

Global tRNA misacylation induced by anaerobiosis and antibiotic exposure broadly increases stress resistance in *Escherichia coli*

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ABSTRACT

High translational fidelity is commonly considered a requirement for optimal cellular health and protein function. However, recent findings have shown that inducible mistranslation specifically with methionine engendered at the tRNA charging level occurs in mammalian cells, yeast and archaea, yet it was unknown whether bacteria were capable of mounting a similar response. Here, we demonstrate that *Escherichia coli* misacylates non-methionyl-tRNAs with methionine in response to anaerobiosis and antibiotic exposure via the methionyl-tRNA synthetase (MetRS). Two MetRS succinyl-lysine modifications independently confer high tRNA charging fidelity to the otherwise promiscuous, unmodified enzyme. Strains incapable of tRNA mismethionylation are less adept at growth in the presence of antibiotics and stressors. The presence of tRNA mismethionylation and its potential role in mistranslation within the bacterial domain establishes this response as a pervasive biological mechanism and connects it to diverse cellular functions and modes of fitness.

INTRODUCTION

Accurate protein synthesis requires high fidelity transfer RNA (tRNA) charging by aminoacyl-tRNA synthetases and accurate tRNA selection via tRNA-mRNA base pairing on the ribosome (1–4). Although protein biosynthesis that deviates from the genetic code (mistranslation) is largely regarded as a deleterious occurrence, partially ambiguous translation continuously occurs in *Candida albicans* due to CUG codon ambiguity (5) and also in *Mycoplasma* due to lost or inactive editing domains in several tRNA synthetases (6). Furthermore, high-levels of natural mistranslation that vary according to growth phase and condition have also been reported in *Bacillus subtilis* (7) and

Mycobacterium smegmatis (8). Although the sheer occurrence of natural mistranslation in conditions that should not directly compromise translational fidelity suggests this process might serve a purpose, there is a significant lack of information regarding the potential benefits of such mistranslation.

The scarcity of described cases of natural mistranslation has prompted the use of artificially constructed mistranslational systems, which rely on mutated tRNAs or tRNA synthetases to engender mistranslation in order to study its effects. Such artificial mistranslation increases resistance to oxidative stress, antifungal agents and phagocytotic killing by macrophages in *C. albicans* (9–11), whereas similarly contrived mistranslation in mycobacteria increases antibiotic resistance (12). Additionally, artificial mistranslation in *Escherichia coli* has been shown to increase tolerance to antibiotic stress (13), oxidative stress (14) and amino acid stress (15). Thus, mistranslation has the potential to provide diverse benefits, but identifying a case of natural mistranslation and subsequently ablating it to elucidate the functional consequence remains a challenge.

Recent evidence has demonstrated that intentional mistranslation via mischarging of non-methionyl-tRNAs with methionine (Met) by the methionyl-tRNA synthetase (MetRS) in mammalian cells is induced by reactive oxygen species and is beneficial for the oxidative stress response (16,17). Similarly, constitutive mistranslation specifically with Met via tRNA misacylation by the MetRS has been described in *Saccharomyces cerevisiae* (18). The archaeon *Aeropyrum pernix* performs an analogous Met mistranslational process through tRNA mismethionylation via MetRS to adapt the proteome to lower growth temperatures (19). Therefore, mistranslation specifically with Met engendered through tRNA mischarging by the MetRS appears to be a pervasive biological mechanism despite being so far undescribed in the bacterial domain of life.

Using the versatile model bacterium *E. coli*, we sought to identify the contribution of natural Met mistranslation to stress tolerance in bacteria. Here, we show that *E. coli*

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readily performs tRNA misacylation with Met like eukaryotes and archaea. In contrast to the mismethionylation previously described in mammalian cells, tRNA misacylation is undetectable under standard laboratory culture conditions. Yet, *E. coli* performs mismethionylation during anaerobic growth conditions reminiscent of its natural colonic habitat. Furthermore, the addition of a subinhibitory concentration of antibiotic also induces mismethionylation. We further elucidate the molecular mechanism that facilitates tRNA mismethionylation, and create strains incapable of this response. These high-fidelity strains are less tolerant to antibiotic exposure and chemical stressors and therefore corroborate the benefits of artificial mistranslation previously described.

MATERIALS AND METHODS

Pulse labeling

A total of 25 ml *E. coli* BW2113 was grown at 37°C at 200 rpm in MOPS EZ Rich (minus)-Met medium (Teknova) with 10 mM glucose in a 250 ml Erlenmeyer flask. Cells were pelleted by centrifugation for 5 min at 2500 rcf after reaching OD₆₀₀ 0.6–0.8 before resuspension in 300 µl of the medium supernatant to resemble normal growth prior to centrifugation. ³⁵S-Met (1 µCi/µl) was added to cell resuspension and incubated for 4 min at 37°C before addition of 400 µl of ice cold tRNA isolation buffer (0.3 M NaOAc/AcOH, 10 mM EDTA pH 4.8) and placement on ice. Cells were pelleted briefly at 4°C and washed once before resuspension in ice cold 400 µl tRNA isolation buffer. For chloramphenicol induction of mischarging, cells were grown with MOPS EZ Rich (minus)-Met 10 mM glucose and 10 µM chloramphenicol was added to resuspended cells directly with the 4 min ³⁵S-Met pulse. For anaerobic pulse labeling, MOPS EZ Rich (minus)-Met medium with 1% glucose was sparged for 45 min with nitrogen gas before being aliquoted into 20 ml anaerobic vials in an anaerobic glove box. *E. coli* was grown anaerobically at 37°C in airtight vials before anaerobic centrifugation for 5 min at 2500 rcf after reaching OD₆₀₀ = 0.3–0.4. For anaerobic respiration experiments, samples were processed identically to the normal anaerobic sample, but 50 mM sodium nitrate was added to anaerobic vials. Pelleted anaerobic cultures were transferred to an anaerobic glovebox for pulse labeling and RNA extraction that was performed identically to the aerobic sample.

RNA extraction

A total of 0.5 ml of 0.1mm glass beads was added to washed cell resuspensions. Mixture was vortexed for 1 min and placed on ice for 1 min four times for lysis. RNA was then extracted from lysed cells a total of three times by adding 400 µl acetate saturated phenol chloroform pH 4.8 followed by 1 min of vortexing and 5 min of centrifugation at 17 000 rcf and 4°C before ethanol precipitation. RNA pellet was suspended in tRNA storage buffer (10 mM NaOAc/AcOH, 1 mM EDTA pH 4.8).

