Pseudomonas aeruginosa EftM Is a Thermoregulated Methyltransferase

Joshua P. Owings, Emory University
Emily G. Kuiper, Emory University
Samantha M. Prezioso, Emory University
Jeffrey Meisner, Emory University
John J. Varga, Emory University
Natalia Zelinskaya, Emory University
Eric Dammer, Emory University
Duc M. Duong, Emory University
Nicholas Seyfried, Emory University
Sebastián Albertí, Universidad de las Islas Baleares

Only first 10 authors above; see publication for full author list.

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Joshua P. Owings,1,2 Emily G. Kuiper,1,3 Samantha M. Prezioso,1,4,4 Jeffrey Meisner,1,5 John J. Varga,1,5 Natalia Zelinskaya,1, Eric B. Dammer,1 Duc M. Duong,1 Nicholas T. Seyfried,1 Sebastián Alberti,1 Graeme L. Conn,1 and Joanna B. Goldberg

From the 1Department of Pediatrics, Division of Pulmonology, Allergy/Immunology, Cystic Fibrosis and Sleep and the 6Emory-Children’s Center for Cystic Fibrosis Research, Emory University and Children’s Healthcare of Atlanta, Atlanta, Georgia 30322, the 4Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, Virginia 22908, the 2Department of Biochemistry and the **Biochemistry, Cell, and Developmental Biology Program and **Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Emory University School of Medicine, Atlanta, Georgia 30322, and the 5Instituto Universitario de Investigación en Ciencias de la Salud, Universidad de las Islas Baleares, Palma de Mallorca, 07122 Spain

E. coli / Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that trimethylates elongation factor-thermo-unstable (EF-Tu) on lysine 5. Lysine 5 methylation occurs in a temperature-dependent manner and is generally only seen when P. aeruginosa is grown at temperatures close to ambient (25 °C) but not at higher temperatures (37 °C). We have previously identified the gene, eftM (for EF-Tu-modifying enzyme), responsible for this modification and shown its activity to be associated with increased bacterial adhesion to and invasion of respiratory epithelial cells. Bioinformatic analyses predicted EftM to be a Class I S-adenosyl-L-methionine (SAM)-dependent methyltransferase. An in vitro methyltransferase assay was employed to show that, in the presence of SAM, EftM directly trimethylates EF-Tu. A natural variant of EftM, with a glycine to arginine substitution at position 50 in the predicted SAM-binding domain, lacks both SAM binding and enzyme activity. Mass spectrometry analysis of the in vitro methyltransferase reaction products revealed that EftM exclusively methylates at lysine 5 of EF-Tu in a distributive manner. Consistent with the in vivo temperature dependence of methylation of EF-Tu, preincubation of EftM at 37 °C abolished methyltransferase activity, whereas this activity was retained when EftM was preincubated at 25 °C. Irreversible protein unfolding at 37 °C was observed, and we propose that this instability is the molecular basis for the temperature dependence of EftM activity. Collectively, our results show that EftM is a thermolabile, SAM-dependent methyltransferase that directly trimethylates lysine 5 of EF-Tu in P. aeruginosa.

Protein post-translational modification adds an additional level of complexity that can influence protein function as well as change the protein charge and tertiary structure. The protein post-translational modification landscape is vast; more than half of the natural amino acids are substrates for chemical modification, and lysine, for example, can be modified with at least 10 different post-translational modifications, including methylation (1).

Although first discovered on the bacterial flagellum (2), the study of lysine methylation in prokaryotes has lagged behind that of eukaryotes. In eukaryotes, the most well studied effect of lysine methylation is within the field of epigenetics, where patterns of methylation form the “histone code,” and serve as another level of DNA transcriptional control (3). In bacteria, methylated lysines have been found on flagella, specific outer membrane proteins, and the ribosome translational machinery; however, for the most part, the functional consequences of these modifications are not known (2, 4 – 8).

