Pyrrolysine in Archaea, a 22nd amino acid encoded through a genetic code expansion

Jean-François Brugère^{1,2}, John F. Atkins^{3,4}, Paul W. O'Toole² & Guillaume Borrel⁵

¹ Université Clermont Auvergne, Clermont-Ferrand, France

² School of Microbiology and APC Microbiome Institute, University College Cork, Cork,
Ireland

³ School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland

⁴ Department of Human Genetics, University of Utah, Salt Lake City, UT, USA

⁵ Evolutionary Biology of the Microbial Cell, Department of Microbiology, Institut Pasteur,
Paris, France

Correspondence to J.-F. B.: 5e CBRV, Facultés de Médecine et de Pharmacie, Université

Clermont Auvergne, Place Henri Dunant, F-63000 Clermont-Ferrand, France

e-mail address: J-Francois.Brugere@uca.fr

ABSTRACT:

The 22nd amino acid discovered to be directly encoded, pyrrolysine, is specified by UAG. Until recently, pyrrolysine was only known to be present in archaea from a methanogenic lineage (*Methanosarcinales*) where it is important in enzymes catalysing anoxic methylamines metabolism, and a few anaerobic bacteria. Relatively new discoveries have revealed wider presence in archaea, deepened functional understanding, shown remarkable carbon source-dependent expression of expanded decoding and extended exploitation of the pyrrolysine machinery for synthetic code expansion. At the same time other studies have shown the presence of pyrrolysine-containing archaea in the human gut and this has prompted health considerations. The article reviews our knowledge of this fascinating exception to the “standard” genetic code.

1. Introduction

It is now more than half a century ago since the genetic code was deciphered (1,2). The understanding gained of how just four different nucleobases could specify the 20 universal amino acids to synthesize effective proteins reveals a fundamental feature of extant life. It is a remarkable feat of information transfer. Correct aminoacylation, supported by aminoacyl-tRNA synthetases (aaRS), and highly discriminatory selection of tRNA whose anticodon is complementary to the codon, are essential for faithful decoding.

Deviations from the genetic code known in most organisms have been gathered under two different types termed “reassignment” and “recoding” (3). In specialized niches, such as certain mitochondria (4,5), the meaning of particular codons is reassigned such that wherever that codon occurs it has the new meaning, and only the new meaning. Many but not all of these reassignments involve UAG or UGA encoding a specific one of the 20 universally encoded amino acids rather than signifying translation termination. This is distinct from context dependent codon redefinition where the new meaning only applies at particular occurrences of the codon. Such redefinition is dynamic with specification of the new meaning being in competition with the standard meaning so that only a proportion of the product reflects the new meaning. It is a type of recoding. When dynamic redefinition occurs at UAG or UGA, which are by far the most frequent codons at which it occurs, one, or several different, of the 20 universal amino acids can be specified. Such occurrences are often termed stop codon readthrough as the identity of the amino acid specified is generally unimportant and functional significance derives from a proportion of the product having a C-terminal extension.

In addition to the 20 amino acids specified in all organisms, 2 additional amino acids, pyrrolysine and selenocysteine, are directly encoded by some organisms but not by others.

52 In the organisms that encode selenocysteine, which includes several archaea (6), such
53 specification is, with few though interesting exceptions (7), by UGA (8,9) and is mRNA
54 context dependent. With just one exception (the 3' region of selenoprotein P mRNA), even
55 when such specification does occur, it is in competition with the release factor mediated
56 termination i.e. the redefinition is dynamic. In contrast, when the 22nd directly encoded
57 amino acid, pyrrolysine, was discovered by Krzycki and colleagues (10,11) to be encoded by
58 UAG in an archaeon, an early question was whether or not all ribosomes decoded all UAG
59 codons in that organism as pyrrolysine, i.e. whether UAG was reassigned to specify
60 pyrrolysine. One alternative considered was that all UAG codons present were sometimes
61 decoded as either pyrrolysine or termination, and another was that a proportion of UAG
62 codons were more prone than others to specify pyrrolysine because of a context feature of
63 their encoding UAGs (a particular mRNA stem loop 3' of such UAG codons). At issue is
64 whether, unlike the selenocysteine specification, pyrrolysine is sometimes specified
65 incidentally and the only selective advantage is a continuous synthesis (UAG reading)
66 without importance to the identity of the amino acid incorporated. Pertinent to this issue is
67 the frequency of utilized UAGs in mRNAs in organisms that encode pyrrolysine. While it was
68 infrequent in the initially studied pyrrolysine-containing archaea, it was frequent in the
69 anaerobic bacteria that also encode pyrrolysine. As discussed below some of the dilemma
70 posed led to an exciting regulatory discovery (12). Related to the natural expansion of
71 genetic decoding exemplified by pyrrolysine encoding, was the excitement that arose with
72 the realization that the nature of pyrrolysine tRNA and of its aminoacylation could be
73 exploited for synthetic code expansion. Another reason for high initial interest in pyrrolysine
74 was the functional role it plays in methylamine-dependent methyltransferases. Recent
75 discoveries of additional organisms, especially archaea that utilize pyrrolysine with