In vitro aminoacylation

In vitro aminoacylation reactions for microarray analysis were performed in 16 µl reactions containing 1 µM MetRS, 20 µg total *E. coli* tRNA and 2.5 µCi/µl ³⁵S-Met, 2 mM ATP and 1 mM DTT in 50 mM HEPES KOH pH 7.5, 100 mM potassium glutamate and 10 mM magnesium acetate. MetRS was succinylated via incubation in 10 mM succinyl-CoA at 37°C for 30 min prior to the tRNA charging reaction. Acetyl-CoA, succinate and Coenzyme-A preincubations with MetRS were performed under the same concentrations and conditions. Succinyl-CoA added to tRNA charging reactions without preincubation was used at 2.5 mM, which was the final succinyl-CoA concentration in the tRNA charging reaction after MetRS succinylation. Reactions were run for 20 min at 37°C and were stopped by adding 45 µl 0.3 M NaOAc/AcOH, 10 mM EDTA pH 4.8 and an equal volume of acetate saturated phenol/chloroform. Mixture was centrifuged for 5 min at 17 000 rcf and 4°C before removal of aqueous layer and ethanol precipitation of the RNA and resuspension in 10 mM NaOAc/AcOH, 1 mM EDTA pH 4.8. *In vitro* aminoacylation time course reactions were performed in 50 mM HEPES KOH pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 10% PEG8000, 1 mM DTT, 2 mM ATP, 100 µM methionine, 1 µCi/µl ³⁵S-Met, 100 µM total *E. coli* tRNA and 100 nM MetRS. Reactions were performed at 37°C and aliquots were taken at the indicated time points and quenched in 10% trichloroacetic acid (TCA). Quenched reactions were spotted on filter disks using a vacuum apparatus and were washed with 2 ml 10% TCA and 1 ml 95% ethanol. Dried filter disks were exposed to a phosphorimaging screen and quantified with Image Lab 4.1 software (Bio Rad).

Plate dilution assay

A standardized inoculum of each strain was serially diluted and 5 µl of each dilution was spotted on LB plates with or without 10 µM chloramphenicol. Plates were incubated at 37°C.

Microarray analysis

tRNA microarray analysis and controls were performed as previously described (17,18,20,21). Briefly, tRNA was deacylated with 0.5 M Tris pH 9.0. For the Met block, 500 pmol of unbound tRNA^{iMet} and tRNA^{eMet} probes were added to the RNA hybridization mix prior to array loading. For the aminopeptidase treatment, 20 µg of RNA was treated with 20 µl Sigma Microsomal Leucine aminopeptidase and the reaction was incubated at room temperature for 25 min. RNA was then extracted with acetate saturated phenol chloroform pH 4.8 and ethanol precipitated before hybridization. RNA (20 µg) was loaded per array. Arrays were exposed to a phosphorimaging screen and quantified with Image Lab 4.1 software (Bio Rad). Percent misacylation was calculated by dividing the total signal obtained from a particular tRNA isoacceptor by the total signal from the initiator (fMet) and elongator tRNA^{Met}.

Protein expression

Recombinant WT or mutant MetRSs were expressed in BL21 *E. coli* from a pET28a expression plasmid containing the MetRS gene under IPTG control. MetRS mutants were created through overlap extension PCR (22) of the WT insert followed by ligation into pET28a. BL21 cells were grown at 37°C at 200 rpm in terrific broth with 50 µg/ml kanamycin. IPTG (1 mM) was added once an OD₆₀₀ of 0.8 was achieved and expression continued for 4 h before harvest.

Recombinant MetRS protein purification

Cells were pelleted and resuspended in 125 mM NaCl, 25 mM Tris pH 7.5, 20 mM imidazole before lysis and removal of cell debris by centrifugation. MetRS was purified via elution from a Ni-NTA column using 75 mM, 200 mM and 500 mM imidazole fractions. Eluted fractions were analyzed via SDS-PAGE and the purest elutions were dialyzed overnight in 125 mM NaCl, 25 mM Tris pH 7.5. Dialyzed samples were concentrated with centrifugal filters.

Proteomics mass spectrometry

E. coli cell pellets were extracted by heating (95°C, 20 min) and vortexing in a reducing and denaturing SDS(1%)/Tris (200 mM, pH 8.0)/DTT(10 mM) buffer and cysteine thiols alkylated with 40 mM iodoacetamide (23). Proteins were purified by a modified eFASP (enhanced filter-aided sample preparation) protocol (24), using Sartorius Vivacon 500 concentrators (30 kDa nominal cutoff). Proteins were digested with MS-grade trypsin (37°C, overnight), and peptides were eluted from the concentrator and purified by micro-SPE (Thermo Pierce C18 tips) and vacuum centrifugation. For LC-MS analysis, peptide samples were separated on a capillary C18 column (Thermo Acclaim PepMap 100 Å, 2 µm particles, 50 µm I.D. × 50 cm length) using a water-acetonitrile + 0.1% formic acid gradient (2–50% AcN over 560 min) at 90 nl/min using a Dionex Ultimate 3000 LC system with nanoelectrospray ionization (Proxeon Nanospray Flex source). Mass spectra were collected on an Orbitrap Elite mass spectrometer (Thermo) operating in a data-dependent acquisition mode, with one high-resolution (120 000 $m/\Delta m$) MS1 parent ion full scan triggering 15 MS2 CID fragment ion scans of selected precursors; the duty cycle for spectral acquisition under these conditions was ~1.9 s. Proteomic mass spectral data were analyzed using the SEQUEST HT and Percolator algorithms implemented in Proteome Discoverer (Thermo Scientific), using the predicted proteome of *E. coli* K12 MG1655 as a search database. Precursor and product ion mass tolerances for SEQUEST searches were set to 10 ppm and 0.6 Da, respectively. False discovery rate for peptide-spectrum matches was controlled by target-decoy searching to 1% at both spectrum and peptide levels. Static cysteine carbamidomethylation and variable methionine oxidation were included as modifications in all searches.

Chromosomal *E. coli* mutations

Chromosomal mutations in *E. coli* were performed as previously described (25). Synonymous wobble changes were

made on two codons upstream and downstream of the mutation of interest to enhance mutagenesis by avoiding mismatch repair. These synonymous wobble changes were maintained in the reverted strains as markers to distinguish them from WT and demonstrate they had no effect on the mutant phenotype.

Chromosomal MetRS purification

A total of 250 ml *E. coli* BW2113 containing a N-terminal His₆-tag in the chromosomal MetRS copy was grown at 37°C at 200 rpm in MOPS EZ Rich (minus)-Met medium (Teknova) with 10 mM glucose in a 2 l Erlenmeyer flask. For chloramphenicol treatment, 30 µg/ml was added 5 min prior to cryogenic lysis. For anaerobic growth, 1 l MOPS EZ Rich (minus)-Met medium with 1% glucose was sparged for 45 min with nitrogen gas before being aliquoted into a 1 l anaerobic serum vial in an anaerobic glove box. Cells were grown until midlog phase (OD₆₀₀ = 0.70 for aerobic, OD₆₀₀ = 0.25 for anaerobic) and were cryogenically lysed as previously described (26). Lysed cell pellets were resuspended in 125 mM NaCl, 25 mM Tris pH 7.5, 20 mM imidazole, 10 mM nicotinamide (desuccinylase inhibitor) and cell debris was removed by centrifugation. MetRS was purified via elution from a Ni-NTA beads using 500 mM imidazole followed by a 125 mM NaCl, 25 mM Tris pH 7.5 buffer exchanges in centrifugal filters.

