Adding pyrrolysine to the *Escherichia coli* genetic code

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Received 23 September 2007; accepted 10 October 2007

Available online 23 October 2007

Edited by Lev Kisselev

Abstract Pyrolysyl-tRNA synthetase and its cognate suppressor tRNA\(^{\text{Pyl}}\) mediate pyrrolysine (Pyl) insertion at in frame UAG codons. The presence of a RNA hairpin structure named Pyl insertion structure (PYLIS) downstream of the suppression site has been shown to stimulate the insertion of Pyl in archaea. We study here the impact of the presence of PYLIS on the level of Pyl and the Pyl analog N-e-cyclopropyl-oxy-carbonyl-\(L\)-lysine (Cyc) incorporation using a quantitative lacZ–lac tandem reporter system in an *Escherichia coli* context. We show that PYLIS has no effect on the level of neither Pyl nor Cyc incorporation. Exogenously supplying our reporter system with d-ornithine significantly increases suppression efficiency, indicating that d-ornithine is a direct precursor to Pyl.

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Keywords: PYLIS; Pyrrolysine; Selenocysteine; Translation termination; Stop codon suppression; *Methanosarcina barkeri*

1. Introduction

In the past 10 years, considerable effort has been invested in devising systems permitting the genetic encoding of modified non-natural amino acids and thus their incorporation into proteins in *Escherichia coli*, yeast and potentially mammalian cells [1,2]. Such systems rely on the presence in the cell of a unique tRNA/aminocyl-tRNA synthetase (aaRS) pair specifically tailored for the recognition and aminoacylation of the non-natural amino acid to be inserted. This special tRNA/aminocyl-tRNA synthetase pair is called orthogonal, as its two components do not interfere with any of the other cellular tRNAs, aaRSs and amino acids. In order to achieve selectivity for the non-natural amino acid, the active site of a number of aminocyl-tRNA synthetases was engineered using a combination of biochemical and elegant in vivo selection methods [1]. The tRNA molecules were also devised so that they are only aminoacylated with the modified amino acid and possess an anticodon nucleotide sequence complementary to UAG or UGA stop codons [1]. Upon aminoacylation onto its cognate tRNA, the non-natural amino acid is delivered by the elongation factor to an in-frame UAG or UGA stop codon located within the coding sequence of a protein of choice. Using this strategy, more than 30 non-natural amino acids have been successfully inserted so far into proteins either in *E. coli* or in yeast [1].

Nature uses a similar approach for the insertion into proteins of selenocysteine (Sec) and pyrrolysine (Pyl), the 21st and 22nd genetically encoded natural amino acids [3,4]. Sec is found in a limited number of organisms from all three domains of life and Pyl is found only in the *Methanosarcinaceae*, a family of methanogenic archaea, as well as in two unrelated bacterial species [5–7]. While Sec is incorporated at UGA stop codons, Pyl is inserted into polypeptides in response to UAG codons [5]. Both modified amino acids utilize a specific tRNA, tRNA\(^{\text{Sec}}\) or tRNA\(^{\text{Pyl}}\), with anticodon sequences complementary to the opal and amber stop codons respectively [3,5]. Since these naturally available orthogonal pairs could be particularly appealing tools for the incorporation of unnatural amino acids, a comprehensive understanding of their recoding mechanism becomes a prerequisite. While the recoding mechanism underlying Sec incorporation into proteins is now fairly well described in both bacteria and eukarya [3,8], the requirements for Pyl insertion at designated UAG codons remain unclear. Genetic and biochemical studies have shown that a hairpin structure termed selenocysteine incorporation structure (SECIS) present immediately 3' of the UGA codon (in bacteria) or in the 3' untranslated region of the mRNA (in archaea and eukarya), is required for the decoding of UGA by tRNA\(^{\text{Sec}}\) [9]. The SECIS element interacts with the carboxy-terminal domain of a specific elongation factor SelB for the binding and delivery of Sec-tRNA\(^{\text{Sec}}\) to the suppression site [8,10]. Similar RNA structures were predicted to exist downstream of the suppression site in the mRNA of some of the Pyl containing *Methanosarcinae* proteins [11,12]. These structures were called PYLIS by analogy to the SECIS elements [11]. However, the stop codon recoding mechanism could be different for Sec and Pyl [13]. No Pyl specific elongation factor seems to be required for delivery of Pyl-tRNA\(^{\text{Pyl}}\) to the ribosome [14–16]. Although a recent study reports that a PYLIS element may facilitate UAG read-through and consequently Pyl incorporation in the monomethylamine methyltransferase in the homologous context of *M. acetivorans* [17], the absence of an identifiable PYLIS in the di- and tri-methylamine methyltransferase mRNAs questions the functional significance of such structure for Pyl recoding [13].
tRNA\textsubscript{Pyl} and PylRS constitute a new natural tRNA/aminoc-tRNA synthetase pair that is orthogonal to the canonical genetic code and could potentially be optimized for the incorporation of unnatural amino acids into proteins. To this end, we recently solved the crystal structure of the PylRS from \textit{Methanosarcina mazei} [18] and elucidated the identity elements of tRNA\textsuperscript{Pyl} [19]. We now would like to investigate whether a specific mRNA context such as PYLIS is required for the insertion into \textit{E. coli} proteins of Pyl or the Pyl structural analogue N-cyclopentylcarboxyl-L-lysine (Cyc), or whether these amino acids could be inserted at any desired position instead. Using the lac\textsubscript{Z}–\textit{luc} dual reporter system [28], we set out to precisely quantify the impact of the PYLIS structure on UAG read-through efficiency. Finally, we attempted to obtain an \textit{E. coli} strain that self-sufficiently produces Pyl, and thus efficiently encodes all 22 known natural amino acids. For this purpose we investigated a number of intermediate metabolites required for Pyl biosynthesis and insertion into \textit{E. coli} proteins.