distinctive traits have substantially extended appreciation of its importance (e.g. climate change and biogenic methane production or potential biofuel production; human health and realization of potential importance of pyrrolysine in gut microbes metabolism), making a review timely.

2. Pyrrolysine, a non-dispensable AA linked to anaerobic methylamines metabolism

From a biochemical perspective Pyl ($C_{12}H_{21}N_3O_3$; N^6 -{[(2*R*,3*R*)-3-methyl-3,4-dihydro-2*H*-pyrrol-2-yl]carbonyl}-L-lysine) is a typical L-lysine amino acid to which a pyrrole ring is branched on the lateral chain through an amide bond (biosynthesis in section 3.1). However, while L-lysine derivatives are already known in proteins, encompassing those from archaea like hypusine or methyllysine (13), Pyl does not originate from post-translational modifications of L-lysine, but is translationally incorporated, representing the 22nd proteinogenic amino acid. This presence has been mostly documented in three different kinds of proteins (MtmB, MtbB and MttB) which are methyltransferases involved in the metabolism of respectively mono-, di- and tri- methylamine (abbreviated in MMA, DMA and TMA). Until recently, the only other known Pyl-containing proteins were certain transposases (14) and a tRNA^{His}-guanylyltransferase Thg1 (15) both present in a subset of *Methanosarcinales*. In methyltransferases, Pyl is found in the active site, thus arguing for a non-dispensable role for biological activity. It captures methylamines (through its imine electrophilic group) before transferring one methyl-group to a Co(I)-corrinoid cofactor of an associated protein (MtmC/MtbC/MttC) (11). Recently, this non-dispensable role of Pyl towards methylamines has been strengthened by the discovery of natural MttB analogs without Pyl (16). These homologues of MttB have been identified in multiple bacteria as well as methanogenic and non-methanogenic archaea. One of these enzymes (named MtgB) has

been characterized in *Desulfitobacterium hafniense* YF51 and catalyses the transfer of a methyl group from glycine-betaine, but not trimethylamine, to a corrinoid protein. In consequence, Pyl in archaea seems tightly linked to methanogenesis from methylamines (MMA, DMA, TMA) and is non-dispensable for such a metabolism (17).

3. Components of the Pyl system

The first indication of unusual decoding of the *mtmB* gene of the archaeon *Methanosarcina barkeri* was that its mRNA contained an in-frame UAG codon that was not interpreted as a stop signal during translation (18). This was also the case in other *Methanosarcinales*, certain anaerobic bacteria and even for genes encoding the different protein families, MtbB and MttB (19). The crystal structure of *Methanosarcina barkeri* MtmB1 revealed the presence of an unknown amino acid, which was named L-pyrrolysine (11). It was determined that it relies on an UAG decoding tRNA (PylT) charged by Pyl (10). With specification of a new non-universally encoded amino acid, this dramatically showed a natural expansion of the genetic code (20).

Pyl synthesis and insertion only relies on 5 genes, *pylTSBCD* (or 6 when tRNA^{Pyl} synthetase is encoded by two genes, see below). In most cases, these genes are gathered in an operon-like structure as shown in Figure 1 (light grey box, upper left) (21,22). However, some exceptions to such genomic organisation have emerged (see some examples upper part of Figure 1) (21,23). Evidence of this minimum set of genes was obtained from generating in *E. coli* an MtmB protein containing Pyl at the correct location by co-expressing *pylTSBCD* from *Methanosarcina barkeri* (24). Figure 1 gives a schematic overview of the Pyl system based on the decoding of UAG as Pyl in the MttB protein of “*Candidatus Methanomethylophilus alvus*”, a *Methanomassiliicoccales* representative.