Western blotting for Lys-succinylation

Purified MetRS was subjected to SDS-PAGE followed by transfer to PVDF membrane (Millipore). PVDF membranes were incubated with anti-succinyllysine antibody (1:100) (PTM Biolabs). After washing, PVDF membranes were incubated with anti-rabbit HRP conjugated IgG (1:30 000) at RT for 30 min. Immune blot bands were visualized by ECL method. As a loading control, the PVDF membrane was stained with Ponceau S to visualize the quantity of MetRS loaded.

Bioinformatic MetRS analysis

All available bacterial MetRS protein sequences were obtained from NCBI and were parsed to the species level. Sequences were aligned with Clustal Omega alignment software and then organized according to phylum and class.

Phenotype microarrays

Phenotype microarray analysis was performed as previously described (27,28). The average read values for the WT strain was subtracted from that of the mutants for each condition and concentration. Conditions without growth of any strain were excluded from data analysis. A particular condition was classified as having growth if at least one strain achieved a higher average read value than the WT strain in the condition without a carbon source. Growth differentials were plotted based on the nutrient being tested or the type of stressor.

RESULTS

E. coli misacylates tRNA with methionine during anaerobic growth and antibiotic stress

We began by examining the fidelity of tRNA charging with Met under various oxygen availabilities to mimic its natural environments in the human colon (anaerobic) and outside of human body (aerobic) using the previously developed ³⁵S-Met pulse labeling and microarray method (17,21). The fidelity of tRNA charging reactions for ³⁵S-Met pulse-labeled samples can be determined by using tRNA microarrays to separate tRNA isoacceptors and subsequent phosphorimaging can determine which tRNAs were aminoacylated with ³⁵S-Met. tRNA mismethionylation was examined during aerobic respiration, fermentation and anaerobic respiration, which represent the metabolic modes important to *E. coli*'s fitness in the environment (29,30). Remarkably, during fermentative anaerobic conditions, Met is constitutively mischarged to several non-methionyl-tRNA species (Figure 1A, B and Supplementary Figure S1). Conversely, during standard aerobic laboratory cultivation, Met misacylation is below our detection limit. As in our mammalian studies (17), several approaches were employed to ensure that the array signals corresponding to nonmethionyl-tRNAs were indeed due to tRNA aminoacylation with ³⁵S-Met. The array controls exclude the possibility of tRNA^{Met} cross hybridization though the addition of free Met probes, exclude the possibility of ³⁵S tRNA nucleotide thio-modifications by removing aminoacylated ³⁵S-Met through chemical deacylation, and exclude the possibility of peptidyl-tRNA engendering spurious mischarging signal through aminopeptidase M (APM) treatment of tRNA (APM treatment also deacylates cognate charged tRNAs and reduces total signal by about 50% under our reaction condition (17) (Figure 1C). We found the mismethionylation response to be specific to anaerobic fermentation (Supplementary Figure S2), since anaerobic growth via nitrate respiration, which obviates the mixed acid fermentation (31), results in minimal Met mischarging (Figure 2A). We also found that tRNA misacylation with Met could be induced aerobically in the presence of antibiotic stress, and sub-lethal concentrations of chloramphenicol were sufficient to immediately induce this response even after only several minutes of exposure (Figure 2B).

We verified that non-methionyl-tRNAs mischarged with Met could indeed be used in protein synthesis by whole proteome mass spectrometry of anaerobically grown *E. coli*, which has high levels of tRNA misacylation (Figure 2C and D). We have shown previously that Leu-to-Met substitutions in the proteins of the hyperthermophilic archaeon *A. pernix* can have significant effects on enzyme activities (19). Other studies on specific protein mutations have also indicated that Leu-to-Met substitutions can markedly change protein properties (32,33). We therefore choose to focus on locating Leu-to-Met substituted peptides in the *E. coli* proteome. Compared to a total of 7264 peptides that exactly match the *E. coli* genome, we found a total of 30 peptides containing Leu-to-Met substitutions in 29 proteins (0.4%, Figure 2D). Interestingly, 24 of these 30 mistranslated peptides (80%) are present in enzymes. This finding suggests

that like *A. pernix*, where Leu-to-Met mistranslated citrate synthase is better adapted to low temperature growth (19), *E. coli* may also use the methionine mistranslation mechanism to alter the activity of its genetically encoded proteins.

E. coli MetRS fidelity is controlled by Lys-succinylation

In order to elucidate the molecular mechanism facilitating tRNA misacylation, we investigated tRNA charging fidelity of the MetRS *in vitro* to determine how the enzyme alters its tRNA substrate specificity. Purified, recombinant MetRS inherently misacylates non-methionyl-tRNA species *in vitro* (20) (Figure 3A), so additional factors must be required to impart the high fidelity observed *in vivo* during aerobic respiration with glucose. We pursued post-translational modifications as a potential mechanism by which MetRS can quickly shift fidelity *in vivo*. We began by assessing whether succinylation or acetylation of lysine residues could confer fidelity to the enzyme since previous proteomic studies have identified multiple succinylation and acetylation sites in *E. coli* MetRS *in vivo* by mass spectrometry (34–36). Accordingly, MetRS was succinylated or acetylated *in vitro* prior to the tRNA charging reaction via succinyl-CoA and acetyl-CoA preincubation, respectively—treatments known to non-enzymatically modify lysine residues in proteins (34,37). Strikingly, the succinyl-modified MetRS did not accept non-methionyl-tRNAs as substrates (Figure 3B). This effect is specific to MetRS succinylation since acetylation, direct addition of succinate or adding succinyl-CoA without prior incubation with MetRS to charging reactions yielded no differences in tRNA charging fidelity (Figure 3B and C).

These *in vitro* tRNA charging results suggest that the MetRS should have reduced Lys-succinylation under anaerobiosis and antibiotic treatment *in vivo* in order to catalyze the aminoacylation of non-methionyl-tRNAs. To determine whether MetRS succinylation is reduced during conditions prompting tRNA misacylation *in vivo*, we constructed a chromosomal N-terminal His₆-tagged MetRS strain that enabled purification of the endogenous MetRS to homogeneity. We then ran the purified MetRS protein from anaerobic, aerobic and antibiotic treated cultures on SDS-PAGE, and Western blotted for lysine succinylation in MetRS (Figure 3D). As hypothesized, the MetRS obtained from anaerobic and antibiotic treated cultures has significantly reduced Lys-succinylation compared to the aerobic control which indicates that cells remove the succinylation modification from the MetRS during anaerobiosis and antibiotic stress. These results reveal that succinylation of MetRS is sufficient to confer high fidelity to the enzyme, although additional fidelity mediating factors may be present *in vivo* to control tRNA mischarging under standard conditions.

