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A Single Genomic Copy of an Engineered Methionyl-tRNA Synthetase Enables Robust Incorporation of Azidonorleucine into Recombinant Proteins in *E. coli*

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The azide and alkyne functional groups have emerged as useful chemical handles in bioconjugation efforts.[1] An efficient way to introduce these functional groups into recombinant proteins is via the incorporation of unnatural amino acids.[2−7] In *E. coli*, unnatural amino acids can be incorporated into proteins in a residue-specific fashion in which the codons corresponding to 1 of the 20 natural amino acids are reassigned to an unnatural amino acid.[8,9] We recently reported a variant of the *E. coli* methionyl-tRNA synthetase (MetRS) that enables high-level, residue-specific, incorporation of the unnatural amino acid azidonorleucine (ANL, Figure S1) into recombinant proteins produced in *E. coli*.[6] This MetRS variant carries a single leucine to glycine mutation (L13G) in its methionine (Met) binding pocket. Kinetic studies on the L13G MetRS demonstrated that it still retained the ability to activate its natural substrate, Met, albeit with a specificity constant (k_{cat}/K_m) 270-fold smaller than that of the wild-type MetRS. Prior ANL incorporation studies were performed in an *E. coli* expression host which carries a genomic copy of the wild-type MetRS gene (*metG*) and a plasmid-borne copy of the mutant synthetase gene (*metG**). We became interested in the question of whether a single genomic copy of the *metG** allele could fulfill the dual roles of supporting cell division via global incorporation of Met into cellular proteins and high-level expression of recombinant proteins containing ANL. A strain carrying *metG** in its genome would be genetically stable relative to plasmid-bearing strains and would represent a "Janus-faced" organism, the genetic code of which changes as a function of its environment.

The *metG** allele was introduced into the Met auxotrophic strain M15MA using the Wanner method.[10] Briefly, a DNA cassette containing the kanamycin resistance (kan^R) gene followed by the first 532 base pairs (bp) of the *metG** gene was assembled and transformed into M15MA cells expressing the λ Red recombinase (Figure 1A). The kan^R marker was subsequently removed using the Flp recombinase generating the M15MA *metG** strain. The presence of the L13G mutation was confirmed by sequencing PCR products amplified from genomic DNA. Deletion of the kan^R marker results in an ∼85 bp "scar" prior to the initiator codon (Figure 1A, Figure S2). RT-PCR experiments confirmed that the scar does not influence transcript levels of *metG*. A growth curve of the M15MA *metG** strain in M9 minimal medium supplemented with 40 mg/L of methionine was generated and compared to that of wild-type M15MA. Remarkably, both strains exhibit the same growth rate and growth yield under these Met-rich conditions (Figure 1B). These results suggest that, under nutrient-rich conditions, the *metG** allele does not have a strong effect on the global protein synthesis capabilities of the cell. Further physiological characterization of the M15MA *metG** strain will be presented elsewhere.

To further probe the protein synthesis capabilities of M15MA *metG**, we used this strain to produce recombinant proteins containing either Met or ANL. The pAJL-60 plasmid[6] encodes a