3.1. Synthesis of Pyl from a standard AA

The synthesis of pyrrolysine relies on three genes, *pylBCD* (22). It was initially hypothesized that L-lysine was one of the precursors, with the pyrrole ring added on its acyl chain derived from either L-isoleucine, L-proline, L-methionine, L-glutamate or D-ornithine. Indeed, supplying D-ornithine stimulates the readthrough of UAG codon of a reporter gene in *E. coli* expressing *pylTSBCD* (25). D-ornithine supply leads however to a desmethylpyrrolysine insertion instead of Pyl due to the respective enzymatic role of *pylCD* products that were identified (26). This suggested that PylB activity was mandatory to get Pyl and led Krzycki and colleagues to postulate that the reaction product catalysed by PylB was (3*R*)-3-methyl-D-ornithine. To obtain such a product, they demonstrated that PylB was an L-lysine mutase inverting the chiral centres in the pyrrole ring (27). Therefore, Pyl has only one kind of precursor, which is surprisingly the common proteinogenic amino acid, L-lysine. It is used twice in a three-reaction pathway (Figure 2). This pathway encompasses first the L-lysine mutase PylB, a radical S-adenosylmethionine (SAM) protein with a TIM barrel (28). The amide bond between a second L-lysine (at ϵ location) is then catalysed by PylC to form (3*R*)-3-methyl-D-ornithine. Finally, PylD initiates an (NAD dependent) oxidation of this molecule to form a semi-aldehyde, intermediary compound that self-reacts to form the pyrrole ring by dehydration (Figure 2).

3.2. Linking Pyl to a specifically dedicated tRNA

The two other genes of the Pyl system, *pylT* and *pylS*, encode respectively a tRNA whose anticodon is complementary to the UAG codon, and the subunit (PylS) of the tRNA^{Pyl} synthetase. This tRNA synthetase / tRNA pair possesses a strict orthogonality towards the

148 other 20 proteinogenic amino acids, leading specifically to link Pyl to this dedicated tRNA
149 (10). The structural analysis of near complete PylS (PylSc from the bacterium
150 *Desulfitobacterium hafniense*, see below) was deduced either from the crystallisation of the
151 apoprotein alone or from its co-crystallisation with tRNA^{Pyl}. It revealed a pair highly distinct
152 from the other ones, even the most closely related ones (29), with both tRNA^{Pyl} synthetase
153 and tRNA^{Pyl} having original features compared to other known equivalents. Moreover,
154 differences are also observable among Pyl-decoding bacteria and archaea, for their
155 (unusually small) tRNA^{Pyl} and for PylS (see below).

156 The active tRNA^{Pyl} synthetase is a homodimer belonging to the class II family of aaRS. While
157 being very different from other class II aaRS, its catalytic core shares common traits with
158 tRNA^{Phe} synthetase, suggesting that these two aaRS are evolutionarily related (section 4.3).
159 This tRNA synthetase is encoded by a unique gene in *Methanosarcinales* (*pylS*) and two in
160 bacteria, one encoding the Cter portion of PylS (*pylSc*) and one encoding ~110 amino acids of
161 PylS corresponding to the Nter portion of *Methanosarcinales* PylS (*pylSn*). The N-ter part of
162 *Methanosarcinales* PylS (homolog to PylSn) is dispensable for correct aminoacylation *in*
163 *vitro*, but is necessary for correct activity *in vivo* (30). Due to its highly hydrophobic nature,
164 likely preventing its *in-vitro* crystallisation, there is no formal structural data on tRNA^{Pyl} with
165 complete PylS or PylSc+PylSn: initial work performed 10 years ago gave however interesting
166 features with several structural data encompassing the catalytic domain structure (C-ter
167 region 185–454) of the archaeal PylS from *Methanosarcina mazei* with Pyl (31) and the
168 bacterial PylSc structure (from *Desulfitobacterium hafniense* (29,32)). PylSn from bacteria
169 has been shown to interact and recognize tRNA^{Pyl} in a specific way, enhancing the affinity /
170 specificity with tRNA^{Pyl} (33). Recently, the crystal structure of the Nter domain of PylS (from
171 the archaeon *Methanosarcina mazei*) with tRNA^{Pyl} gave precious new insights, and notably