Post-translational modification of proteins involved in protein synthesis has the potential to exert a significant effect on bacterial gene expression. Lysine methylation of components of the translational machinery, including essential translation factors such as elongation factor-thermo-unstable (EF-Tu),6 which binds to and delivers aminoacylated tRNA to the ribo-

6 The abbreviations used are: EF-Tu, elongation factor thermo-unstable; DTMk, di/trimethyl lysine; EFm, EF-Tu modifying enzyme; ITC, isothermal titration calorimetry; LB, lysogeny broth; N-His, amino-terminal hexahistidine; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine.
some, has been identified (9). In *Escherichia coli*, for example, EF-Tu lysine 57 is monomethylated during logarithmic growth phase (10), but as cells transition to stationary phase, there is a gradual conversion of monomethyl lysine to dimethyl lysine (11). Although methylation of EF-Tu at lysine 57 does not significantly alter its interaction with GTP, GDP, EF-Tu, or aminoacylated tRNAs, there is a 2-fold decrease in GTP hydrolysis when EF-Tu is 20% monomethylated and 80% dimethylated compared with unmethylated (11). These findings suggest that when EF-Tu is 20% monomethylated and 80% dimethylated noacylated tRNA, there is a 2-fold decrease in GTP hydrolysis.

EftM is a Thermoregulated Methyltransferase

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Primers**—The strains and plasmids used in this study are listed in Table 1. All DNA oligonucleotide primers used for cloning and sequence analysis in this study are listed in Table 2.

**Plasmid Construction**—For complementation experiments, the PAHM23 *eftM* gene (with G148C mutation encoding a G50R amino acid substitution) was cloned into the broad-host-range expression vector pUCP18ApGw, as described previously for the wild-type gene from PAO1 (13). Briefly, the coding sequence was amplified using purified PAHM23 genomic DNA as template with primers oJPO18 and oJPO19 (Table 2). The amplicon was cloned into the Gateway entry vector pENTR/SD/D-TOPO (Life Technologies, Inc.) following the manufacturer’s instructions. Gateway LR Clonase II enzyme mix (Life Technologies) was then used to clone from the entry vector into the destination vector pUCP18ApGw (13) to generate plasmid pJPO7. The nucleotide sequence of the plasmid insert was verified using automated DNA sequencing.

To create plasmids for overexpression of wild-type and K5A substituted EF-Tu, the coding sequence of PAO1 *tufB* (PA4277) was amplified from PAO1 genomic DNA with an engineered amino-terminal hexahistidine (N-His$_6$) affinity tag using primer pairs tufBF and tufBR or tufBFK5A and tufBR, respectively (13). Amplicons were Gateway cloned as described above into the destination vector pDEST14 (Life Technologies) to generate expression plasmids pJPO4 and pJPO5 containing N-His$_6$ *tufB* and N-His$_6$ *tufB* K5A, respectively. Similarly, for EftM, the coding sequences of PAO1 *eftM* (PA4178) and PAHM23 *eftM* (G50R) were amplified from PAO1 and PAHM23 genomic DNA, respectively, using primers oJPO20 and oJPO21 (Table 2). PCR products were digested with NdeI and HindIII (New England Biolabs) and cloned into pCOLD II (Takara Bio) in-frame with a N-His$_6$ affinity tag to generate expression plasmids pJPO1 and pJPO2, respectively.

