Published in final edited form as:

Curr Opin Microbiol. 2011 June ; 14(3): 342–349. doi:10.1016/j.mib.2011.04.001.

Functional context, biosynthesis, and genetic encoding of pyrrolysine

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Summary

In *Methanosarcina* spp., amber codons in methylamine methyltransferase genes are translated as the 22nd amino acid, pyrrolysine. The responsible *pyl* genes plus amber-codon containing methyltransferase genes have been identified in four archaeal and five bacterial genera, including one human pathogen. In *E. coli*, the recombinant *pylBCD* gene products biosynthesize pyrrolysine from two lysine and the *pylTS* gene products direct pyrrolysine incorporation into protein. In the proposed biosynthetic pathway, PylB forms methylornithine from lysine, which is joined to another lysine by PylC, and oxidized to pyrrolysine by PylD. Structures of the catalytic domain of pyrrolysyl-tRNA synthetase (archaeal PylS or bacterial PylSc) revealed binding sites for tRNA^{Pyl} and pyrrolysine. PylS and tRNA^{Pyl} are now being exploited as an orthogonal pair in recombinant systems for introduction of useful modified amino acids into proteins.

Introduction

Methanogenesis is a process unique to the Archaea. Befittingly, many unusual enzymes, cofactors, and metabolites were first encountered in methanogens and only later in Bacteria [1]. One such find is pyrrolysine, the 22nd amino acid to be encountered in the natural genetic code [2,3].

In Archaea, the pyrrolysyl residue is known to occur only in the family *Methanosarcinaceae*. Unlike most other methanogens, members of this group can use methylamines as precursors to methane. Metabolism of trimethylamine, dimethylamine, or monomethylamine is respectively initiated by the pyrrolysine-containing proteins MttB, MtbB, or MtmB (Fig. 1) [4–6]. These proteins each methylate a cognate corrinoid protein (MttC, MtbC, or MtmC) in the Co(I) state. Adventitious oxidation of the corrinoid proteins to Co(II) can inactivate the methyltransferase reactions, but the iron-sulfur protein RamA can reactive the corrinoid proteins via ATP dependent reduction [7]. The methyl-Co(III) corrinoid proteins are substrates of MtbA which methylates coenzyme M (CoM) [8,9]. From here, methyl groups can be converted directly to carbon dioxide, and cell carbon.

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MttB, MtbB, or MtmB have no significant sequence similarity, but each of their genes contains a single in-frame amber codon [10,11] that is translated [12,13]. The crystal structure of MtmB revealed pyrrolysine as the UAG-encoded residue [3,14]. Mass spectral studies demonstrated that the UAG-encoded residues of MttB and MtbB are also pyrrolysine [13]. Pyrrolysine was observed in the crystal structure to bind ammonia at the carbon of the imine bond [3,14], and it is hypothesized that a pyrrolysine-methylammonium adduct serves to activate and orient methylamines as substrates for nucleophilic attack by the Co(I) corrinoid protein (Fig. 1) [3,15].

Pyrrolysine is synthesized and incorporated into the methylamine methyltransferases through the combined actions of the products of the *pyl* genes (Fig. 1). The *pylT* gene encodes tRNA^{Pyl} whose CUA anticodon allows for amber codon translation [2,16]. The *pylS* gene produces the pyrrolysyl-tRNA synthetase that charges tRNA^{Pyl} directly with pyrrolysine [17,18]. The synthesis of pyrrolysine is carried out by the *pylBCD* gene products [19].

Incorporation of pyrrolysine into protein under the direction of amber codons does not require specific signals in the gene, as in-frame amber codons inserted into the *E. coli uidA* gene for β -glucuronidase are translated as pyrrolysine at 20–30% efficiency in *M. acetivorans* [20,21]. Translation of UAG as pyrrolysine even in foreign genes with an introduced amber codon suggests this level of translation occurs by a mechanism analogous to that underlying amber suppression, with tRNA^{Pyl} acting as a suppressor tRNA. On the other hand, UAG translation in the methanogen with *mtmB1* transcripts appears much more efficient, with little UAG-termination product detectable. Substitution of sequence immediately downstream of the UAG codon dramatically increases the UAG-termination product, but with still a relatively high level of UAG translation as pyrrolysine. While the deleted region may act as a sequence enhancing translation over termination with *mtmB1* transcripts, previous suggestions from several laboratories of an obligate pyrrolysine insertion element or tRNA^{Pyl} specific translation factors have not been borne out [20–24].