the determinants of its high specificity against canonical AAs not based on anticodon recognition (34). These interactions are susceptible to nucleotides mutations of tRNA^{Pyl} that are not involved in PylSc interactions with tRNA^{Pyl} (for example in the T-arm and variable loop). PylS Cter part / PylSc are responsible at least for the catalytic amino-acylation and partly for the recognition of tRNA^{Pyl}. Nonetheless, anticodon UCA is not directly involved in its recognition by PylS (31), while only few specific bases on anticodon stem and loop nucleotides of tRNA^{Pyl} seem necessary, like for example the bases immediately adjacent to the anticodon in the archaeon *Methanosarcina barkeri* (35). However, recent genomic identification of the Pyl system in archaeal lineages other than *Methanosarcinales*, especially in the *Methanomassiliicoccales* gives indications that these properties are not shared by all Pyl systems (see section 4.2).

4. Recent Pyl discoveries

As mentioned, work performed more than a decade ago showed that at least some Pyl gene cassettes from archaea or bacteria are fully functional when expressed in *E. coli* (i.e. UAG specification of Pyl) (24). Moreover, desmethylpyrrolysine was incorporated when D-ornithine was provided to *E. coli* expressing a Pyl system, indicating a low susceptibility of the side chain for aminoacylation of tRNA^{Pyl} by PylS. These properties were therefore used to divert Pyl system from its natural function, allowing non-canonical amino acids (ncAAs) to be included in proteins, and so in organisms, by expressing (wild type or mutated) PylS / tRNA^{Pyl} in the presence of (free) ncAAs in the growth medium. Achievements and strategies for using Pyl system components as a genetic code expansion tool have been recently reviewed (e.g. (36-39)). To date, more than 100 ncAAs have been successfully engineered and incorporated, in a broad phylogenetic spectrum of organisms ranging from bacteria and

unicellular eukaryotes (e.g. *Saccharomyces cerevisiae* (40)) to metazoans, like *Caenorhabditis elegans* (41), *Drosophila melanogaster* (42), the Zebrafish (43) or the mammal *Mus musculus* (44).

4.1. Pyl encoding, a widespread feature in the archaeal world?

For a long time, all known methanogens were part of six orders gathered into two large classes (Class I: *Methanobacteriales*, *Methanococcales*, *Methanopyrales*; Class II: *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*) (45). Recent data based on culture and metagenomic approaches have largely modified our vision of the diversity of the methanogens. Indeed, methanogenesis was predicted, and sometimes experimentally confirmed, in several recently discovered lineages that are only distantly related to the Class I and Class II methanogens: the *Methanomassiliicoccales* (46-47), the *Methanofastidiosa* (48), possibly the *Bathyarchaeota* (49), the *Verstraetearchaeota* (50) and the *Methanonatronarchaeia* (51) (Figure 3). A striking characteristic of the members of all these lineages is their dependence on methyl-compounds for methanogenesis, a metabolism present in a minority of previously known methanogens. Along with the methyl-dependent hydrogenotrophic methanogenesis, the Pyl system was also found to be more widespread, being present in some of these new methanogen lineages and for the first time in non-methanogenic archaea (Figure 3). Among these lineages, the Pyl system was first described in the *Methanomassiliicoccales* where it exhibits several unusual characteristics compared to previously characterized Pyl systems (see section 4.2). In addition, one of the five currently known genomes of the *Verstraetearchaeota* harbours the genes for the synthesis and encoding of Pyl (Table 4 in supplementary data of (50)). In this genome, PylS is mentioned as being encoded by two genes, *pylSn* and *pylSc*. This is different from *Methanosarcinaceae*

