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The genetic incorporation of *p*-azidomethyl-L-phenylalanine into proteins in yeast

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ABSTRACT

The noncanonical amino acid *p*-azidomethyl-L-phenylalanine can be genetically incorporated into proteins in bacteria, and has been used both as a spectroscopic probe and for the selective modification of proteins by alkynes using click chemistry. Here we report identification of *Escherichia coli* tyrosyl tRNA synthetase mutants that allow incorporation of *p*-azidomethyl-L-phenylalanine into proteins in yeast. When expressed together with the cognate *E. coli* $tRNA^{Tyr}_{CUA}$, the new mutant tyrosyl tRNA synthetases directed robust incorporation of *p*-azidomethyl-L-phenylalanine into a model protein, human superoxide dismutase, in response to the UAG amber nonsense codon. Mass spectrometry analysis of purified superoxide dismutase proteins confirmed the efficient site-specific incorporation of *p*-azidomethyl-L-phenylalanine. This work provides an additional tool for the selective modification of proteins in eukaryotic cells.

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The genetic incorporation of noncanonical amino acids (ncAAs) into proteins provides useful tools for the study of protein structure and function, as well as a method for the selective modification of proteins with a variety of agents including spectroscopic probes, oligonucleotides and drugs.[1] Two such useful ncAAs are *p*-azido-l-phenylalanine (pAzF) and *p*-azidomethyl-l-phenylalanine (pAMF). The azido group, a functional group not found in the canonical amino acids, provides a useful IR probe, photo-affinity label and has been successfully used in many instances for the biorthogonal labelling of proteins through a 1,3-dipolar cycloaddition with strained olefins and alkynes.[2,3] Of note, when pAMF was introduced into proteins by Open Cell Free Synthesis system, pAMF enabled more efficient conjugation reactions than pAzF,[4] as expected by the enhanced reactivity of an alkyl azide moiety. Although biorthogonal tRNA/aminoacyl tRNA synthetase (aaRS) pairs have been evolved for the recombinant incorporation of pAzF and pAMF into proteins in bacteria,[5,6] and for pAzF into proteins in eukaryotic cells,[7] no such a pair has been generated for genetically encoding pAMF in eukaryotic cells. Here we describe development of new mutant aaRS variants that efficiently incorporate pAMF into proteins in *Saccharomyces cerevisiae.*

To evolve an aaRS capable of incorporating pAMF into proteins in yeast, a previously reported *E. coli* amber suppressor TyrRS/$tRNA^{Tyr}_{CUA}$ pair was used.[8] This pair is orthogonal in yeast, i.e., $tRNA^{Tyr}_{CUA}$ does not cross-react with any of the endogenous aminoacyl-tRNA synthetases in yeast, and *E. coli* TyrRS does not accept any endogenous yeast tRNAs as substrates.[8] This orthogonality is a consequence of the distinct acceptor stem identity elements for tRNA-aaRS recognition in prokaryotic and eukaryotic organisms, and has been exploited to generate a number of orthogonal pairs for the incorporation of ncAAs into proteins in eukaryotic cells and organisms.[9] In addition, it has been shown that an tRNA/aaRS pair evolved in yeast can be used to efficiently encode the same ncAA in mammalian cells.[7,10] Indeed, the *E. coli* TyrRS/$tRNA^{Tyr}_{CUA}$ pair has been used to incorporate a number of structurally diverse amino acids into proteins with high fidelity and good efficiency in yeast and mammalian cells,[10,11] but no such aaRS variant identified to date can incorporate pAMF into proteins in significant yields.