2. Materials and methods

2.1. Bioinformatics and databases

The available sequences of the mono-, di- and tri-methylamine methyltransferases genes (\textit{mttB}, \textit{mttB} and \textit{mttB}, respectively) were collected from Pyl utilizing organisms' genome database. The sequences of those genes and related information including loci, class, length, strand, were loaded into a database, which can be accessed at http://www.lri.fr/~zhouyu/pylis/. Two strategies were used to identify putative PYLIS in the Ply-associated genes. The first one was to find similar structures compared to experimentally validated structure[12] in those genes. The second strategy was to use the programs, which could find common structures in a set of unaligned sequences like RSEARCH [20], Infernal [21], RNAMotif [22], PatScan [23], Foldalign [24], CARNA [25] and RNAProfile [26] were tried. Significance of a candidate structure was inferred from statistically measured P-value, based on the probability of finding the structure in well-hit random sequences from GenRGenS [27].

2.2. \textit{E. coli} strains

Most of the experiments were performed using \textit{E. coli} BL21(DE3) strain (Invitrogen). Control experiments were performed with the \textit{E. coli} DH5\textalpha{} strain, which carries a chromosomal copy of the glutamine \textsuperscript{supE44} amber suppressor.

2.3. Cloning of \textit{pylT} and \textit{pylS} genes

The \textit{pylT} and \textit{pylS} genes were cloned into the pETDuet-1 (Novagen) for co-expression. The \textit{pylS} gene was amplified by PCR from a previous construct, cloned into the pCR2.1-TOPO vector (Invitrogen), sequenced and subcloned into the multi-cloning site one of pETDuet-1 between BamHI and SalI. The \textit{pylT} gene was cloned downstream of \textit{pylS} using the single HindIII restriction site. The nucleotide sequence and orientation of PCR was verified by automated sequencing.

2.4. Cloning of \textit{pylB}, \textit{pylC}, and \textit{pylD} genes

The \textit{pylB}, \textit{pylC} and \textit{pylD} genes were cloned into the pRSFDuet-1 vector (Novagen) for co-expression. The \textit{pylD} gene was amplified by PCR from \textit{M. Barkeri} fusaro genomic DNA, cloned into the pCR2.1-TOPO vector (Invitrogen), sequenced and sub-cloned into the multi-cloning site one of pRSFDuet-1 between NcoI and HindIII. The \textit{pylB} and \textit{pylC} genes were co-amplified by PCR from \textit{M. Barkeri} fusaro genomic DNA. The amplified piece of DNA was cloned into the pCR2.1-TOPO vector (Invitrogen), sequenced and subcloned into the multi-cloning site two of pRSFDuet-1 between BglIII and Kpnl.