members, that have a single gene coding for both PylSn and PylSc domains, but similar to what was observed in bacteria so far. Recently, the Pyl system was also reported from the *Methanonatronarchaeia*, a class composed of extreme halophiles (51). In all these novel lineages, the occurrence of the Pyl system was coupled with the presence of one or several Pyl-containing methyltransferases (i.e. MtmB, MtbB, MttB) needed for methylamine utilization. This strengthens previous observations that the Pyl system is dedicated to the incorporation of Pyl in these methyltransferases and thus associated to methylamine utilisation. Other methanogens missing the Pyl-containing methyltransferases (e.g. *Methanosphaera* spp. (52), *Methanofastidiosa* spp. (48)), also lack the Pyl system, which argues that this system is more linked to methylamine methyltransferases than to archaea relying on a methanogenesis based on methyl-compounds. In addition, several members of the candidate division MSBL-1 (or *Persephonarchaea*) harbour a complete set of genes for Pyl synthesis and encoding, as well as *mtmB*, *mtbB* and *mttB* genes, as determined from single-cell genomics (53). Interestingly, genomic analyses of these uncultured archaea revealed that they are likely not methanogens, but rather sugar-fermenters. Moreover, the components of the Pyl system in MSBL-1 are phylogenetically related to the bacterial ones, and more specifically with the Pyl system found in the bacteria *Acetohalobium arabaticum* (53). This possibly indicates a horizontal gene transfer (HGT) between these organisms living in the same kinds of environment (hypersaline) and gives new missing pieces to the puzzle of the unknown history, evolution and dissemination of Pyl. The main conclusions to be drawn from these results are that i) archaea with a Pyl-encoding capacity do not only belong to the *Methanosarcinales* but have a much wider distribution in the archaeal phylogeny, probably not limited to the recently discovered lineages, and that ii) Pyl is not necessarily associated to methanogenesis in archaea .

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245 4.2. The case of Pyl in *Methanomassiliicoccales*

246 The Pyl system of *Methanomassiliicoccales* was the first archaeal one found outside of the
247 *Methanosarcinaceae* (54) and it revealed several originalities compared to
248 *Methanosarcinaceae* or to bacteria (23). It is not shared by all members of this methanogen
249 order, even among closely related species living in an identical environment, the human gut
250 (55). This patchy distribution of the Pyl system in the *Methanomassiliicoccales* is most likely
251 due to its complete loss in some representatives. In this order, all *pyl* genes are not always
252 grouped together in an operon-like structure. This is, for example, the case in “*Candidatus*
253 *Methanomethylophilus alvus*”, in which *pylB* is distant (more than 600 kbp) from *pylTSCD*
254 (Figure 1). It is notable that important genes for methanogenesis are present within a few
255 kilobases from either *pylB* or *pylTSCD*, those of the methyl-coenzyme M reductase catalysing
256 methane formation, and those of the methyltransferases dedicated to methylamines,
257 respectively. (23,56). Also, *Methanomassiliicoccus luminyensis* is the only known organism to
258 possess more than one copy of all *pyl* genes (23). The most amazing feature of the
259 *Methanomassiliicoccales* Pyl system concerns the nature of both tRNA^{Pyl} and PylS. Indeed,
260 the (UAG-recognizing) tRNA^{Pyl} shows a low number of universally conserved bases among
261 tRNA^{Pyl} (21,23). It has the usual cloverleaf secondary structure of tRNAs, although in a
262 condensed manner (Figure 4), and a longer anticodon stem (23). In *Methanomassiliicoccales*,
263 tRNA^{Pyl} shows the surprising feature of having one or two mismatches in the anticodon
264 “stem” (23) (Figure 4). This is a special feature among all natural tRNAs.
265 *Methanomassiliicoccales* PylS corresponds to the Cter part of *Methanosarcinaceae* PylS or to
266 the PylSc of Bacteria. No homolog of the Nter part of PylS / PylSn has been identified in all
267 complete genomes of *Methanomassiliicoccales* analysed so far. The structure of PylS in

Methanomassiliicoccales is thus likely different than those of *Methanosarcinaceae* members and bacteria. This case seems also present in other archaea (e.g. *Persephonarchaea* (MSBL-1)). Phylogenetic analyses revealed that the *Methanomassiliicoccales* Pyl system has an intermediary position between the *Methanosarcinales* and the bacteria/MSBL-1 (57). In view of these very atypical features, the functionality of the Pyl system in *Methanomassiliicoccales* was therefore questionable. However, the utilization of methylamines as a methanogenesis substrate by several members of the *Methanomassiliicoccales* strongly suggests that they possess a functional Pyl system. TMA, DMA and MMA utilization has been reported for a pure culture of *Methanomassiliicoccus luminyensis* (58), as well as MMA utilisation by an enrichment culture of “*Ca. Methanoplasma termitum*” (59) and TMA utilisation by “*Ca. Methanomethylophilus alvus*” (55). The remaining question is therefore, how these organisms can accommodate such a different tRNA^{Pyl} and the absence of Nter part of PylS / PylSn. One possibility is that in this lineage, PylSc-like / shortened PylS efficiently binds and links Pyl to its dedicated tRNA^{Pyl} in absence of the PylSn domain. Another one is that it requires an unknown factor that would functionally replace PylSn and/or enhance the effectiveness of this restricted system. In any case, this indicates that other mechanisms than those described so far are possible. Finally, it seems that the presence of the Pyl system in *Methanomassiliicoccales* has led to different genomic adaptations among clusters of *Methanomassiliicoccales* (56), some of which are mentioned in section 4.4.