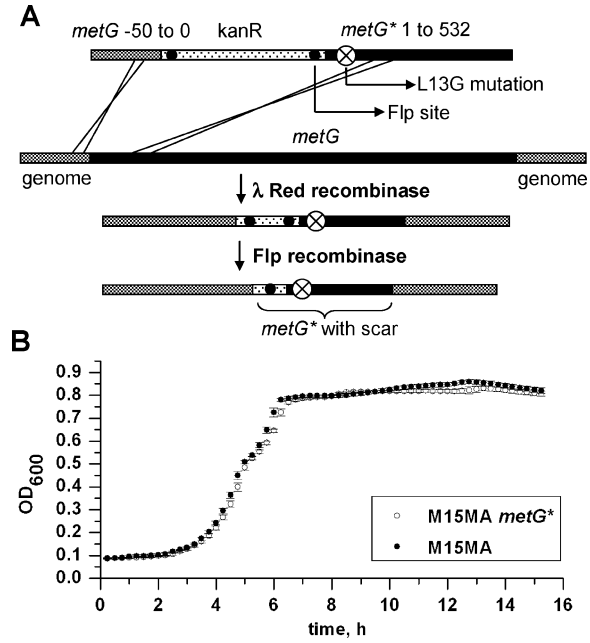

Figure 1. (A) Strategy for introduction of the L13G MetRS gene into the *E. coli* M15MA genome. The new strain produced via this method includes the *metG** allele and an 85 bp DNA scar. (B) M15MA and M15MA *metG** have similar growth rates and growth yields in M9 minimal medium supplemented with 20 natural amino acids.

histidine-tagged, IPTG-inducible murine dihydrofolate reductase (DHFR) gene as well as a copy of the high affinity *lac* repressor $lacI^q$ to ensure tight regulation of protein expression. pAJL-60 was transformed into three different expression hosts: M15MA, M15MA *metG**, and M15MA *metG** pREP4. The pREP4 plasmid encodes an additional copy of $lacI^q$, and we hypothesized that the addition of extra $lacI^q$ would improve the extent of incorporation of ANL by reducing leaky expression. DHFR production was carried out using a medium shift procedure:[9] the cells were grown to midlog phase in media containing all 20 natural amino acids and then shifted to media with 19 amino acids, lacking Met. The culture was then divided and supplemented with 40 mg/L (0.27 mM) Met, 320 mg/L (1.86 mM) ANL, or water as a negative control. Total cell lysates from each of the three strains were examined using SDS-PAGE (Figure 2A). M15MA only produced protein when supplemented with Met, while the M15MA *metG** strains produced protein when either Met or ANL was added. Despite the reduced aminoacylation activity of M15MA *metG**, this strain qualitatively produces the same amount of Met-containing protein (DHFR-Met) as the wild-type strain (compare lanes 4, 8, and 12 of Figure 2A). Remarkably, the M15MA *metG** strains also produce DHFR

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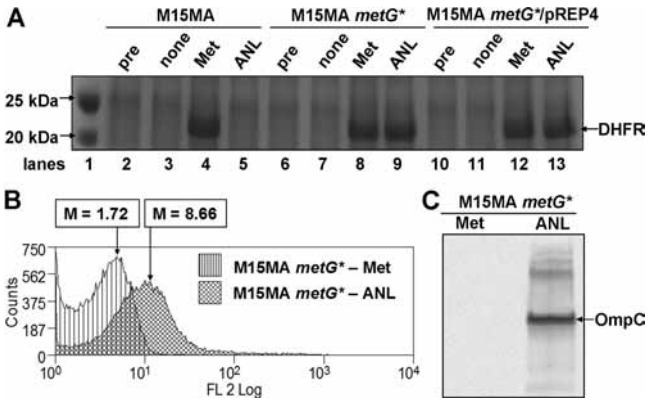

Figure 2. (A) SDS-PAGE analysis of total cell lysates of M15MA and M15MA *metG** expressing DHFR. Lanes marked pre are lysates prior to induction with IPTG, while all other lanes are lysates after 3 h of induction in media supplemented with the indicated amino acid. (B) Fluorescence histograms of M15MA *metG** cells bearing OmpC-Met or OmpC-ANL that have been biotinylated using CuAAC and stained with streptavidin-phycoerythrin. M: median fluorescence. (C) Western blot (streptavidin-HRP) of outer membrane protein fractions of cells biotinylated as in (B).

containing ANL (DHFR-ANL) in amounts comparable to DHFR-Met (compare lanes 8 and 12 to lanes 9 and 13).