We next employed mass spectrometry to determine which specific succinylated lysine residues were mediating high fidelity tRNA charging after *in vitro* succinylation. Based on the mass spectrometry result and the known crystal structure of *E. coli* MetRS (38), we tested 11 lysine residues with scattered MetRS positions as potential regulatory sites for MetRS fidelity (Lys217, 248, 295, 316, 342, 362, 388, 402, 439, 492, 528). The veritable lysine regulatory sites were

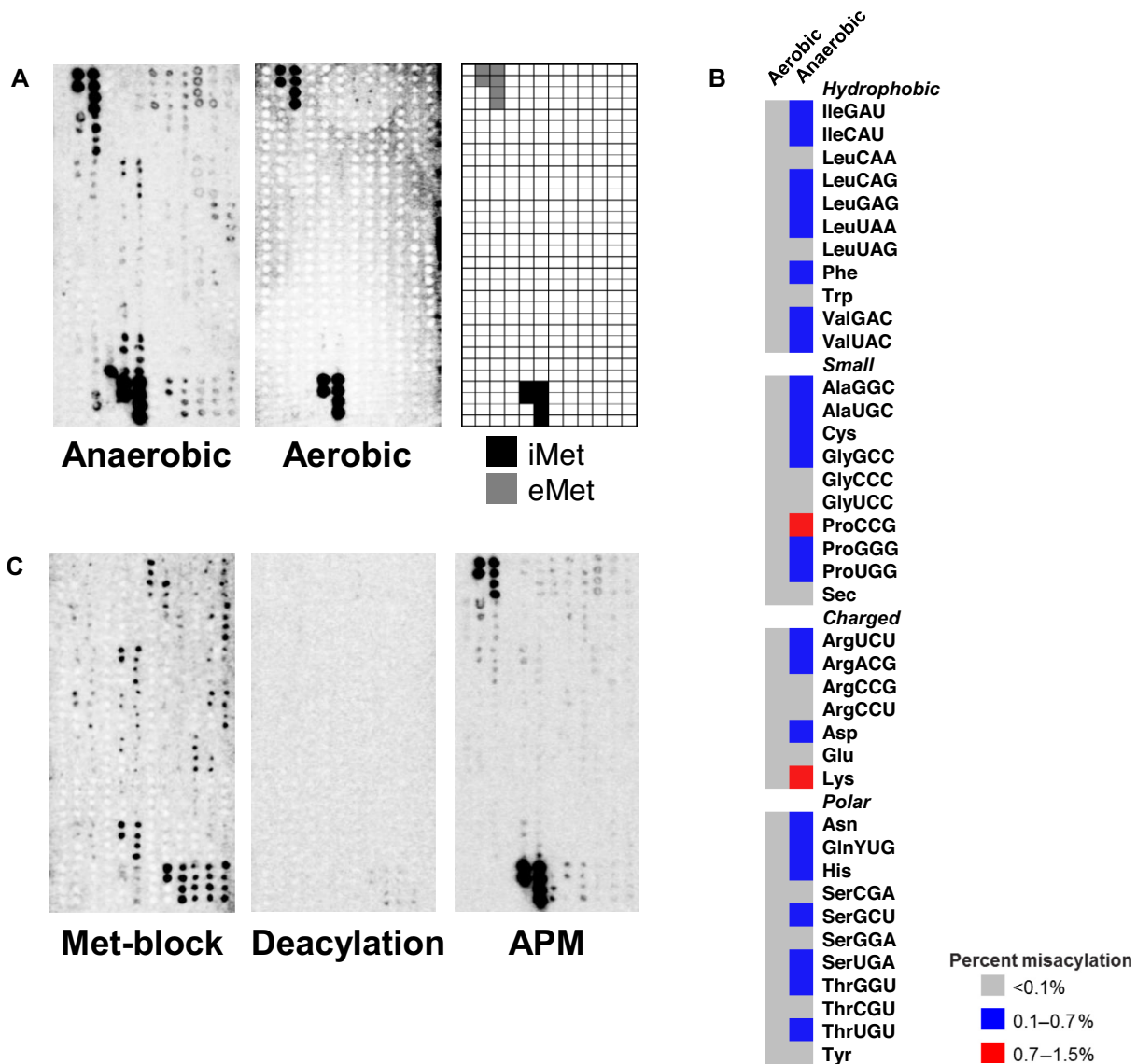


Figure 1. Non-methionyl-tRNAs are constitutively misacylated with methionine during anaerobic fermentation. (A) *E. coli* tRNA microarrays showing tRNA misacylation during anaerobic fermentation, but not during aerobic respiration with glucose. Array map shows the probe locations of *E. coli* tRNA^{iMet} and tRNA^{eMet}. Percent misacylation is calculated by dividing the total signal obtained from a particular tRNA isoacceptor by the total signal from the tRNA^{iMet} and tRNA^{eMet}. (B) Semi-quantification of total array spot signals for anaerobic tRNA misacylation to individual tRNA species, averaged from 3 arrays. Variations of individual tRNAs are within 1.5-fold between arrays. The detection limit is ~0.1% for an individual tRNA. Since the array results are semi-quantitative, we choose to present the data in heat maps rather than precise values with error bars. (C) Array controls to validate ³⁵S-methionine signal from non-methionyl-tRNA probes show exclusion of tRNA^{Met} cross hybridization with addition of free Met probes, exclusion of ³⁵S tRNA nucleotide thio-modifications by removing aminoacylated ³⁵S-Met through chemical deacylation attached to tRNA, and exclusion of peptidyl-tRNA with aminopeptidase M (APM) treatment of tRNA.

identified through glutamic acid mutations in these lysine sites followed by subsequent assessment of the tRNA charging fidelity of the mutant enzymes. Glutamic acid substitutions should work to irreversibly imitate the terminal carboxyl group of succinyllysine. This method of employing glutamic acid to mimic succinylation at specific lysine positions sites has been successfully utilized previously (39). Remarkably, Glu substitutions at Lys362 and Lys388 were sufficient to independently confer high fidelity to the MetRS during *in vitro* tRNA charging (Figure 4A and B). Furthermore, the mutant MetRSs are capable of charging tRNA

with Met at a similar rate and to a similar extent as the wild-type enzyme (Figure 4C). A Lys362 to alanine MetRS mutant, which mimics acetyl-lysine, still mischarges non-methionyl-tRNAs (Figure 4D). This result validates the distinctive role of Lys-succinylation (which converts Lys to a negatively charged side chain) compared to acetylation (which converts Lys to a neutral side chain) for mediating high fidelity tRNA charging. Intriguingly, the *E. coli* MetRS crystal structure without tRNA bound suggests that Lys362 and Lys388 are located in close proximity on the putative tRNA binding interface of the MetRS, thus pointing