4.3. About the origin and evolution of Pyl

Recent data have revealed that both methanogenesis based on methylamines (methylophilic one and methyl-dependant hydrogenotrophic one) (60) and Pyl-encoding

behaviour are more widespread than stated just a few years ago (Figure 3). The hypothesis that methanogenesis is a very ancient metabolism that arose before the last common ancestor of *Archaea* has been strengthened by these data (61) and in a same way, a similar hypothesis can be made for the origin of the 22nd amino acid.

It is generally accepted that LUCA (Last Unique Common Ancestor) did not have Pyl and that the genetic code based on 20 proteinogenic amino acid catalogue was already established. Among the hypotheses of the presence of Pyl in various and extremely distant lineages, Gogarten and colleagues postulated that Pyl could have been encoded by cells living at the time of LUCA, whose descendants are however either extinct or not yet discovered (62). In this hypothesis, Pyl and associated coding behaviour persisted until present time because, before their extinction, some of the descendants of this hypothetical lineage would have spread the Pyl system by HGT to some LUCA descendants, an archaeon (leading to Pyl-encoding in the lineage *Methanosarcinales*), and a bacterium. Molecular and structurally-based phylogenetic studies seem to indicate that PylS arose from a duplication of PheRS-coding gene anterior to LUCA (31). The extra copy of this gene could have been the matrix for Pyl invention, once in one lineage, or possibly independently in several lineages, later during the evolution (31). The evidence of Pyl in *Methanomassiliicoccales* and the nature of its encoding may indicate a unique origin of the Pyl-system (in either archaea or bacteria) and its later spread to the other domain of life by HGT(s). This would make its origin subsequent to LUCA (23). The recent discovery and the nature of the Pyl system in *Persephonarchaea* (MSBL-1) also supports this view. Irrespective of such considerations, current data strongly suggests that Pyl-encoding is a very ancient trait, which arose likely just once as a post LUCA invention possibly linked to methanogenesis. It could have then evolved and been retained in some organisms for which methylamine metabolism was key to

survival and could have been further transferred across the bacterial and archaeal domains. While our understanding is likely to deepen further with the probable discovery of new Pyl-containing organisms, interesting questions can now be asked about how organisms have adapted to this genetic code expansion. One type of informative analysis has come from studying the consequences of deleting a functional Pyl system (63).

4.4. Implications and consequences of the natural presence of a UAG decoding tRNA

While genetic studies with mutants of tRNA (*amber* suppressors) that permit decoding of UAG in competition with release factors have provided decoding insights, the organisms studied have not adapted over long evolutionary time to the presence of the UAG-decoding tRNA. In contrast, such adaptation has occurred in Pyl-containing organisms. Though the situation in such organisms is not as extreme as the ciliate *Condolysoma magnum* where UAG, UAA and UAG have been reassigned to be sense codons, with termination being specified by a recoding event (64,65), since whole organisms are involved, the issues are nevertheless more substantial than in the case of decoding small genomes such as mitochondria.

The mechanism used for UGA specification of selenocysteine was first considered as a potent paradigm from which Pyl insertion could be extrapolated. UGA specification of selenocysteine is dependent on special features of the mRNA 3' of the UGA codon. UGA codons that do not have the recoding signals involved function in the standard manner of specifying termination (66,67). A counterpart Pyl insertion sequence (PYLIS) element was first postulated (25). However, consistent with a detailed bioinformatics analysis (14), such a *cis*-acting element was found to be not necessary for insertion of Pyl in engineered *E. coli* (68, 25). The resulting hypothesis is that Pyl insertion relies “simply” on a competition