As additional controls, we expressed DHFR in M15MA and M15MA *metG** harboring the multicopy plasmid pAJL-61,[6] which carries the *metG** allele but is otherwise identical to pAJL-60. This experiment allows us to assess the effectiveness of incorporating ANL using a single genomic copy of *metG** as compared to multiple, plasmid-borne copies. To determine the protein yield and extent of ANL incorporation, DHFR-Met and DHFR-ANL were purified to homogeneity using Ni-NTA chromatography. Protein yields were measured using the bicinchoninic acid (BCA) assay, and the extent of Met replacement by ANL was measured using Edman degradation. High levels of DHFR production (>20 mg/L of culture) were achieved under all conditions tested (Table 1). Consistent with previous findings,[6] the multicopy expression of *metG** led to effectively complete (96%) replacement of Met with ANL in DHFR produced in both M15MA and M15MA *metG** (entries 4 and 5 in Table 1). Remarkably, the extent of ANL incorporation in DHFR-ANL produced from M15MA *metG**, which carries only a single copy of the *metG** gene, is 90% (entry 2, Table 1). The addition of extra copies of *lacI*^{q} via the pREP4 plasmid does not affect the incorporation extent of ANL in M15MA *metG** and actually adversely affects the overall DHFR yield (entry 3, Table 1). Electrospray mass spectrometry on intact DHFR-ANL also confirmed the high extent of Met replacement with ANL. The major peak in all samples of DHFR-ANL appeared at $m/z = 24\,224$ corresponding to substitution of all eight Met residues of DHFR with ANL (Figure S3).

Table 1. Purified Protein Yield and Extent of Incorporation of ANL into DHFR Produced in M15MA and M15MA *metG**

entry	strain	plasmid(s)	amino acid	DHFR yield, mg/L[a]	extent of incorporation %[b]
1	M15MA	pAJL-60	Met	27.5	ND[c]
2	M15MA *metG**	pAJL-60	ANL	27.4	90
3	M15MA *metG**	pAJL-60, pREP4	ANL	20.2	89
4	M15MA	pAJL-61	ANL	33.5	96
5	M15MA *metG**	pAJL-61	ANL	31.9	96

[a] By BCA assay, [b] By Edman degradation, [c] Not determined.

To demonstrate the utility of the M15MA *metG** strain in producing azide-labeled proteins for bioconjugation, the strain was transformed with the plasmid pAJL-20, which encodes an IPTG-inducible variant of the outer membrane protein OmpC.[11] The expression of OmpC was induced in media supplemented with either Met or ANL, and the intact cells were subjected to labeling with biotin-PEO-propargylamide **2** (Figure S1) using copper-catalyzed azide−alkyne cycloaddition (CuAAC).[12,13] After labeling, the cells were stained with fluorescent streptavidin (streptavidin-phycoerythrin) and subjected to flow cytometry. Cells expressing OmpC containing ANL (OmpC-ANL) exhibited a 5-fold increase in median fluorescence relative to cells expressing OmpC-Met, which exhibit fluorescence equivalent to *E. coli* autofluorescence (Figure 2B). The biotinylated cells were also subjected to outer membrane protein preparation,[14] and the resulting proteins were examined using Western blotting with streptavidin-HRP. Only cells expressing OmpC-ANL exhibited labeling with the biotin probe (Figure 2C). Collectively, these results demonstrate that azide-labeled proteins produced by M15MA *metG** can be covalently modified using CuAAC.

Here we demonstrate that a single genomic copy of an engineered MetRS gene, *metG**, is sufficient in both supporting robust cell growth and enabling high levels of expression of recombinant proteins containing the unnatural amino acid ANL. The *metG** mutation renders MetRS defective with regards to Met, so our results demonstrate that the cell is robust to this insult on its protein synthesis machinery. Furthermore, the M15MA *metG** strain is genetically stable as compared to *E. coli* strains carrying plasmids and does not require antibiotic selection to maintain the allele. A recent report demonstrated selective labeling of bacterial proteins with ANL in a model host−pathogen system composed of macrophages and *E. coli*.[15] Stable genomic strains like M15MA *metG** may enable further host−pathogen studies of this type as well as more fundamental investigations into the robustness of cellular protein synthesis.

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Supporting Information Available: Detailed materials and methods, supplementary figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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