2.5. Construction of the dual lac\textsubscript{Z}–\textit{luc} fusion reporter system

The commonly used yeast dual reporter system pAC99 [28] has been adapted for expression in \textit{E. coli}. The lac\textsubscript{Z}–\textit{luc} fusion was amplified by PCR from the pAC99 with the phusion (finzyme) polymerase and primers lac.w (5'-TCACCTGCCTCGTTCCTACAGC-3') and luc.c (5'-TTTACAATGTTGCTTCCGCTTCCCT-3'). After gel purification, the PCR product was cloned at the ZraI site into the pCL1 plasmid. pCL1 is a pCDF-Duet (Novagen) based plasmid where the fragment NcoI-Avrl was replaced by a linker (5'-CATGATTCCAGACGGCTGCAGTTCACAGC-3') with compatible ends. This allowed the insertion of the ZraI BamHI and HindIII restriction sites and the deletion of pCDF-Duet-1 second T7 promoter. The resulting plasmid was called pCL99. It carries the lac\textsubscript{Z}–\textit{luc} fusion under the control of an inducible T7 promoter, the streptomycin resistance gene and the CloDF13-derived CDF replicon. The various stem loop structure sequences tested for their impact on suppression efficiency were inserted by oligo-cloning at the Mscl restriction site of pCL99, placing them 3' of the suppression site, at the junction between the lac\textsubscript{Z} and \textit{luc} genes. An in-frame control was constructed, replacing the UAG stop codon by an AAG (Lys) codon, for all the tested stem loop structures. Sequence of all the constructs was verified by automated sequencing.

2.6. Quantification of UAG stop codon read-through

\textit{E. coli} BL21 competent cells were transformed with the pETDuet-1 borne \textit{M. Barkeri} pyl\textsubscript{S} and pyl\textsubscript{T} genes and a series of pCL99 construct carrying the various stem loop sequences structures tested for their impact on suppression efficiency. The transformants were grown overnight at 30°C in 1 ml of Luria Broth in the presence of 1 mM of IPTG, 20 mM of Cyc (Sigma, as indicated in figure legends) and the appropriate antibiotics. For each culture, a 500 \textmu{}l aliquot was split into two tubes and centrifuged for 2 min at room temperature. Each cell pellet was resuspended in 100 \textmu{}l of luc buffer [29]; followed by 50 \textmu{}l of acid-washed glass beads (SIGMA). Cells were opened up by vortexing for 20 min at 4°C, 1 ml of luc buffer was added at the end. Both luciferase and \beta-galactosidase activities were quantified using 1 \mu{}l and 5 \mu{}l of the cell extracts, respectively [29]. The efficiency of stop codon read-through was calculated as the ratio of luciferase activity over \beta-galactosidase activity reported to the ratio of luciferase activity obtained with the corresponding in-frame construct [28,29]. Each value is the median of at least five independent experiments. In order to determine the impact of the addition of a series of \textit{d} amino acids (\textit{d}-proline, \textit{d}-isoleucine, \textit{d}-glutamate and \textit{d}-ornithine), a concentrated solution of each of these amino acids was added to the growth media (in order to reach a final concentration of 5 mM) at the time of the IPTG induction. UAG read-through was quantified as described above.

3. Results and discussion

3.1. PYLIS sequence is only present in \textit{mtmB} genes

Pyrolysine is found in only five archaean organisms from the \textit{Methanomasarinae} and two unrelated bacterial species [5,7]. Previous bioinformatics analysis led to the identification of all the archaean genes with an in-frame UAG codon, which may encode Pyl in the translated gene products [13]. From this analysis, Pyl is likely present in a set of monoo-, di- and tri-methylamine methyltransferases (coded by \textit{mttB}, \textit{mttB} and \textit{mttB}, respectively), which is consistent with experimental evidence [30,31], as well as in some transposases [13]. Searches for sequences and secondary structures similar to PYLIS in the read-through regions (140 nt downstream of the \textit{Pyl UAG codon}) of all the genes containing a potential Pyl codon, using various programs (RSEARCH [20], Infernal [21] or CARNAC [25]) showed that PYLIS-like structures could only be found in the nine \textit{mtmB} sequenced genes which is consistent with an earlier study [13].