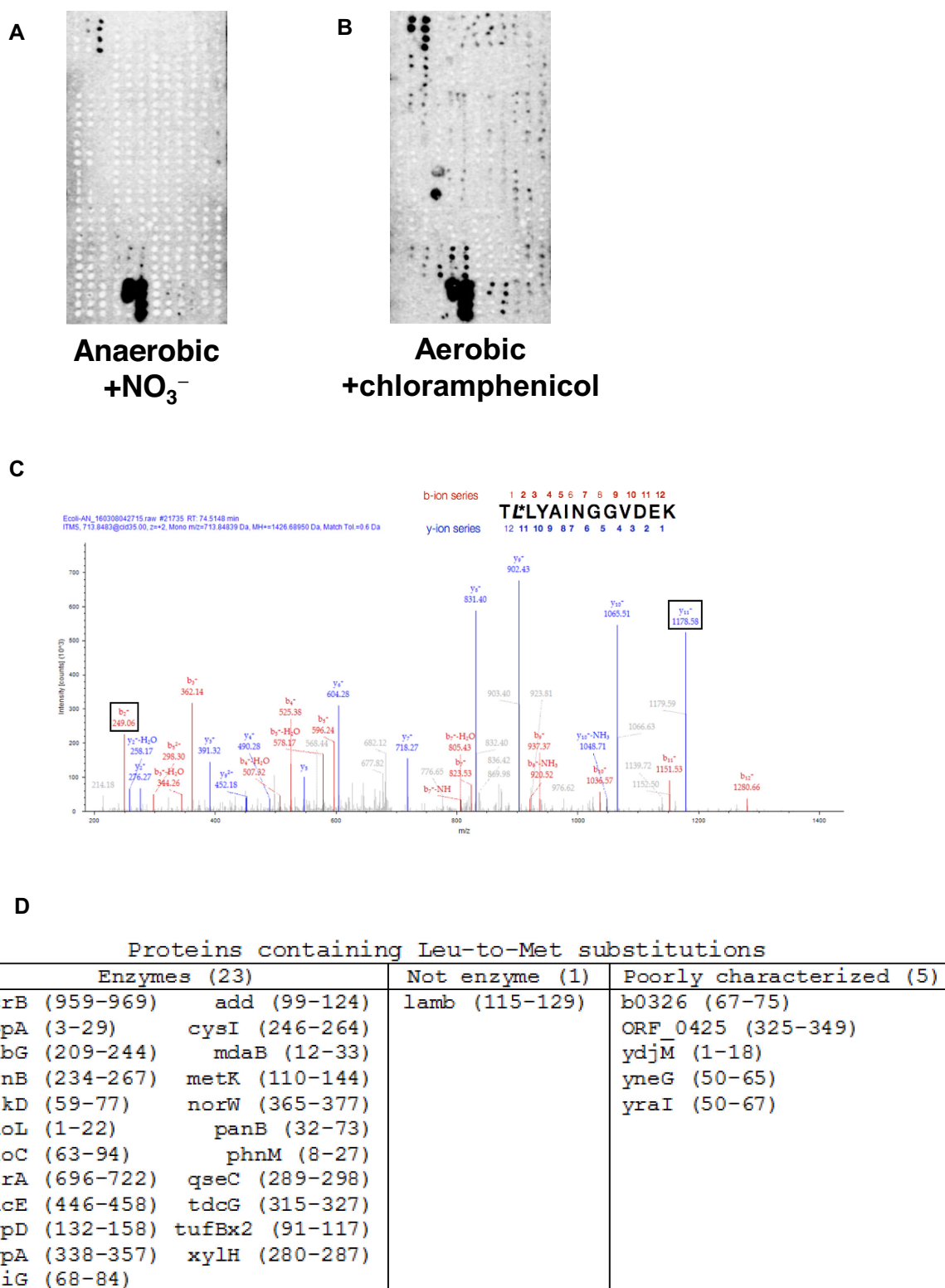


Figure 2. Other conditions that induce tRNA misacylation and identification of mistranslated peptides. (A) tRNA misacylation during anaerobic respiration with nitrate. (B) Misacylation after 10 μ M chloramphenicol was added with the ³⁵S-methionine pulse label. (C) Example fragmentation spectrum indicating leucine-to-methionine mistranslation in peptide TL*LYAINGGVDEK (from TdcE, 2-ketobutyrate/pyruvate-formate lyase), where L2 has been replaced with M, here detected as the sulfoxide. Matched b-ions (red) and y-ions (blue) are indicated by colored peaks in the spectrum and bold numbers in the sequence legend, and site-determining ions for the mistranslation at L2 (b2+ and y11+) are highlighted. (D) Gene names and the peptide locations (in parenthesis) of the Leu-to-Met substituted peptides grouped according to their functions as enzymes, non-enzymes or unknown.

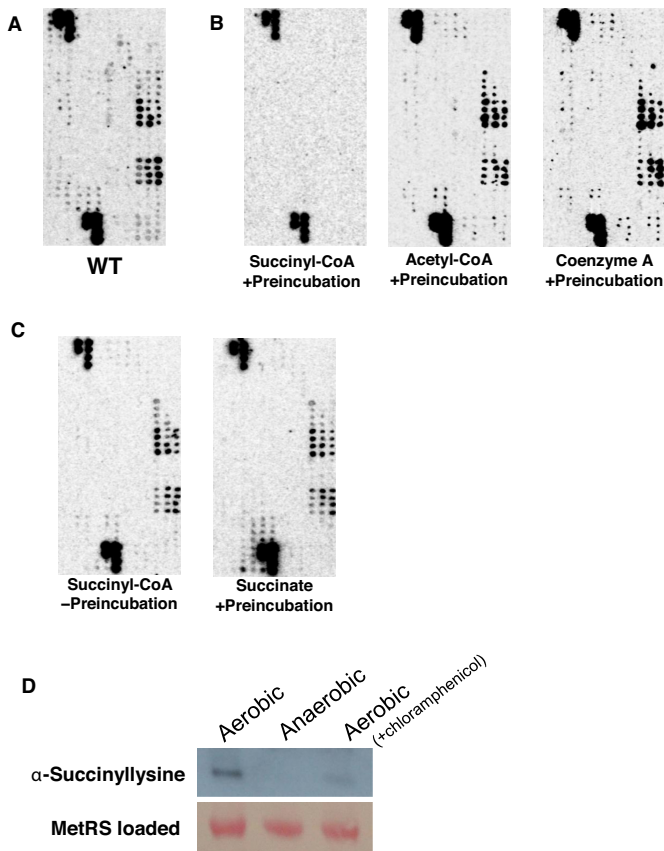


Figure 3. Succinyl-lysine modifications in *E. coli* MetRS confer high tRNA charging fidelity to the promiscuous, unmodified enzyme. (A) tRNA microarray showing tRNA misacylation *in vitro* by the purified, recombinant MetRS. (B) *In vitro* tRNA charging by purified MetRS that has been pre-incubated with succinyl-CoA, acetyl-CoA or coenzyme A prior to the charging reaction. (C) *In vitro* tRNA charging by purified MetRS in the presence of succinyl-CoA without preincubation, or in the presence and with preincubation with succinate. (D) Western blot for succinyl-lysine on purified chromosomally his-tagged MetRS that was obtained from *E. coli* growing aerobically, anaerobically and with antibiotic exposure. MetRS loading control was visualized via Ponceau S staining of the membrane following exposure.

to a role for both residues in non-methionyl-tRNA discrimination (Figure 4E). Since tRNAs are negatively charged, it is possible that the positively charged lysine residues along the tRNA binding interface of the unmodified MetRS mediate non-specific tRNA binding and/or aminoacylation. Consequently, the negative charges imparted by succinyllysine at these MetRS sites may increase its specificity for tRNA binding and/or aminoacylation.