Sequenced genomes with *pyl* genes

At the time of the discovery of pyrrolysine, only *Methanosarcina* spp. and the bacterium *Desulfitobacterium hafniense* were known to possess *pyl* genes [2]. Recent sequencing has now expanded the count to six bacterial and six archaeal species belonging to nine genera (Fig. 2). In the archaea, *pyl* genes are still limited to members of the *Methanosarcinaceae*, but now also include psychrotrophic methanogen *Methanococcoides burtonii*, and two halophilic methanogens, *Methanohalophilus mahii* and *Methanohalobium evestigatum*. The *pyl* genes are also found in select species of the bacterial groups Clostridia or the Deltaproteobacteria. The latter group includes symbionts of multicellular organisms, such as a gut inhabitant of a marine worm identified in a metagenomic study [25,26]. Most recently, *pyl* genes were annotated in the genome of the human intestinal bacteria, *Bilophila wadsworthia* (Fig. 2). The unannotated *pylT* gene was identified by the authors some distance from the other *pyl* genes (Fig. 2, Fig. S1). This organism has been a common isolate in cases of gangrenous appendicitis and abscesses in a variety of bodily locations [27], and represents the first known human symbiont, and pathogen, to have pyrrolysine genes.

While amber codons have been identified in *Thg1* and transposase genes of one or two *Methanosarcina* spp., these are likely to be mutations stable in the context of tRNA^{Pyl} and PylS, as homologs of these genes without amber codons exist in other *Methanosarcinaceae* [21,28,29]. In sharp contrast, each of the organisms possessing the five *pyl* genes also possess one or more homologs of *mtmB*, *mtbB*, and/or *mttB* with conserved in-frame amber codons, as well as the genes for associated proteins such as the corrinoid proteins and RamA

(Fig. 2). Each organism is an anaerobic respirer found where methylamines or their precursors are available. The correlation runs both ways, as no amber-codon containing methylamine methyltransferase is found in any genome lacking the five *pyl* genes. The *Desulfobacterium autotrophicum* genome is interesting in this regard, as *pylSn*, *pylT*, and *pylB*, are present, but not *pylSc*, *pylC*, or *pylD*. A transposase gene homolog within the remaining *pylTSnB* cluster suggests the *pyl* gene cluster was disrupted [21]. Accordingly, although many *mttB* homologs are found in this genome, all lack an in frame amber codon.

Such homologs of methyltransferase genes without amber codons are found in many genomes. Particularly numerous are *mttB* non-amber homologs. A recent BLASTP search by the authors in ~1500 microbial genomes kept at NCBI revealed 278 *mttB* homologs in sequenced genomes. A similarity tree shows the bacterial and archaeal MttB homologs whose genes have amber codons form a single clade not following the rRNA phylogenetic tree (J. Krzycki, unpublished data). The pyrrolysine-containing MttB are well separated from the great majority of MttB homologs whose encoding genes lack the pyrrolysine amber codon. However, one clade of MttB homologs without pyrrolysine branches very close to the pyrrolysine-containing MttB family. This raises the question as to whether such MttB homologs without pyrrolysine are actually TMA methyltransferases, or homologs evolved to use, for example, other N-methyl substrates.

Substrate recognition by PylS

The archaeal pyrrolysyl-tRNA synthetase is a homodimer whose subunits have two functional domains. The C-terminal domain contains the three motifs of the class-II aminoacyl tRNA synthetases [2,30,31], while the N-terminal domain is not similar to proteins with known function [32]. In bacteria, N- and C-terminal domains of archaeal PylS are respectively represented by two independent proteins, PylSn and PylSc (Fig. 2) [2, 32].

Crystal structures of the C-terminal domain of archaeal PylS and bacterial PylSc have revealed the catalytic site that accommodates pyrrolysine and ATP [30,31,33–35]. The pyrrolysine ring is accommodated by a hydrophobic pocket closed by a mobile loop bearing a tyrosine which may H-bond the imine nitrogen of pyrrolysine [30], and/or provide stability to the formed pyrrolysyl-adenylate prior to tRNA binding [33]. While pyrrolysine has the most favorable kinetics for amino acid activation, analogs having an oxygen atom replacing the imine nitrogen are favored over those with carbon, suggesting H-bonding to the imine nitrogen could play a role in substrate binding [36]. Loss of the loop tyrosine does not inactivate the enzyme, but kinetic parameters have not yet been determined [33].

Even with the limited sample size of *pyl* containing organisms, considerable diversity is apparent in tRNA^{Pyl} with only 26 residues universally conserved (Fig. 3). While the sequences of the four archaeal tRNA^{Pyl} homologs from the Methanosarcinaeae are 87% identical, the five bacterial tRNA^{Pyl} molecules are more divergent, exhibiting only ~ 45% sequence identity. All tRNA^{Pyl} examples share unusual secondary structure features, including a three-base variable loop, small D-loop, long anticodon stem, and a single base between the D- and acceptor stems [2,35,37]. Nonetheless, tRNA^{Pyl} assumes the typical tRNA L-shaped tertiary structure, albeit with a smaller core [35,37]. In the co-crystal structure [35], PylSc approaches tRNA^{Pyl} from the major groove interacting with the D-stem residues in the core as well as the acceptor stem (Fig. 3).