Since the PYLIS structure is strictly conserved in only three of the nine \textit{mtmB} genes ([Fig. 1A] [12], we tried to find a more general consensus. Using RNAMotif [22] we determined the minimal sequence-structure requirements so that a consensus can account for the PYLIS found in all nine \textit{mtmB} genes.
We then tested the significance of the resulting structure by searching for its presence in random sequences. Three sets of 10000 sequences were generated with GenRGens [27]. Since a PYLIS-like structure could be found in only 0.44–0.71% of the random sequences tested we conclude that the proposed PYLIS element (Fig. 1B) is significant owing to its rare occurrence in random sequences. However, even with the minimal consensus structure, no PYLIS-like stem loop can be found in the UAG downstream sequence of the archaeal mtbB or mttB genes.

3.2. PYLIS is not needed for efficient read-through of UAG codons in E. coli

The recent in vivo results obtained in M. acetivorans [17] together with our bioinformatic analysis seem to indicate that the presence of the PYLIS structure in mtmB genes is relevant for improving the recoding efficiency of the in-frame UAG codon as a Pyl sense codon. Therefore, we investigated the effect of the mtmB1 PYLIS element on the efficiency of UAG read-through in the presence of the M. barkeri PylRS:tRNAPyl suppressor orthogonal pair with an exogenous supply of Cyc. In order to precisely measure the read-through efficiency associated with each of the tested PYLIS variants, we adapted the lacZ–luc dual reporter system in E. coli. In this system, an in-frame UAG stop codon is inserted between the lacZ and luc genes (coding for β-galactosidase and luciferase respectively). While termination of translation at the UAG codon produces a regular β-galactosidase protein, suppression of the in-frame UAG codon produces a β-galactosidase–luciferase fusion protein. Each of these two activities can then be measured. The ratio of the luciferase activity over that of the internal control provided by the β-galactosidase activity reported to the same ratio obtained when the UAG codon is a sense lysine codon. The reporter system produces statistically relevant and reproducible measurements even when read-through efficiency is low. We inserted a series of stem loop structures downstream of the suppression site in the reporter system. The different constructs include M. barkeri mtmB1 wild type PYLIS sequence, a series of M. barkeri mtmB1 PYLIS variants in which point mutations designed to modify the PYLIS secondary structure were inserted and finally a stem loop structure from the infectious bronchitis virus (IBV) known to affect the movement of the ribosome along the mRNA in E. coli [32,33] (Fig. 2). Precise quantification of the β-galactosidase and luciferase activities for each constructs shows that suppression can reach

![Fig. 1. Sequence alignment of mtmB genes (A). Shaded regions with four different colors correspond to the four PYLIS stems. Cartoon representation for the structural descriptor of the revised consensus PYLIS (B). “Mis-pair = 2” means that the maximum number of mis-pairs allowed in the stem is 2; “ends = mm” means that the mis-pair could be on either strand of the stem.](image-url)
up to 60% in the presence of an exogenous supply of Cyc (Fig. 3); while no suppression is detectable in the absence of Cyc (Fig. 3). Our results show that the suppression levels remain unchanged upon mutation or deletion of PYLIS compared to the wild-type PYLIS (Figs. 2 and 3). This demonstrates that, in an E. coli context, the PYLIS sequence has no impact on the efficiency of the UAG suppression by tRNA Pyl. Moreover, no significant difference was observed either when the PYLIS sequence was replaced by an unrelated IBV stem loop structure, indicating that efficient UAG read-through can be achieved in virtually any mRNA context (Fig. 3). Previous suppression assays using a genome encoded lacZ reporter system in the presence of pylS and pylT genes which were encoded on low copy number plasmids, yielded suppression efficiency in the 20% range [16], thus clearly indicating that PylRS and tRNA Pyl expression levels are limiting factors. Under these limiting conditions, the presence of the PYLIS element did not result in the stimulation of the read-through efficiency either (data not shown). Finally, we compared the behavior toward the PYLIS structure of the tRNAPyl suppressor to that of the glutamine suppressor tRNA encoded in the genome of E. coli DH5α strain. Our results show that the tRNA\textsubscript{Cua}\textsuperscript{Glu} allows an UAG suppression efficiency of 12% (Fig. 3) and as for tRNA\textsuperscript{Pyl} suppression efficiency is not affected by the presence of the PYLIS structure downstream the stop codon (Fig. 3). Our data does not necessarily contradict the fact that the PYLIS structure might enhance read-through in archaea. Although our E. coli based reporter system has been extensively used to characterize the sites of internal ribosome re-entry during translation (IRES), a phenomenon occurring in eukaryotic cells [11], it is possible that the bacterial translation machinery is not able to recognize PYLIS and therefore trigger the same effect observed in archaea. Further studies in a homologous archaeal system will be needed to determine in a quantitative fashion the impact of PYLIS on Pyl insertion. This would further progress towards the understanding of the UAG recoding mechanism in these fascinating organisms.