Since glutamic acid mutations at either lysine position were sufficient to confer high fidelity to the MetRS, the two sites appear to be redundant in their ability to discriminate against non-methionyl-tRNAs. Succinylation at Lys362 in MetRS was detected *in vivo* by two independent investigations that globally identified succinylation sites in *E. coli* (34,35). Although succinylation at site 388 has not been identified *in vivo* in these studies, succinylation data from the *in vivo* investigations vary considerably and this site was readily succinylated *in vitro*. Furthermore, our study implicates Lys388 as being rapidly desuccinylated in response to

stress, which could have unknowingly occurred in the previous proteome wide Lys-succinylation studies.

Strikingly, lysine occupies both regulatory positions 362 and 388 of MetRS in 92% of all Gammaproteobacteria and 98% of all Betaproteobacteria (Figure 5). This high level of conservation indicates that succinylation mediated tRNA mismethionylation may be a pervasive mechanism in these large bacterial classes that comprise a wide array of pathogens, commensals and other bacteria inhabiting diverse environments.

Ablating tRNA mismethionylation results in reduced stress tolerance

To evaluate the cellular processes that may be associated with tRNA mismethionylation, strains were created with chromosomal Glu substitutions at the Lys362 or Lys388 sites of MetRS in *E. coli* to irreversibly confer higher fidelity to the MetRS. When subjected to anaerobiosis, the mutant strains showed substantially higher tRNA^{Met} charging fidelity than wild type (Figure 6A). Reversion of the Glu mutations to Lys (strains E362K and E388K, which are distinguished from the wild type through the preservation of synonymous codon mutations in the MetRS gene made in the K362E and K388E mutants) restored tRNA misacylation to WT levels (Figure 6A). These results validate the role for Lys-succinylation in mediating MetRS fidelity *in vivo*. We next subjected the wild-type and mutant strains to Biolog phenotype microarray analysis which analyzed growth in ~2000 different metabolic and stress conditions (Figure 6B). Phenotype microarray conditions assaying growth in various carbon, nitrogen, phosphorus and sulfur sources yielded results that are dependent on the specific mutant strain, indicating that the mutant strains have varying abilities to grow on different nutrients and supplements. In contrast, both mutants had a striking and unvarying growth deficiency when exposed to a multitude of antibiotics (including chloramphenicol) and chemical stressors at different concentrations. To validate one phenotype of the mutant strains observed in the phenotype microarrays, all mutants and reverted strains were plated with and without a sub-lethal concentration of chloramphenicol (Figure 6C), which readily induces tRNA misacylation in the WT strain (Figure 2B). While all strains grew comparably without chloramphenicol, strains incapable of tRNA mismethionylation had growth deficiencies in the presence of this antibiotic and were not capable of growth at high dilutions. These results demonstrate that natural tRNA misacylation with methionine and its potential utilization in subsequent mistranslation are associated with increased resistance to antibiotics and stressors. These results augment the previously established benefits identified for mistranslation from previous studies (9,11,12,14).

DISCUSSION

This work establishes that Met mistranslation at the level of tRNA mischarging via MetRS is a widespread biological phenomenon that is conserved from bacteria to eukaryotes. In addition, we have identified a function for the recently identified Lys-succinylation in mediating MetRS fidelity