To evolve a new aaRS/ $tRNA^{Tyr}_{CUA}$ pair capable of incorporating pAMF into proteins in yeast, we initially tested the previously evolved OMeY RS[8] for its ability to charge $tRNA^{Tyr}_{CUA}$ with pAMF. First, we transformed a yeast strain that harbored the *HIS3* and *URA3* genes under the control of the *GAL4* promoter (MAV203, ThermoFisher) with the pGADGAL4-2TAG plasmid.[8] This plasmid harbors a mutant *GAL4* transcription activator gene that has two in-frame TAG stop codons at the permissive positions 44 and

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110.[8] As a result, the full length Gal4 protein is not expressed, and the transformed MaV203(pGADGAL4-2TAG) strain is auxotrophic for histidine (Fig. 1). Transformation of the MaV203:pGADGAL4-2TAG strain with the plasmid pESC, which harbors the OMeYRS and $tRNA^{Tyr}_{CUA}$ genes, allows the MaV203(pGADGAL4-2TAG, pESC) strain to grow on media lacking histidine, but only when the growth medium is supplemented with OMeY (Fig. 1). The presence of this ncAA in the medium enables suppression of the nonsense codon, and translation of the full length Gal4 transcription factor that further drives expression of His3 and mediates conditional histidine prototrophy.

The ncAA-responsive MaV203(pGADGAL4-2TAG, pESC) yeast strain allows facile assessment of the ability of OMeYRS to charge $tRNA^{Tyr}_{CUA}$ with an ncAA, and incorporate it into proteins in yeast. It also allows semi-quantitative assessment of ncAA incorporation efficiency by scoring yeast strain growth on media with different concentrations of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3. When 3-AT is included in the growth medium at a high concentration, an ncAA has to be incorporated into the Gal4 protein efficiently to enable yeast growth. Using this ncAA-responsive yeast reporter strain, we found that OMeYRS enables incorporation of pAMF into proteins in yeast only poorly, and pAMF cannot support growth of the MaV203(pGADGAL4-2TAG: pESC) strain on a medium lacking histidine when 3-AT is present at 100 mM concentration (Fig. 1).

To evolve an aaRS that can incorporate pAMF into yeast proteins efficiently, we first generated an OMeYRS mutant library by error-prone PCR mutagenesis, since active site-directed libraries failed to identify the desired variant. The conditions of mutagenic PCR were adjusted to introduce on average 5 nucleotide substitutions per one OMeYRS gene copy. A library comprising $\sim 10^7$ of such mutants in pCR2.1 plasmid (Invitrogen) was generated in *E. coli*. The mutant library DNA was then digested with *Hin*dIII-Xho1 restriction endonucleases on a preparative scale, and the library DNA fragment was co-transformed into the MaV203 yeast strain together with linearized pESC vector and pGADGAL4-2TAG plasmid. The yeast library transformants were submitted to three rounds of positive/negative selection on media containing pAMF in order to identify mutants selectively incorporating pAMF into the Gal4 protein.[8] Positive selection was carried out on minimal medium lacking histidine and supplemented with 1 mM pAMF and 100 mM 3-AT. To eliminate RS mutants that can charge $tRNA^{Tyr}_{CUA}$ with one of 20 canonical amino acids, we applied also a negative selection by growing transformants on a medium lacking pAMF, but containing 0.1% 5-fluoroorotic acid. Repeated rounds of positive and negative selection resulted in the identification of 6 independent clones that were able to grow in the absence of histidine when pAMF was present in the growth medium (Fig. 2A).

Next, the ability of the clones to incorporate pAMF into proteins in yeast was tested by assessing the expression of the His6-tagged human superoxide dismutase gene (hSOD-33TAG-His6) containing an amber stop codon at a permissive site (Trp33TAG). All six

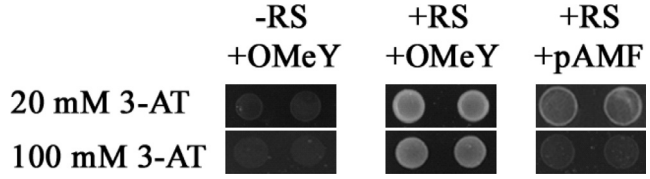

Fig. 1. ncAA-responsive yeast reporter strain expressing O-methyl L-tyrosine-selective RS (+RS) is auxotrophic for histidine when yeast growth medium lacking histidine is supplemented with *p*-azidomethyl L-phenylalanine (+pAMF) instead of O-methyl L-tyrosine (+OMeY). To suppress yeast growth due to the basal level of *HIS3* gene expression, 3-amino-1,2,4-triazole was added to plates at 20 mM or 100 mM concentration.