3.3. d-Ornithine is a precursor of pyrrolysine.

The lack of specific mRNA requirements for the M. barkeri PylRS:tRNA\textsuperscript{Pyl} suppressor mediated read-through of UAG codon constitutes a valuable advantage as it should permit the incorporation of Cyc and Pyl in virtually any desired E. coli proteins. Transformation of E. coli with pylB, pylC and pylD, could expand the repertoire of amino acids used for protein synthesis to the 22 known co-translationally inserted natural amino acids, since E. coli already utilizes Sec. In order to achieve this goal, we cloned and expressed the M. barkeri pylB, pylC and pylD genes in E. coli and monitored UAG suppression efficiency using the lacZ–luc construct. Precise quantification clearly demonstrated Pyl incorporation of 6% (Fig. 4B), which is significantly different from the low background level observed in the absence of the three genes (P-value = 0.003) but remains lower than when Cyc was added to the cells. This result suggests that the intracellular Pyl supply is low either because PylB, PylC and PylD are poorly expressed as suggested by a recent study [17], or perhaps because a Pyl...
metabolic precursor is not abundant enough in \textit{E. coli}. Retrosynthetic analysis of pyrrolysine led us to propose \textit{d}-glutamate, \textit{d}-isoleucine, \textit{d}-proline and \textit{d}-ornithine as possible precursors for \textit{d}-1-pyrroline-4-methyl-5-carboxylate, the Pyl pyrroline ring \cite{34}. While the addition of 5 mM of \textit{d}-glutamate, \textit{d}-isoleucine or \textit{d}-proline to our \textit{E. coli} based reporter system left the PylRS/tRNA\textsuperscript{Pyl} mediated suppression level unchanged, addition of 5 mM of \textit{d}-ornithine increased the suppression efficiency to 48\% (Fig. 4B), a value close to the efficiency obtained upon addition of Cyc to the growth media. Conversion of ornithine to 1-pyrroline-5-carboxylate is not an unknown chemistry, since this is the first step of one of the two possible paths to \textit{l}-proline, shown to exist in a number of bacteria \cite{34}. Formation of 1-pyrroline-5-carboxylate requires the oxidation of \textit{l}-ornithine to glutamate semialdehyde by \textit{l}-ornithine amino transferase, a PLP dependent enzyme. While the Pyl biosynthetic proteins share some level of sequence homology to known proteins (biotin synthase and \textit{d}-alanine-\textit{d}-alanine ligase for PylB and PylC, respectively) no homology to \textit{l}-ornithine amino transferase can be detected. Further studies will determine the role of each of the Pyl biosynthetic genes as well as the precise chemistry underlying Pyl biosynthesis.