**Protein Expression and Purification**—Expression of N-His$_6$-tagged EF-Tu or EF-Tu K5A was accomplished in *E. coli* BL21-A1 cells transformed with pIP04 or pIP05, respectively, in 1-liter cultures of ZYM-5052 medium (18) supplemented with 0.2% arabinose (w/v) and carbenicillin (100 µg/ml). Cultures were incubated with shaking at 25 °C for 14 h. Cells were pelleted at 12,000 × g for 10 min at 4 °C and resuspended in 25 ml of lysis buffer (GoldBio Bacterial Cell Lysis Buffer and 10 mM imidazole) with 100 µg/ml DNase I (10 mg/ml; GoldBio), 300 µl of ProBlock Gold protease inhibitor (GoldBio), and 0.2 mg/ml lysozyme (Roche Applied Science). Cell suspensions were lysed using a French pressure cell (American Instrument Co.) at 16,000–18,000 p.s.i. Lysates were then subjected to centrifugation at 12,000 × g for 10 min at 4 °C to remove cellular debris before applying the supernatants to columns containing HisPur nickel-nitrilotriacetic acid resin (Thermo Scientific). The columns were washed three times with 1 ml of wash buffer (50 mM sodium phosphate, 150 mM sodium chloride, 20% glycerol (v/v), 6 mM β-mercaptoethanol, and 50 mM imidazole, pH 7.4) and eluted in four sequential 1-ml fractions of elution buffer (wash buffer with 250 mM imidazole).
### TABLE 1
Summary of strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics or Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>Wild-type</td>
<td>(14)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1ΔeftM</td>
<td>Deletion of <em>eftM</em> derived from PAO1</td>
<td>(14)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAHM23</td>
<td>Chronic infection isolate with G50R mutation in <em>eftM</em></td>
<td>(15)</td>
</tr>
<tr>
<td><em>E. coli</em> BL21</td>
<td><em>fhuA2 [floc] ompT gal [dcm] ΔhsdS</em></td>
<td>New England Biolabs</td>
</tr>
<tr>
<td><em>E. coli</em> BL21-A1</td>
<td>F′ <em>ompT hsdS</em>&lt;sub&gt;9&lt;/sub&gt;(r&lt;sub&gt;K&lt;/sub&gt;&lt;sup&gt;r&lt;/sup&gt;)gal dcm araB::T7RNP-tetA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> Top10</td>
<td>F′ <em>mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD189 Δ(ara-leu)7697 galU galK rpsL (Str&lt;sup&gt;R&lt;/sup&gt;) endA1 nupG</em></td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α Library Efficiency</td>
<td>F′ <em>φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r&lt;sub&gt;K&lt;/sub&gt;&lt;sup&gt;r&lt;/sup&gt;,m&lt;sub&gt;K&lt;/sub&gt;&lt;sup&gt;r&lt;/sup&gt;)phoA supE55 thi-1 gyrA96 relA1 λ</em></td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
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<tr>
<td>pUCP18ApGw</td>
<td>Gateway compatible broad-host-range vector</td>
<td>(14)</td>
</tr>
<tr>
<td>pENTR/SD/D-TOPO</td>
<td>Gateway® compatible directional cloning entry vector with Shine-Dalgarano sequence; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>pUCP18ApGw (eftM)</td>
<td>pUCP18ApGw + PAO1 <em>eftM</em> C-terminal FLAG fusion</td>
<td>(14)</td>
</tr>
<tr>
<td>pUCP18ApGw (tufB)</td>
<td>pUCP18ApGw + PAO1 tufB N-His, tag</td>
<td>(14)</td>
</tr>
<tr>
<td>pJPO7</td>
<td>pUCP18ApGw + PAHM23 (G50R) <em>eftM</em> C-terminal FLAG fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pCOLDII</td>
<td>Cold shock expression vector; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Takara</td>
</tr>
<tr>
<td>pDEST14</td>
<td>Gateway® compatible arabinose inducible expression vector; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>pG-Tf2</td>
<td>Tetracycline inducible chaperone plasmid containing <em>groES-groEL-tig</em>; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Takara</td>
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<tr>
<td>pJPO1</td>
<td>pCOLDII + <em>eft</em> M N-His, fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pJPO2</td>
<td>pCOLDII + <em>eft</em> M G50R N-His, fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pJPO4</td>
<td>pDEST14 + tufB N-His, fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pJPO5</td>
<td>pDEST14 + tufB K5A N-His, fusion</td>
<td>This study</td>
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### TABLE 2
Summary of DNA oligonucleotides

