl, 1 mM MgCl₂, 5 mM ATP, 0.5 mM dithiothreitol and 50 μM synthetic pyrrolysine in 10 mM HEPES buffer pH 7.2, and 8 μg *M. acetivorans* tRNA pool preparation or 40 nM of tRNA_{CUA} transcript. The reaction was terminated after 5–30 min at 37 °C with an equal volume of 0.3 M sodium acetate, 8 M urea pH 5.0. Charged and uncharged tRNA were separated by acid-urea acrylamide gel electrophoresis²⁵, blotted to nitrocellulose and probed with a 5' ³²P-end-labelled, 72-base oligonucleotide complementary to tRNA_{CUA}. Radioactivity was analysed with a STORM Phosphorimager (Amersham Biosciences).

The pyrophosphate exchange reaction was performed after the method of ref. 13. The 100–200-μl reactions incubated at 37 °C typically contained 0.3–1 μM PylS-His₆, 10 mM MgCl₂, 25 mM KCl, 1 mM KF, 4 mM dithiothreitol, 2 mM ATP, 100 μM pyrrolysine, 2 mM ³²P-PP_i (4–10 d.p.m. pmol⁻¹; PerkinElmer, Boston, Massachusetts) in 20 mM HEPES-KOH pH 7.2.

Pyrrolysine-dependent amber suppression in *E. coli*

The *M. barkeri* MS *mtmB1* (GenBank accession number AF013713) was removed with *NdeI* and *EcoRV* from plasmid pCJ09 (ref. 6), and ligated into MCS2 of pET-Duet to create pEC01 (Novagen). The *pylS* and *pylT* genes were PCR amplified from genomic *M. acetivorans* DNA (GenBank accession number NC 003552) and *pylT* cloned into the *XbaI* site directly upstream of MCS1 in pEC01 to create pEC02. The *pylS* gene was inserted into the *NcoI* and *BamHI* sites of MCS1 of pEC02 to create pEC03. The *pylT XbaI* fragment was excised from pEC03 to create pEC05. All constructs were confirmed by restriction mapping and sequencing. To test for amber suppression, overnight cultures were grown in Luria-Bertani broth (3 ml) with 100 μg ml⁻¹ ampicillin. Subsequently, 200 μl was inoculated into 1 ml fresh medium and grown to an OD₆₀₀ of 0.6. The culture (100 μl) was then transferred to a polypropylene tube and induced for 4 h with 1 mM isopropyl β-D-thiogalactoside in the presence or absence of 1 mM pyrrolysine. The *mtmB1* gene products in equivalent amounts of lysates were then analysed by immunoblotting of a SDS 12.5% polyacrylamide gel with affinity-purified rabbit anti-MtmB antibody⁶. The Rainbow Molecular Weight markers (Amersham Biosciences) were used.

To isolate recombinant MtmB for mass spectrometry, *E. coli* bearing pEC03 (10 ml) was used with 0.75 mM pyrrolysine. The *mtmB1* gene products were inclusion bodies.

Sequentially washing the pellet from a French-pressed cell lysate with 0, 1, 3, 5 and 7 M urea in 50 mM MOPS pH 7 yielded purified *mtmB1* gene products in 7 M urea; these were separated by SDS gel electrophoresis. The 50-kDa MtmB was subjected to in-gel chymotrypsin digestion and peptide sequencing by tandem mass spectrometry²⁷.