PylSc does not have direct interaction with residues in the anticodon stem or T-stem [35]. However, mutations in the T-stem and residues flanking the anticodon have relatively strong effects on *in vitro* aminoacylation of the *M. barkeri* tRNA^{Pyl} by PylS, in the apparent absence of a structural role for the targeted residues [38]. In further contrast to archaeal PylS, PylSc has no detectable *in vivo* activity in *E. coli* in all but the most sensitive assays

for amber suppression [35,39], and PylSc binds cognate tRNA^{Pyl} with relatively low affinity. A possible resolution to this conundrum is that PylSc lacks the N-terminal domain of the methanogen enzyme, represented by bacterial PylSn [2,32]. As a homolog of the archaeal PylS N-terminal domain, PylSn may be responsible for strengthened interaction with tRNA^{Pyl}.

PylS and PylT as an orthogonal pair for chemical biology

Amber codons in *E. coli* bearing *pylTS* can translate UAG using exogenous pyrrolysine [17]. This suggested the possible utility of PylS and tRNA^{Pyl} as an orthogonal pair, and this potential has now been demonstrated in bacterial [40], yeast [41], and mammalian cells [42,43]. The hydrophobic pocket in PylS can accommodate other moieties, so long as they are in amide linkage to ^εN of lysine, leading to notable substrate flexibility [34]. Either wild type or mutated PylS have been employed to insert unnatural residues such as: acetyl-N^ε-lysine, with potential applications in histone research [40]; N^ε-(o-azidobenzoyloxycarbonyl)-L-lysine, allowing fluorescent tagging [34]; tetrahydrofuran-^εN-lysine [36] modified with an alkyne group, allowing modification for FRET analysis [44]; D-cysteinyl-^εN-lysine, allowing site-specific chemical ubiquitination of protein [45], and finally α-hydroxy acid derivatives, inserting a backbone ester bond for easy hydrolysis [46]. Most recently, PylS was used to incorporate a photocaged lysine derivative into p53 in a mammalian cell line, giving control over the timing of a specific protein's transport into an organelle [43].

Pyrrolysine Biosynthesis

The relationships of PylB, PylC, and PylD to enzymes with known metabolic functions fueled the idea they functioned in pyrrolysine biosynthesis [2]. This has proved the case, as *E. coli* expressing archaeal *pylTSBCD* can incorporate biosynthesized pyrrolysine into protein, with *pylBCD* required to make the PylS substrate [20].

Hypothetical pathways for pyrrolysine biosynthesis generally considered amide formation between the ^εN of lysine and a ring or precursor derived from ornithine, glutamate, isoleucine, or proline [15,20,47]. Stable isotope labeling experiments using the *E. coli* recombinant *pyl* system did indeed show that the acyl portion of pyrrolysine derives from lysine [48]. However, the methylated pyrroline ring *also* derives from lysine. All six carbons of two molecules of lysine are retained in pyrrolysine. One ^εN is lost from the two lysine molecules, presumably as one lysine becomes the ring precursor [48].

How is lysine converted into the methylated pyrroline ring? An important clue is that addition of D-ornithine allows *pyl* transformed *E. coli* to make desmethylpyrrolysine (dmPyl), a pyrrolysine analog lacking the ring methyl group [48]. Formation of dmPyl requires only PylC and PylD. PylC carries out ligation of D-ornithine to the terminal amine of lysine, as cells transformed with *pylC* produce D-ornithyl-^εN-lysine dependent on exogenous D-ornithine. PylC has similarity to members of the carbamoyl phosphate synthetase family, including D-ala, D-ala ligase, and amide formation is in keeping with this phylogeny (Fig. S2) [2]. PylD is similar to several different dehydrogenases binding FAD or NAD (Fig. S3), and may carry out oxidation of the terminal amine of the PylC product, leading to dehydration and formation of the dmPyl ring [48].

However, synthesis of pyrrolysine itself is not D-ornithine dependent, but instead requires PylB, in addition to PylC and PylD. This suggests that PylB produces a D-ornithine derivative (Fig. 1). PylB is related to members of the radical S-adenosyl-L-methionine (SAM) family (Fig. S4) [48], and possesses the characteristic CXXXCXXC motif binding the Fe₄S₄ cluster that initiates reactions through the reductive cleavage of SAM to generate a 5'-deoxyadenosine radical [49]. Subsequent hydride abstraction from the substrate with

radical SAM enzymes can lead to difficult reductions or mutase reactions, depending on the enzyme. For PylB, we hypothesize that exchange of the glycyl radical and a hydride between the beta and gamma carbons of lysine could create *R,R*-3-methylornithine, which is ligated to another lysine by PylC then oxidized by PylD to form pyrrolysine (Fig. 1) [48]. The proposed lysine mutase reaction of PylB would be the first time a radical SAM enzyme has been implicated in this type of mutase reaction involving carbon backbone rearrangement. Analogous reactions are performed by coenzyme B₁₂ enzymes, such as glutamate mutase, or methylmalonyl-CoA mutase [50].