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<td><em>tufBF</em></td>
<td>CACCATGATCATCATCATCATCATCATATGGCTAAAGAAAATTTGA</td>
<td>See Ref. 14</td>
</tr>
<tr>
<td><em>tufBR</em></td>
<td>TTATTCGATGATCTTGGCAACC</td>
<td>See Ref. 14</td>
</tr>
<tr>
<td><em>tufBF</em>K5A</td>
<td>CACCATGATCATCATCATCATCATCATATGGCTAAAGAAAAGCATTTGA</td>
<td>See Ref. 14</td>
</tr>
<tr>
<td>oIP18</td>
<td>CACCATGTCGCAACCGCCCTTGTGCCTCAGCGAC</td>
<td><em>eftM</em> forward Gateway</td>
</tr>
<tr>
<td>oIP19</td>
<td>CATCATATGTCGCAACCGCCCTTGTGCCTCAGCGAC</td>
<td><em>eftM</em> reverse C-FLAG</td>
</tr>
<tr>
<td>oIP20</td>
<td>CATATATGTCGCAACCGCCCTTGTGCCTCAGCGAC</td>
<td><em>eftM</em> forward Ndel</td>
</tr>
<tr>
<td>oIP21</td>
<td>CATATATGTCGCAACCGCCCTTGTGCCTCAGCGAC</td>
<td><em>eftM</em> reverse HindIII</td>
</tr>
<tr>
<td>oIP120</td>
<td>CATATATGTCGCAACCGCCCTTGTGCCTCAGCGAC</td>
<td><em>eftM</em> forward Ncol</td>
</tr>
<tr>
<td>oIP121</td>
<td>ATGAAGCTTTCTGATGATCATCATATGGCTAAAGAAAATTTGA</td>
<td><em>eftM</em> reverse FLAG HindIII</td>
</tr>
</tbody>
</table>
EftM is a Thermoregulated Methyltransferase

For overexpression of N-His<sub>6</sub>-tagged EftM (PAO1) and N-His<sub>6</sub>-tagged EftM G50R (PAHM23), chemically competent BL21(DE3) cells were transformed with pJPO1 or pJPO2, respectively, along with pG-Tf2 helper plasmid encoding groES-groEL-tig (Takara Bio) and cultured overnight at 37 °C in lysogeny broth (LB) containing chloramphenicol (20 µg/ml) and carbenicillin (100 µg/ml). LB (1 liter) supplemented with carbenicillin, chloramphenicol, and tetracycline (5 ng/ml) was inoculated with 14 ml of overnight culture and then incubated in a 15 °C water bath for 30 min before the addition of 0.5 ml of methylation buffer and the Bio-Rad ChemiDoc MP imager. All images were analyzed using Image Lab version 5.1 (Bio-Rad). Methyltransferase assays with heat pretreatment were performed in gel filtration buffer and contained 10 µM EftM, 16 µM EF-Tu, and 1 mM SAM. EftM was preincubated at either 25 or 37 °C for 0, 5, 10, and 20 min prior to addition to the methyltransferase assay. Reactions were incubated at 25 °C for 20 min and inactivated as described above. Reactions were incubated at 25 °C for 20 min and inactivated as described above. Samples were run on 14% SDS-polyacrylamide gels and stained with Coomassie or analyzed by immunoblotting using a DTmK antibody (Upstate Biotechnology). Immunoblotting analysis was used to assess EF-Tu methyltransferase activity. In vivo and in vitro assays performed under conditions designed to produce partial or complete methylation. For in vivo methylation, recombinant N-His<sub>6</sub>-tagged EF-Tu was purified from P. aeruginosa strain PAO1 grown at 25 °C, as described by Barbier et al. (13), and digested directly with chymotrypsin for MS analysis. Partial methylation of EF-Tu was accomplished using products of in vitro methyltransferase assays performed with excess SAM and EF-Tu but limiting EftM (0.6 µM) and short incubation times (5 and 10 min). Products of in vitro assays were run on a 10% SDS-polyacrylamide gel (Bio-Rad) and stained with GelCode Blue (Thermo Scientific) for protein visualization. Bands were excised, and protein was digested in-gel with chymotrypsin. The resulting peptides were extracted with a solution of 5% formic acid and 50% acetonitrile and speed-vacuumed to dryness.