between tRNA^{Pyl} and aRF1 on UAG codon during mRNA translation at ribosome. This is likely promoted by a peculiar aRF1, as some distinct patterns are observed in Pyl coding *Methanosarcinaceae* compared to other archaeal aRF1 (14). In such a case, one can imagine that UAG codon that does not need to be reassigned to a Pyl would be counter-selected in favour of the other two non-sense codons. In agreement with this hypothesis, it is observed that the UAG codon usage in Pyl-coding archaea is low (14,69) with *Methanosarcinaceae* having only about 4 to 6 % CDSs with an in-frame UAG codon (12). Recent data indicate that UAG usage is even lower in some *Methanomassiliicoccales* (in-frame UAG predicted in 2.8% of CDSs in “*Ca. M. alvus*”). However, the UAG usage varies among *Methanomassiliicoccales*, independently of GC% of considered genomes (56). More surprisingly, members of *Methanomassiliicoccales* are predicted, through bioinformatics analysis, to use Pyl in a larger repertoire of proteins than the usual methyltransferases described above (56). This encompasses 16 non-MT proteins in “*Ca. M. alvus*” (56) and more than 40 non-MT proteins in another representative (methanogenic archaeon ISO4-H5 (70)). This questions a possible “genetic code domestication” of Pyl in *Methanomassiliicoccales* corroborating the hypothesis of an on-going irreversible conversion of UAG sense from a stop sense to a Pyl-encoding one proposed in *Methanosarcinales* (69). Indeed, the peculiar properties observed in some *Methanomassiliicoccales* may reflect the on-going establishment of a genetic code having 21 amino acids encoded by 62 codons and only 2 codons instead of 3 being non-sense. This 21 proteinogenic AA repertoire would be currently invading the proteome of some of these organisms by neutral selection on most proteins, while it cannot be excluded that some cases are providing / will provide benefits for the cell.

The frequency of UAG codons is much higher in Pyl-coding bacteria than in archaea, with about 20-28% noted in one study (14,12). In the absence of evidence for mRNA context

dependency for the natural Pyl specification that occurs in some bacteria, how, if it is, can specification of Pyl by the numerous UAG codons be avoided? For *Acetohalobium arabaticum*, a member of the phylum *Clostridia*, a fascinating resolution to the dilemma has been discovered. While UAG specifies Pyl when the cells are grown in trimethylamine, when instead the cells are grown on pyruvate as a carbon source, UAG only specifies termination (12). This presumably means that when the cells are grown on trimethylamine, many proteins for which pyrrolysine has no specific function have C-terminal extensions with several amino acids encoded after pyrrolysine. Indeed, some such proteins have been identified (12). Doubtless, selection has had ample time to adjust, where significant, further downstream stop codon position. However, for most genes such selective pressure may be weak since where analysed there is an abundance of proteases that act on incorrectly folded peptides, and the efficiency of newly isolated genetically selected *E. coli* amber suppressors with little or no discernible growth rate. Nevertheless, a decoding-wide strategy has evolved for when trimethylamine is absent.

5. Conclusion

Recent data about Pyl in archaea have renewed our comprehension of this original and still enigmatic exception in the “universal” genetic code. It was recently uncovered that this system is not confined to the *Methanosarcinales* in the domain Archaea, but present in at least 4 other orders, and not only methanogens. In members of those novel lineages, the divergences from canonical Pyl systems offer new opportunities to understand its functioning. These advances will likely facilitate improved utilization of the Pyl system as a biotechnological tool for engineering of proteins / organisms by incorporating ncAAs in their proteins. Also, Pyl is mandatory for methylamines utilisation under anoxic conditions which

may explain its presence and maintenance in diverse and phylogenetically distant organisms. One of these methylamines, TMA, was recently shown to be a by-product of human intestinal bacteria, and the unique precursor of plasma TMAO, an important atherosclerogenic factor (71). Thus, autochthonous archaea able to metabolise TMA in the gut (some *Methanomassiliicoccales* members), or engineered microbes with a Pyl-encoding behaviour to “trap” TMA in the gut may be valuable auxiliaries for preventing cardiovascular diseases in humans (58). Besides these biotechnological aspects, its study will likely facilitate understanding of the genetic code evolution among lineages and the intimate mechanisms of such important biological functions as translation. All of these bode well for increasing interest in Pyl and its host organisms in the coming years.