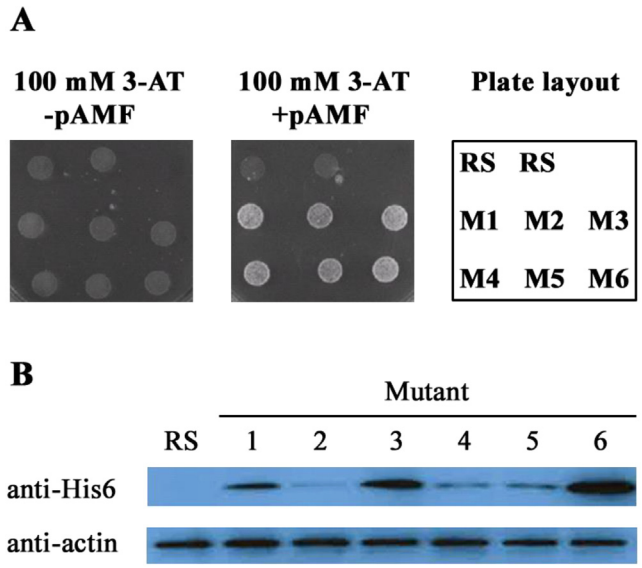

Fig. 2. (A) Six independent (M1-M6) O-methyl L-tyrosine-selective RS mutants support *p*-azidomethyl L-phenylalanine-dependent yeast growth (−/+pAMF) on medium lacking histidine; RS-O-methyl L-tyrosine-selective RS (B) All 6 mutants resulted in expression of His6-tagged hSOD gene harboring an TAG codon when grown in medium supplemented with pAMF. Yeast actin was used as a yeast cell lysate loading control.

mutants showed pAMF-dependent expression of hSOD to varying degrees (Fig. 2B).

Sequencing of six mutant RS genes revealed that M1, M2, M3, M4 and M6 RS genes harbored different mutations, while M5 was identical to M4 (Table 1). Among all mutants, the yeast strain harboring the M6 mutant RS showed the highest level of hSOD synthesis (Fig. 2B), and it was characterized further. Surprisingly, none

Table 1
Amino acid mutations identified in 6 mutant RS genes that enabled incorporation of pAMF into hSOD in yeast.

Mutant	Mutations
1	L49S
2	F146Y, D267G
3	Q63R, Q159R, N134S, V101A, S207T
4	F256L
5	F256L
6	E27G, L49S, A123E, D393V

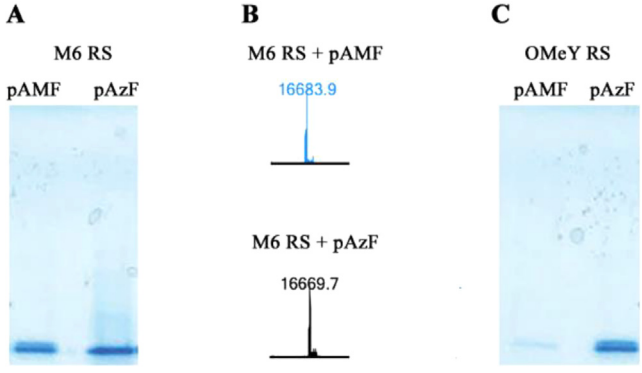

Fig. 3. (A) His6-tagged hSOD proteins isolated by Ni-NTA affinity chromatography from yeast expressing M6 RS and grown in medium containing pAMF or pAzF. (B) ESI-QTOF analysis of His6-tagged hSOD proteins purified from yeast expressing M6 RS incorporating pAMF and pAzF. (C) His6-tagged hSOD proteins isolated by Ni-NTA affinity chromatography from yeast expressing OMeY RS and grown in medium containing pAMF or pAzF.

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