An equal volume of each peptide sample was resuspended in loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile), and peptide eluents were separated on a 1.9 µM C18 (Dr. Maisch, GmbH, Ammerbuch, Germany) self-packed column (15 cm × 75 µm; New Objective) on a nanoACQUITY UPLC system (Waters) and monitored on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific). Elution was performed over a 120-min gradient at a rate of 325 nl/min with buffer B ranging from 3 to 80% (buffer A: 0.1% formic acid and 5% DMSO in water; buffer B: 0.1% formic and 5% DMSO in acetonitrile). The mass spectrometer cycle was programmed to collect one full MS scan followed by 10 data-dependent tandem mass spectrometry (MS/MS) scans. The MS scans were collected at a resolution of 35,000 (300–1800 m/z range, 1,000,000 automatic gain control, 100-ms maximum ion time), and the MS/MS spectra were acquired at a resolution of 17,500 (2 m/z isolation width, 30% collision energy, 10,000 automatic gain control target, and 50-ms maximum ion time). Dynamic exclusion was set to exclude previous sequenced peaks within a 10-ppm window for 30 s.

The SageN Sorcerer SEQUEST version 4.3 algorithm was used to search and match MS/MS spectra to a complete semi-chymotryptic E. coli database harboring the recombinant EF-Tu sequence from P. aeruginosa PAO1 strain (total with 11,541 entries), including pseudoreversed E. coli decoy sequences (21, 22). Searching parameters included mass toler-
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![Image of Western blot analysis](image_url)

**FIGURE 1. Wild-type but not G50R substituted EftM is able methylate EF-Tu in vivo.** Shown is Western blotting analysis of whole cell lysates from the indicated *P. aeruginosa* strains as well as a previously described *eftM* deletion mutant, PAO1ΔeftM, complemented with a plasmid (pUCP18ApGw (*eftM*)) containing the wild-type PAO1 EftM (+eftM) or a plasmid (pJ07) containing the G50R EftM (+eftM G50R) blotted with both anti-DTmK (top) and anti-RpoA (bottom) antibodies.

ance of precursor ions (±20 ppm) semichymotryptic restriction, dynamic modifications for oxidized Met (+15.9949 Da), trimethyl lysine (+42.0470 Da), four maximal modification sites, and a maximum of two missed cleavages. Only b and y ions were considered for scoring (Xcorr), and Xcorr along with Cn were dynamically increased for groups of peptides organized by a combination of chymotrypticity (fully or partial) and precursor ion charge state to remove false positive hits along with decoys until achieving a false discovery rate of <5% (<0.25% for proteins identified by more than one peptide) (23).

Following discovery-based identification of the trimethylated EF-Tu lysine 5 peptide (m/z = 398.231) and corresponding unmodified peptide (m/z = 377.208), both were quantified on an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific) using a targeted MS approach essentially as described (24). A user-defined precursor mass tolerance of ±20 ppm was employed for extracted ion chromatogram-based quantification. For time course assays, fully chymotryptic peptides representing unmodified (AKEKF, m/z = 311.68), monomethylated (AKEKmeF, m/z = 318.69), dimethylated (AKEKme₂F, m/z = 325.70), and trimethylated (AKEKme₃F, m/z = 332.71) EF-Tu lysine 5 were directly monitored and quantified by extracted ion chromatogram on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific).

**Isothermal Titration Calorimetry—**SAM and *S*-adenosyl-L-homocysteine (SAH) (Sigma-Aldrich) were each dissolved in gel filtration buffer to 1 mM final concentration and titrated into EftM (30–50 μM) in 16 2.4-μl injections using an Auto-iTC200 microcalorimeter (Malvern/MicroCal) at 25 °C. After accounting for the heat of dilution by subtraction of the residual heat measured at the end of the titration, the data were fit using a model for one set of sites to determine the binding affinity (Kᵦ). Values reported are the average of at least two independent titrations and the associated S.D. between these measurements.

**Circular Dichroism Spectroscopy—**Analysis of EftM (10 μM, in gel filtration buffer) unfolding by circular dichroism (CD) was recorded using a Jasco J-810 spectropolarimeter with a Peltier temperature controller. Data were collected using a 1-mm path length cuvette at 218 nm, collecting data every 0.5 °C, and a heating rate of 1 °C/min from 20 to 45 °C.