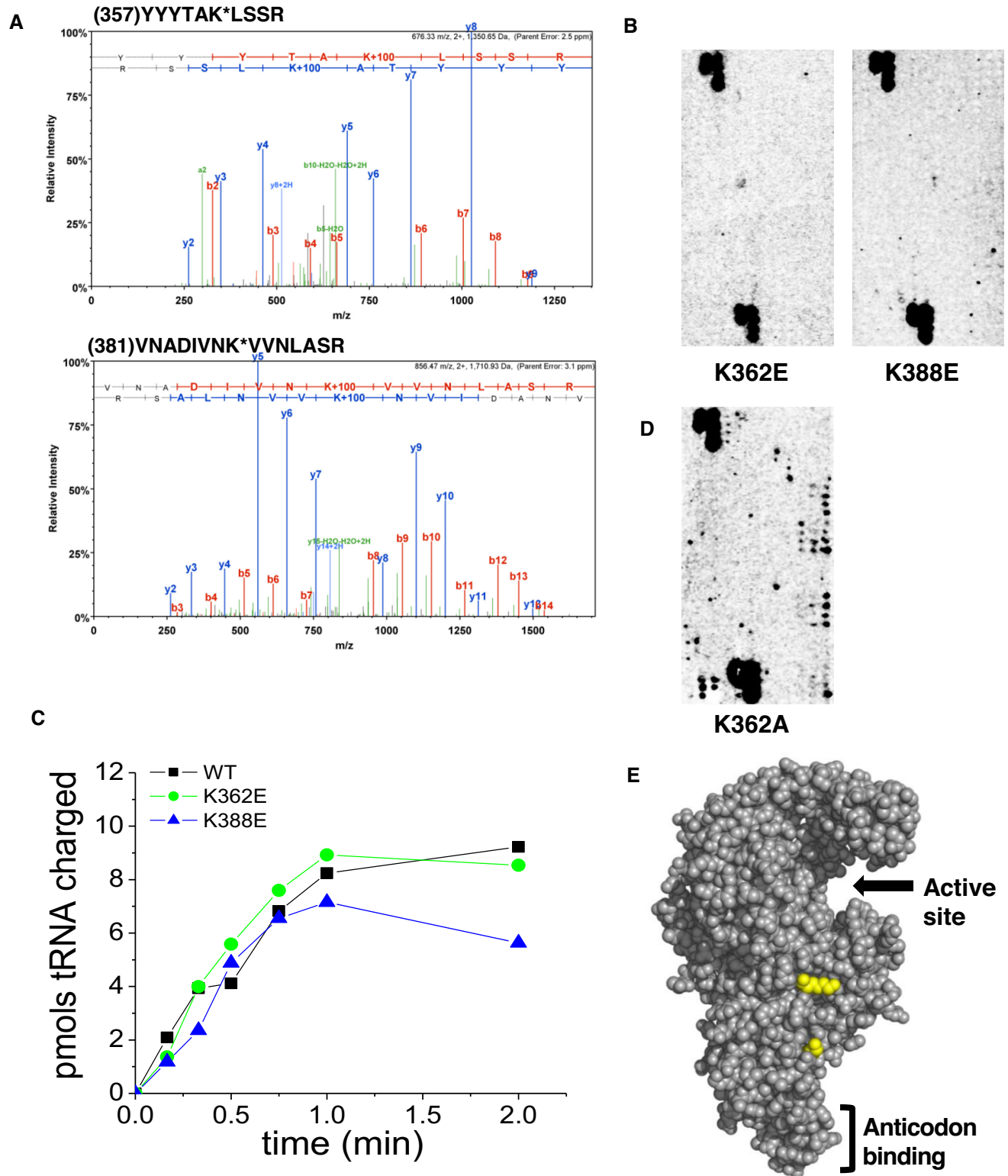


Figure 4. Succinylation at Lys362 or Lys388 is sufficient to confer high fidelity to *E. coli* MetRS. (A) Mass spectrometry spectra showing succinylation at Lys362 (top) and Lys388 (bottom) in MetRS during the *in vitro* succinylation reaction. (B) *In vitro* tRNA charging by mutant MetRS enzymes containing Glu substitutions at lysine 362 or 388. (C) tRNA charging with Met by WT and mutant MetRSs at 37°C with 100 nM MetRS and 100 μM total *E. coli* tRNA. (D) *In vitro* tRNA charging by the mutant MetRS containing alanine substitution at Lys362. (E) *E. coli* MetRS crystal structure highlighting the regulatory sites 362 (top residue) and 388 (bottom residue) along the putative tRNA binding interface.

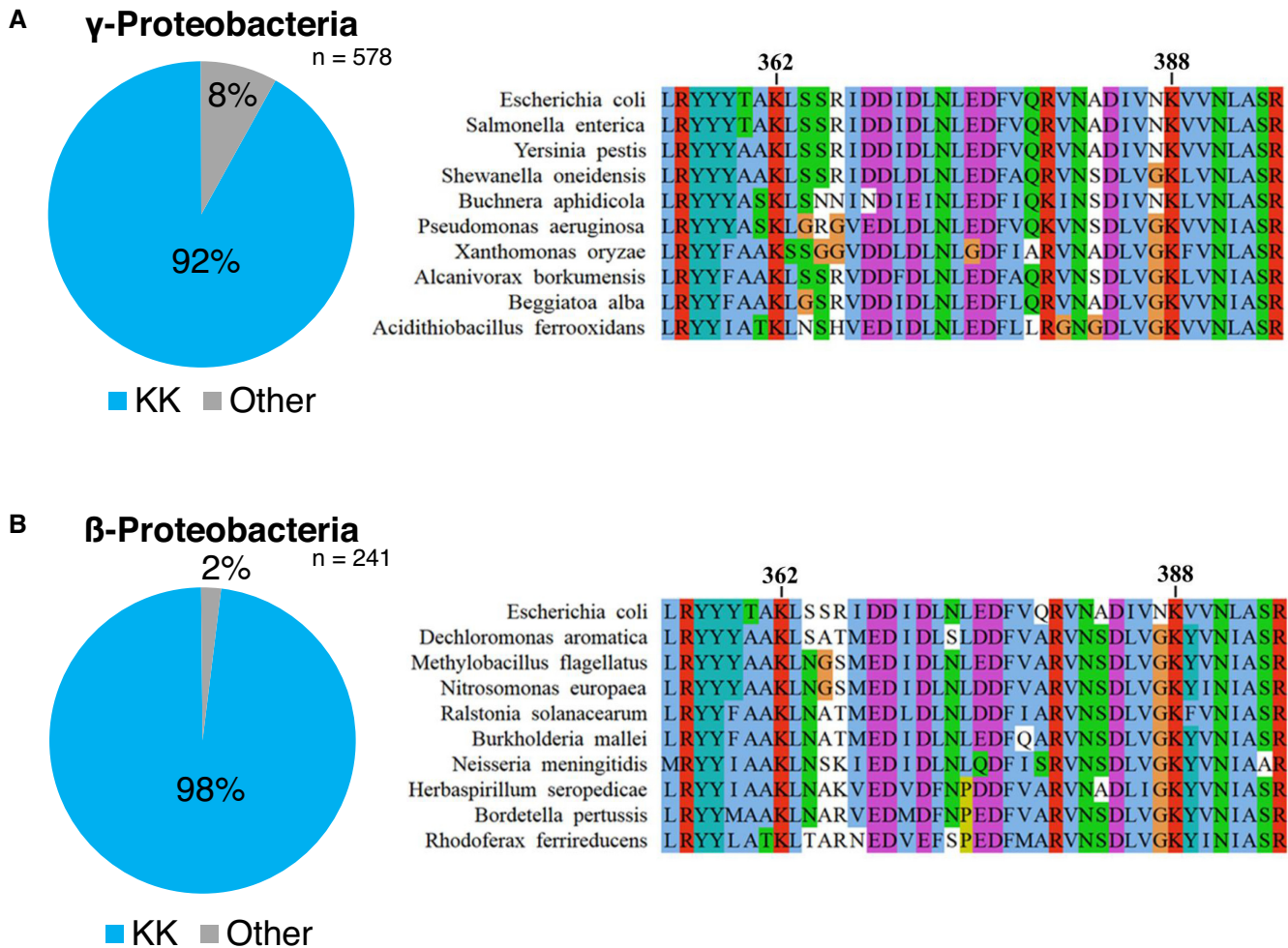


Figure 5. Lysine conservation at the homologous MetRS regulatory sites. Amino acid identity of homologous MetRS regulatory sites 362 and 388 in (A) Gammaproteobacteria and (B) Betaproteobacteria with representative sequence alignments showing *E. coli* as a reference.

and therefore playing a large role in global tRNA mis-methionylation. It remains to be determined which exact cellular processes are responsible for installing and removing the succinyllysine modifications on MetRS. Succinyl-CoA, a product of the TCA cycle, is capable of non-enzymatically modifying lysine residues (34) and one enzyme has been identified in *E. coli* that is capable of removing succinyllysine modifications in proteins (35). These factors likely play a role in regulating tRNA mismethionylation. We found that two distinct conditions that prompt tRNA misacylation (anaerobiosis and antibiotic treatment) similarly reduce MetRS succinylation *in vivo*. However, the precise means by which tRNA misacylation is induced under diversely activating conditions requires further investigation. Identifying succinylation as a veritable fidelity mediating factor for MetRS allowed us to irreversibly render MetRS accurate *in vitro* and *in vivo* through succinylation imitating lysine to glutamic acid mutations. By substantially increasing the fidelity of tRNA charging in the mutant strains, we show that the inability to perform tRNA misacylation is associated with multiple phenotypic weaknesses in *E. coli*.