Fig. 3. Effect of PYLIS on UAG read-through efficiency. Solid black bars represent the median value of at least 10 independent experiments in \textit{E. coli} BL21 strain carrying pyl\textit{S} and pyl\textit{T} genes, the dual \textit{lacZ–luc} reporter system and grown in the presence of 20 mM Cyc in the media. The white bars represent the median value of four independent experiments carried out in the same conditions than previously except that Cyc has been omitted in the media. The solid grey bars are the median value of five independent experiments performed in \textit{E. coli} DH5\textit{a} strain carrying the dual \textit{lacZ–luc} reporter system and a chromosomal copy of sup\textit{E44} (suppressor tRNA\textsuperscript{Gln}). The star indicates that read-through efficiency has not been determined.

\begin{figure}
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\caption{Fig. 3. Effect of PYLIS on UAG read-through efficiency. Solid black bars represent the median value of at least 10 independent experiments in \textit{E. coli} BL21 strain carrying pyl\textit{S} and pyl\textit{T} genes, the dual \textit{lacZ–luc} reporter system and grown in the presence of 20 mM Cyc in the media. The white bars represent the median value of four independent experiments carried out in the same conditions than previously except that Cyc has been omitted in the media. The solid grey bars are the median value of five independent experiments performed in \textit{E. coli} DH5\textit{a} strain carrying the dual \textit{lacZ–luc} reporter system and a chromosomal copy of sup\textit{E44} (suppressor tRNA\textsuperscript{Gln}). The star indicates that read-through efficiency has not been determined.}
\end{figure}

\begin{figure}
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\caption{Fig. 4. \textit{M. barkeri} pyl\textit{B}, pyl\textit{C} and pyl\textit{D} genes products are sufficient to allow Pyl biosynthesis in \textit{E. coli}. (A) UAG read-through quantification was performed in \textit{E. coli} BL21 strain containing the dual \textit{lacZ–luc} reporter system with the \textit{Δpyl} sequence, plasmid borne pyl\textit{S} and pyl\textit{T} genes and either pRSF-Duet-1 borne pyl\textit{B}, pyl\textit{C}, pyl\textit{D} genes or the empty pRSF-Duet-1 plasmid. Suppression efficiency in the presence of the pyl\textit{B}, pyl\textit{C} and pyl\textit{D} genes is highly significant in comparison with the background level (\textit{P}-value = 0.003). (B) UAG read-through has been performed as described previously. Different \textit{d}-amino acids, which are potential precursors were added to the culture at a 5 mM final concentration (NO: no precursor added, \textit{d}-PRO: \textit{d}-proline, \textit{d}-ORN: \textit{d}-ornithine, \textit{d}-GLU: \textit{d}-glutamic, \textit{d}-ILEU: \textit{d}-isoleucine). A 7-fold increase of the UAG read-through is specifically observed in presence of \textit{d}-ornithine, indicating that this is the limiting precursor of PYL biosynthesis in \textit{E. coli}.}
\end{figure}
4. Concluding remarks

Our data clearly demonstrate that UAG read-through mediated by the M. barberi PylRS:tRNAPyl suppressor orthogonal pair (i) is very efficient since up to 60% read-through can be reached when 20 mM of Cyc is added to the growth medium (ii) suppression efficiency is not influenced by the downstream mRNA context of the UAG codon in E. coli. We also showed earlier that since PylRS does not recognize the tRNAPyl anticodon, Cyc could also be incorporated at UGA codons and virtually any undesired codons [19]. Taken together our data clearly show that the natural M. barberi PylRS:tRNAPyl suppressor orthogonal pair is particularly appealing for protein engineering and incorporation of any desired modified amino acid upon mutation of PylRS active site.

Furthermore, we confirm that no archaeal protein other than PylB, PylC and PylD is needed to provide E. coli with a low but sustainable source of Pyl. We further show that D-ornithine is the pyrroline ring precursor and addition of this metabolite to the growth medium allows very efficient insertion of Pyl into protein, therefore successfully adding Pyl to the E. coli genetic code. Understanding Pyl biosynthetic route is a first step towards engineering a methanogenesis pathway in E. coli and to expand the methanogenic substrates to include methyamines in organisms such as M. maripaludis.

Acknowledgements: We thank Ian Brierly for his help at the initial steps of this work. This work was supported by grants from the National Institute of General Medical Sciences (Grant GM 22854 to D.S.), the Agence Nationale pour la Recherche (Contract ANR-06-LAN-0391-01 to J.P.R.) and the Association pour la Recherche sur le Cancer (Contract 3849 to J.P.R.).

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