**Differential Scanning Fluorimetry—**The ability of SAM to stabilize the EftM structure was assessed using differential scanning fluorimetry essentially as described by Neisen et al. (25). Briefly, the change in fluorescence arising from binding of SYPRO Orange dye (5000-fold dilution) to hydrophobic residues upon unfolding of EftM (24 μM) was measured over a linear temperature gradient (0.5 °C/min from 25 to 75 °C) in the presence of SAM (150 μM) in a StepOne Plus real-time PCR instrument (Applied Biosystems) and compared with control experiments containing SAM and dye alone. The first derivative of the melting curve was calculated using GraphPad Prism software to determine the melting temperature (Tₘ) corresponding to 50% unfolded protein.

**Results**

**EftM Is a SAM-dependent Methyltransferase—**We have previously demonstrated that the laboratory *P. aeruginosa* strain PAO1 shows EF-Tu methylation at 25 °C but not at 37 °C (Fig. 1). Deletion of *eftM* (PAO1ΔeftM) results in no methylation of EF-Tu at either temperature. When PAO1ΔeftM was complemented with *eftM* under the control of a constitutive promoter, EF-Tu methylation was present at both temperatures, probably due to the overexpression of *eftM* (Fig. 1) (13). Through the screening of clinical isolates, we identified the strain PAHM23, which failed to methylate EF-Tu at either temperature. Sequencing of *eftM* from PAHM23 revealed a single nucleotide change (G148C) resulting in a single amino acid substitution, G50R. To determine whether this amino acid substitution impacted EftM function, we attempted to complement the EF-Tu methylation-deficient phenotype of PAO1ΔeftM with the PAHM23 *eftM* gene. In contrast to previous experiments with the wild-type PAO1 *eftM* gene (13), the *eftM* gene from PAHM23 was unable to complement PAO1ΔeftM (Fig. 1). This result thus identifies an amino acid residue critical for EftM activity.
To define the function of EftM, we analyzed the amino acid sequence of EftM using the remote homology detection program HHpred (19). This search revealed that EftM shares predicted structural similarity with Class I SAM-dependent methyltransferases and identified the best homology modeling template as DesVI (Protein Data Bank entry 3BXO), an N,N-dimethyltransferase found in *Streptomyces venezuelae* (26).

The EftM homology model has a core domain consisting of seven β-strands with three α-helices on either side of the β-sheet (Fig. 2A), characteristic of the Class I methyltransferase fold. This fold loosely divides the protein into two lobes, one of which is responsible for binding to SAM and the other for target substrate specificity (27). Augmentations of the conserved Class I methyltransferase core fold vary both in size and architecture. Based on the DesVI template, EftM is predicted to possess an auxiliary variable domain composed of an α-helix derived from the EftM N terminus and a C-terminal four-stranded anti-parallel β-sheet. This domain forms a structure that covers the SAM-binding cleft of the methyltransferase core fold and could thus define substrate specificity and/or access to the bound SAM cosubstrate.

To experimentally test the structural prediction that EftM is a SAM-dependent methyltransferase and whether SAM and SAH have similar binding affinities (\(K_d\)) for EftM, we used isothermal titration calorimetry (ITC). These analyses revealed that EftM binds SAM and SAH with \(K_d\) values of 20 ± 10 and 26 ± 19 \(\mu\)M (Fig. 2, B and C), respectively. Although relatively weak, these binding affinities are comparable with other bacterial methyltransferases (28, 29).

EftM residue Gly-50 is located in a SAM-binding motif (motif I, (E/D)XG) conserved among Class I methyltransferases (30), corresponding to residues 46–52 of EftM. To test whether a defect in SAM binding results in the inactivity of the EftM G50R variant from PAHM23, we again used ITC to assess the EftM G50R-SAM interaction. EftM G50R affinity for SAM was dramatically reduced, falling below the limit of detection by ITC (greater than low millimolar range) (Fig. 2D). This result confirms the importance of Gly-50 for interaction with SAM and explains the observation that PAHM23 EftM G50R fails to modify EF-Tu (Fig. 1).