The most striking and common phenotype obtained from the mutant strains is their compromised ability to grow in the presence of antibiotics and chemical stressors, which may be explained in three, possibly overlapping ways (40–42). Firstly, mistranslated proteins can have novel functions or altered susceptibilities. For example, specific methionine mistranslated CamkII protein kinases can have drastically distinct enzymatic activities in response to oxidative or ATP activation compared to the wild-type CamkII, and these mistranslated proteins can enhance the CamkII derived phenotype in human cells (43). In addition, methionine mistranslated citrate synthase in the hyperthermophile *Aeropyrum pernix* has enhanced activity at lower temperatures to accommodate the conditional low temperature growth of this archaeon (19). Similar to the genetic diversification of a population, protein diversification with methionine may ensure that a subset of the mistranslated proteins will always be active, even in pernicious growth conditions. Secondly, mistranslation has been shown to induce secondary stress responses such as the general stress responses in *E. coli* (14), which could also contribute to a broad increase in tolerance to stressors (44). Thirdly, mismethionylated tR-

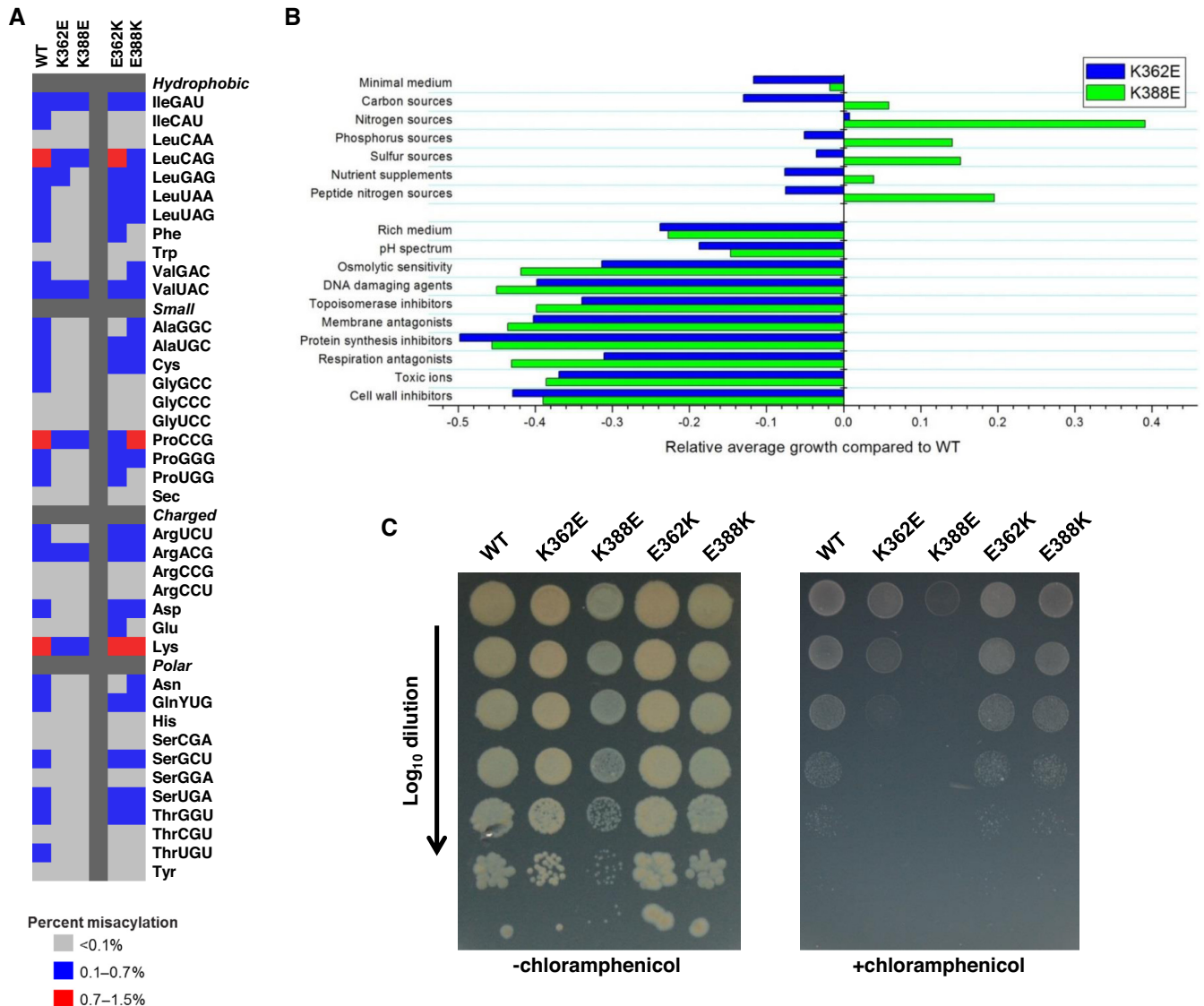


Figure 6. Strains incapable of tRNA misacylation with Met are less adept at growth in the presence of antibiotics and stressors. (A) Semi-quantitative heatmap of anaerobic tRNA misacylation with Met to individual tRNA species in the WT, misacylation deficient mutant strains and their revertants. The revertants were distinguished from the WT strain through the preservation of the synonymous codon mutations at two adjacent codons to the respective Lys362 or 388 site in the MetRS gene made in the mutants. (B) Mutant growth quantification compared to WT in Biolog phenotype microarrays organized by condition or stress types. All individual compounds and media conditions used can be found under http://www.biolog.com/products-static/phenotype_microbial_cells_use.php. (C) LB plate dilution assay showing growth of WT, mutants and revertants with and without 10 μ M chloramphenicol—the same concentration used to induce mismethionylation in Figure 2B.

NAs may play a distinct, yet unknown functional role not involved in translation.

An intriguing finding in this study shows that *E. coli* constitutively performs tRNA mismethionylation during fermentative anaerobic conditions. One possible rationalization for this response stems from the fact that *E. coli* is a facultative anaerobe and can benefit from improved growth via anaerobic respiration and microaerophilic growth during colonization and persistence in the colon (29,30,45). Hence, when *E. coli* is restricted to survival exclusively through fermentation, it may benefit from the phenotypic advantages associated with tRNA mismethionylation and its potential utilization in mistranslation. *E. coli* has also been shown to

be capable of making anticipatory responses (46). It is therefore possible that *E. coli* has evolved to make a preemptive mistranslational response to anaerobiosis or in anticipation of stresses associated with colonization and competition in the mammalian colon.

In summary, our work has uncovered a readily activated response in *E. coli* that is associated with diverse phenotypic benefits. The broad conservation of the two succinylated lysine residues in γ and β Proteobacteria suggests that tRNA mismethionylation may also be employed for beneficial functions in other diverse bacteria including pathogens that may use mistranslation to promote their virulence.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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