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- Hao, B. *et al.* A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science* **296**, 1462–1466 (2002).
- Srinivasan, G., James, C. M. & Krzycki, J. A. Pyrrolysine encoded by UAG in Archaea: charging of a UAG-decoding specialized tRNA. *Science* **296**, 1459–1462 (2002).
- Atkins, J. F. & Gesteland, R. The 22nd amino acid. *Science* **296**, 1409–1411 (2002).
- Paul, L., Ferguson, D. J. & Krzycki, J. A. The trimethylamine methyltransferase gene and multiple dimethylamine methyltransferase genes of *Methanosarcina barkeri* contain in-frame and read-through amber codons. *J. Bacteriol.* **182**, 2520–2529 (2000).
- Burke, S. A., Lo, S. L. & Krzycki, J. A. Clustered genes encoding the methyltransferases of methanogenesis from monomethylamine. *J. Bacteriol.* **180**, 3432–3440 (1998).
- James, C. M., Ferguson, T. K., Leykam, J. F. & Krzycki, J. A. The amber codon in the gene encoding the monomethylamine methyltransferase isolated from *Methanosarcina barkeri* is translated as a sense codon. *J. Biol. Chem.* **276**, 34252–34258 (2001).
- Polycarpo, C. *et al.* Activation of the pyrrolysine suppressor tRNA requires formation of a ternary complex with class I and class II lysyl-tRNA synthetases. *Mol. Cell* **12**, 287–294 (2003).
- Ibba, M. & Söll, D. Aminoacyl-tRNAs: setting the limits of the genetic code. *Genes Dev.* **18**, 731–738 (2004).
- Hao, B. *et al.* Reactivity and chemical synthesis of L-pyrrolysine – the 22nd amino acid. *Chem. Biol.* (in the press).
- Ho, Y. S. & Kan, Y. W. *In vivo* aminoacylation of human and *Xenopus* suppressor tRNAs constructed by site-specific mutagenesis. *Proc. Natl Acad. Sci. USA* **84**, 2185–2188 (1987).
- Varshney, U., Lee, C. P. & RajBhandary, U. L. Direct analysis of aminoacylation levels of tRNAs *in vivo*. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutamyl-tRNA synthetase. *J. Biol. Chem.* **266**, 24712–24718 (1991).
- Schimmel, P. & Söll, D. Aminoacyl-tRNA synthetases: general features and recognition of transfer RNAs. *Annu. Rev. Biochem.* **48**, 601–648 (1979).
- Cole, F. & Schimmel, P. R. On the rate law and mechanism of the adenosine triphosphate-pyrophosphate isotope exchange reaction of aminoacyl-transfer ribonucleic acid synthetases. *Biochemistry* **9**, 480–489 (1970).
- Francklyn, C., Perona, J. J., Puetz, J. & Hou, Y. M. Aminoacyl-tRNA synthetases: versatile players in the changing theater of translation. *RNA* **8**, 1363–1372 (2002).
- Burke, S. A. & Krzycki, J. A. Reconstitution of monomethylamine:coenzyme M methyl transfer with a corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. *J. Biol. Chem.* **272**, 16570–16577 (1997).
- Smith, M. W., Tyreman, D. R., Payne, G. M., Marshall, N. J. & Payne, J. W. Substrate specificity of the periplasmic dipeptide-binding protein from *Escherichia coli*: experimental basis for the design of peptide prodrugs. *Microbiology* **145**, 2891–2901 (1999).
- LaRiviere, E. J., Wolfson, A. D. & Uhlenbeck, O. C. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science* **294**, 165–168 (2001).
- Stadtman, T. C. Selenocysteine. *Annu. Rev. Biochem.* **65**, 83–100 (1996).
- Commans, S. & Bock, A. Selenocysteine inserting tRNAs: an overview. *FEMS Microbiol. Rev.* **23**, 335–351 (1999).
- Wang, L., Brock, A., Herberich, B. & Schultz, P. G. Expanding the genetic code of *Escherichia coli*. *Science* **292**, 498–500 (2001).
- Doring, V. *et al.* Enlarging the amino acid set of *Escherichia coli* by infiltration of the valine coding pathway. *Science* **292**, 501–504 (2001).
- Kiick, K. L., Weberskirch, R. & Tirrell, D. A. Identification of an expanded set of translationally active methionine analogues in *Escherichia coli*. *FEBS Lett.* **502**, 25–30 (2001).
- Chin, J. W. *et al.* An expanded eukaryotic genetic code. *Science* **301**, 964–967 (2003).
- Paul, L. & Krzycki, J. A. Sequence and transcript analysis of a novel *Methanosarcina barkeri* methyltransferase II homolog and its associated corrinoid protein homologous to methionine synthase. *J. Bacteriol.* **178**, 6599–6607 (1996).
- Jester, B. C., Levensgood, J. D., Roy, H., Ibba, M. & Devine, K. M. Nonorthologous replacement of lysyl-tRNA synthetase prevents addition of lysine analogues to the genetic code. *Proc. Natl Acad. Sci. USA* **100**, 14351–14356 (2003).
- Sowers, K. R. & Schreier, H. J. in *Archaea, a Laboratory Manual* (eds Sowers, K. R. & Schreier, H. J.) 459–489 (Cold Spring Harbor Laboratory Press, Plainview, New York, 1995).
- Wilm, M. *et al.* Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466–469 (1996).

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