**EftM Is Necessary and Sufficient to Methylate EF-Tu**—To determine whether EftM is sufficient to directly methylate EF-Tu in the presence of SAM, an in vitro methyltransferase assay was employed. Purified EftM was incubated with and without SAM and purified EF-Tu at 25 °C. Modified EF-Tu was detected by immunoblotting analysis using an anti-di-/trimethyl lysine antibody only when EftM, SAM, and EF-Tu were all present in the reaction (Fig. 3A). The EftM G50R substitution in EftM ablated methylation of EF-Tu in the assay (Fig. 3A), consistent with in vivo observations (Fig. 1) and the effect of the substitution on SAM binding affinity in vitro (Fig. 2D). Additionally, no modification was detected using a K5A variant of EF-Tu, consistent with exclusive in vitro methylation at this residue (Fig. 3A).

LC-MS/MS analysis was used to further confirm that the amino acid methylated by EftM in the in vitro assay was lysine 5, as observed for in vivo methylated EF-Tu (13). EF-Tu from in vitro reactions with and without EftM was subjected to LC-MS/
FIGURE 3. In vitro lysine 5 trimethylation and quantification on recombinant EF-Tu. A, a representative in vitro methylation assay \((n = 3)\) with wild-type (WT) PAO1 EftM or EftM G50R and WT PAO1 EF-Tu or EF-Tu K5A shows that EftM requires SAM and methylates EF-Tu exclusively at lysine 5. Top, Western blotting of methyltransferase assay products detected with D7mK antibodies. Bottom, Coomassie-stained gel of the same reaction products. B, chymotrypsin-digested EF-Tu from in vitro reactions examined by LC-MS/MS. Representative MS/MS spectra are shown of the EF-Tu doubly charged \((M + 2H)^{2+}\) unmodified (top) and EftM-modified (bottom) peptide sequence corresponding to residues 1–6. Fragment ions \(y_2\) and \(y_3\) (green) are shifted by 42 Da in mass, confirming lysine 5 trimethylation. Precursor (MS1 scans) extracted ion chromatograms (measured as the percentage intensity using ±20 ppm mass tolerance) for the unmodified EF-Tu peptide \((m/z = 377.208; m/z = 377.2018\) theoretical) (C) and the trimethylated EF-Tu lysine 5 peptide \((m/z = 398.231; m/z = 398.2253\) theoretical) (D) are shown for EftM-treated and -untreated samples (top and bottom in each panel, respectively). The x axis indicates the retention time when the peptide eluted from the LC column. In each panel, peptide intensities were normalized to 100% for the sample with the most intense signal.
MS, and the peptide corresponding to EF-Tu amino acids 1–6 was identified in both reactions. However, in the reaction containing EftM, a mass shift of 42 Da was observed on the y2 and y3 ions in the MS/MS spectrum (Fig. 3B, bottom) compared with the unmodified peptide spectrum (Fig. 3B, top). Comparison of the complete MS data showed no other differential methylation of EF-Tu. Taken together, these results confirm our previous observations that the site of lysine trimethylation in EF-Tu was specifically on residue 5 (13) and further that both purified EftM and its co-substrate SAM are necessary to specifically and exclusively methylate Lys-5.

**EF-Tu Methylation by EftM Is Distributive—**Examination of the precursor (MS1 scans) extracted ion chromatograms showed that trimethylated lysine 5 peptide \( m/z = 398.231 \) was observed only when EftM was present in the *in vitro* reaction (Fig. 3C, right). However, whereas exclusively unmodified EF-Tu peptide \( m/z = 377.208 \) was detected in the untreated sample, some unmodified peptide was also found in the EftM-treated sample (Fig. 3C, left). In these samples from an end point methylation reaction, no mono- or dimethylated lysine 5 was detected. This result could indicate that EftM acts in a processive manner such that trimethyl lysine 5 is the only product of the enzyme. Alternatively, the observation could reflect a complete reaction with a remaining fraction of recombinant EF-Tu that is not an active substrate of EftM (e.g., due to misfolding). In this scenario, whether EftM could act in a processive or distributive manner would be ambiguous. To address this question, we performed similar MS analyses on *in vivo* methylated EF-Tu and with an *in vitro* methylation time course designed to capture intermediate species should they exist.