Conclusions

Much has been learned about pyrrolysine since its discovery, but certain areas remain relatively unexplored. How pyrrolysine interacts with PylS for ligation to tRNA^{Pyl} is now better understood, but the role of the N-terminal domain of PylS (and PylSn) in *in vivo* charging of tRNA^{Pyl} remains unknown. The recent proposal of the first empirically based pathway of pyrrolysine biosynthesis must be coupled to *in vitro* demonstration of the reactions and properties of PylB, PylC, and PylD. Such studies may provide a new route to useful genetically encoded unnatural residues produced from modified biosynthetic precursors. Finally, the function of pyrrolysine in methylamine methyltransferase remains largely a hypothesis, and direct tests of the proposed mechanism and alternatives are highly desirable. In a similar vein, investigation of homologs of the methanogen methylamine methyltransferases lacking pyrrolysine may help to determine if pyrrolysine is essential for the function as a methylamine methyltransferase. The answers to such questions as these may provide insight into the forces that have brought and maintained pyrrolysine in the genetic code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work in our laboratory is supported by National Institutes of Health Grant GM070663 and Department of Energy Grant DE-FG0202-91ER200042. The authors wish to thank Charles Daniels and John Reeve for helpful discussions.

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Fig. 1.

Schematic of pyrrolysine and methylamine metabolism in *Methanosarcina* spp. Pyrrolysine is made from two molecules of lysine. In the proposed pathway, PylB converts one lysine into a methylated D-ornithine derivative, which is then ligated to another lysine by PylC. The resultant dipeptide is oxidized by PylD, which results in spontaneous elimination of water and formation of pyrrolysine. Pyrrolysine is then ligated to tRNA^{Pyl} by PylS. The pyrrolysyl-tRNA^{Pyl} is carried to the ribosome by the usual elongation factor for co-translational incorporation into one of the three methylamine methyltransferases, MtmB, MtbB, or MttB. Below each methyltransferase is indicated its particular methylamine substrate. Pyrrolysine in the catalytic site is hypothesized to form an adduct with that methylamine which orients and activates it for methyl group transfer to the Co(I) form of the corrinoid cofactor bound to either MtmC, MtbB, or MttB. Each corrinoid protein interacts preferentially with the methyltransferase indicated below it. Adventitious oxidation of the corrinoid protein can result in inactivation, and a single protein, RamA, can return the corrinoid protein to the Co(I) state. All three methylated corrinoid proteins can serve as substrates for MtbA, which methylates the thiol of CoM (HSCoM). Methyl-CoM can then serve to directly generate methane, or to enter pathways leading to carbon assimilation and carbon dioxide production. Further details are provided in the text.

Fig. 2.

Local context of homologs of *pyl* and amber methylamine methyltransferase genes identified in completely sequenced genomes curated at the National Center for Biotechnology (NCBI). Colors indicate known functions of the homologous gene in *Methanosarcina* spp.: blue, pyrrolysine biosynthesis; red, amber decoding as pyrrolysine; green, methylamine metabolism; and gray, function unknown or not known to be involved in pyrrolysine or methylamine metabolism in *Methanosarcina* spp. All organisms with a full set of the *pyl* genes also have at least one *mttB*, *mtbB*, *mtmB* gene with the conserved amber codon (TAG), not all amber-methyltransferase genes are shown for each species. Numbers below genes indicate nucleotide location in genome. The *Bilophila wadsworthia pylT* was identified on contig 1.181 (NZ_ADGP01000181.1), and the genome numbering is from the wgs scaffold at NCBI.

Fig. 3.

Secondary structure of tRNA^{Pyl} from *Methanosarcina acetivorans* and *Desulfitobacterium hafniense*. A). The tRNA^{Pyl} common to *M. acetivorans*, *M. barkeri* Fusaro and *M. mazei* is shown. Arrows indicate base substitutions found in *M. burtonii* (upper case), *M. mahii* (lower case), and *M. evestigatum* (lower case italics). Asterisks indicate positions at which base substitution resulted in less than 50% of UAG codon translation in a recombinant system [38]. B). The *Desulfitobacterium hafniense* tRNA^{Pyl} secondary structure. Colored residues indicate: dark orange, identical in archaeal and bacterial tRNA^{Pyl}; light orange, identical in bacterial tRNA^{Pyl}; and green, conserved (>70% identity) in bacterial tRNA^{Pyl}. Asterisks indicate bases contacting protein residues in *D. hafniense* PylSc:tRNA co-crystal [35].