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Declaration of interest

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Author contribution statement

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609

610

611 **ABBREVIATIONS:**

612 AA: amino acid

613 aaRS: amino-acyl tRNA synthetase

614 aa-tRNA: amino-acyl charged tRNA

615 DMA: Dimethylamine

616 HGT: Horizontal gene transfer

617 IUBMB: International union for biochemistry and molecular biology

618 IUPAC: International Union of Pure and Applied Chemistry

619 LUCA: Last Unique Common Ancestor

- 620 MMA: monomethylamine
- 621 ncAA: non-canonical AA
- 622 Pyl: Pyrrolysine
- 623 PYLIS: Pyl insertion sequence
- 624 Sec: Selenocysteine
- 625 SECIS: Sec insertion sequence
- 626 TIM: Triose phosphate isomerase
- 627 TMA: trimethylamine

FIGURE LEGENDS

FIGURE 1: Overview of the components and functioning of the Pyl-system.

Some examples of genomic organisation of genes involved in Pyl synthesis and encoding are provided in the upper part of the figure (adapted from (21), (23) and (53)). Examples from Archaea and Bacteria are given respectively in a red delimited box at left and a green delimited box at right. The “canonical” organisation in archaea is given in the upper light grey box. The functioning of the Pyl-system is depicted here taken the example of Pyl insertion by readthrough of UAG codon contained in *mttB* mRNA of the *Methanomassiliicoccales* “*Candidatus* Methanomethylophilus alvus”. (A) It relies first on the biosynthesis of Pyl (depicted with more details in Figure 2). Pyl is then engrafted on a specific tRNA (encoded by *pylT*) whose anticodon recognizes UAG codons. This implies the specific reaction catalysed by PylRS (in this example encoded by a truncated version of *pylS* corresponding to *PylSc* (see section 4.2 for more details). (B) All the genes encoding the (mono- di- or tri-) methylamine-corrinoid methyltransferases (respectively *mtmB*, *mtbB* and *mttB*) show an in frame UAG codon, which should typically lead to a translational stop, i.e. a truncated protein. (C) Due to tRNA^{Pyl} able to interact with UAG codon, translation is going on by readthrough this codon which leads to insertion of Pyl in the growing polypeptide. Functioning of such a system at the genomic and cellular level is discussed in section 4.4.

FIGURE 2: Biosynthesis of Pyl.

The complete biosynthesis pathway of L-pyrrolysine from 2 lysines catalysed by PylB, PylC and PylD is depicted. This corresponds respectively to reactions #13074, #13080 and #13084 adapted from MetaCyc (72), as determined from original data in (26,27).

651

652 **FIGURE 3:** Distribution of the Pyl-encoding microorganisms in the domain Archaea, as
653 indicated by red dots. Lineages in bold contain predicted (based on genome analysis) or
654 experimentally characterized methanogens. “H₂ + R-CH₃” indicates that some members (e.g.
655 *Methanosphaera* in *Methanobacteriales*, *Methanosarcinaceae* in *Methanosarcinales*) or all
656 members of the lineage can gain energy through methyl-dependent hydrogenotrophic
657 methanogenesis. Depending on the methanogen species, one or several methylated
658 compounds (R-CH₃, e.g. methanol, TMA, DMA, TMA, methanethiol, glycine betaine) can be
659 used. Some of the names and the topology of the tree are derived from (56).

660

661 **FIGURE 4:** Sequence diversity among tRNA^{Pyl}.

662 The sequence and structure of tRNA^{Pyl} are depicted here (with a cloverleaf representation)
663 using selected examples of known sequences, encompassing a bacterial one (green box) and
664 four archaeal ones (red box). Compared to other tRNAs, tRNA^{Pyl} is smaller and has marked
665 differences among Pyl lineages, especially between *Methanosarcinaceae* and other recently
666 discovered Pyl-encoding archaeal lineages. The MSBL-1 tRNA^{Pyl} shows a larger variable loop
667 (green arrow) and D-loop (blue arrow), than *Methanomassiliicoccales* tRNA^{Pyl} shown here
668 that has the unique characteristics of mismatches / a loop in their anticodon « arm » (red
669 arrow). This is especially evident in *M. luminyensis* and “*Ca. M. intestinalis*” (23). This figure
670 is an adaptation of the data available in (23,53). Only one of the 2 tRNA^{Pyl} recovered from *M.*
671 *luminyensis* genome is indicated (tRNA^{Pyl} #1 according to (23)).

Bacteria

Desulfitobacterium hafniense

Archaea

M. barkeri

Methanosarcinaceae

Methanomethylophilus alvus

Methanomass. luminyensis

Methanomassiliicoccales

MSBL1 - SAG AAA382A20

Persephonarchaea