To determine whether species other than trimethylated lysine 5 exist *in vivo*, recombinant EF-Tu was purified from *P. aeruginosa* grown at 25 °C and analyzed by MS. This analysis revealed that although the majority of purified EF-Tu (79%) was indeed trimethylated at lysine 5, each of the un-, mono-, and dimethylated species could also be detected, albeit at much smaller percentages of the purified EF-Tu: 6, 5, and 10%, respectively. To determine whether such intermediates, indicative of a distributive enzyme action, are also observed in a controlled *in vitro* methylation reaction, additional EftM methyltransferase assays were performed using 10-fold lower enzyme concentration compared with our standard assay. Under these conditions, Western blotting analysis of reaction time course showed a modest level of modification at 5 min, which was higher after 10 min (Fig. 4). MS analysis of EF-Tu modification at each of these time points revealed that the majority of EF-Tu was not trimethylated. Concomitant with a decrease in unmethylated lysine 5, the amount of mono-, di-, and trimethylated lysine 5 was greater after 10 min compared with 5 min (Fig. 4). This distribution of methyl species indicates that EftM functions in a non-processive (distributive) manner.

**EftM Methyltransferase Activity Is Thermosensitive—**In the wild-type strain PAO1, methylation of EF-Tu was observed exclusively at 25 °C and not at 37 °C (Fig. 1). To assess not only whether EftM was sufficient for the methylation of EF-Tu, but in addition whether the temperature-dependent phenotype is recapitulated using purified proteins in our defined *in vitro* methylation assay, EftM was preincubated at either 25 or 37 °C for between 0 and 20 min and then used in the *in vitro* methyltransferase assay at 25 °C. Preincubation of EftM at 25 °C at all times tested had no effect on its activity. In contrast, pretreatment of EftM at 37 °C for 5 min resulted in a sharp decrease in EF-Tu modification; by 10 min, no *in vitro* modified product was detected (Fig. 5). Amounts of both EftM and EF-Tu present in the reaction were verified, and the levels of each remained constant in each of these reactions (Fig. 5, bottom), indicating that protein degradation was not responsible for the decreased activity. When EftM was subjected to preincubation at 37 °C in the presence of 2 mM SAM, methyltransferase activity was still lost (data not shown), suggesting that SAM does not stabilize the activity.

**Structural Thermolability Regulates EftM Activity—**Because EftM methyltransferase activity is abolished at 37 °C both *in vivo* and *in vitro*, we investigated, using CD spectroscopy, whether temperature was affecting the enzyme secondary structure, which might account for this temperature sensitivity. At 25 °C, the CD spectrum of recombinant EftM is consistent with a well folded protein of mixed α/β secondary structure. To determine the \( T_m \) of EftM, we monitored the CD signal at 218 nm (CD218) over a linear temperature gradient. The EftM structure remained stably folded over the range 20–30 °C but subsequently began unfolding as the temperature was increased further. The decrease in strongly negative CD218 signal is indicative of the secondary structure unfolding with an estimated melting temperature (50% unfolded, \( T_m \)) of 37 °C (Fig. 6A). The observed unfolding was irreversible; the alteration in CD spectrum was retained upon cooling and induced visible precipitation of the protein. We conclude from these results that the temperature sensitivity of EftM activity is due to irreversible protein unfolding at elevated temperature.

We next asked whether EftM is stabilized by its obligatory co-substrate SAM. Because the addition of SAM complicates CD analysis, we used differential scanning fluorimetry to measure the thermal stability of EftM in the presence of the co-substrate (Fig. 6B). Differential scanning fluorimetry experiments
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monitor the binding of SYPRO Orange dye to hydrophobic amino acids as they become progressively exposed during protein unfolding at higher temperatures (25). The $T_m$ for EftM unfolding in the absence of ligand is comparable with that derived from the CD melt, with a value of 30.5 °C. The difference in measured $T_m$ is probably due to the dye binding and stabilizing an unfolded form of EftM, resulting in a lower $T_m$ compared with CD analysis. Adding SAM (150 μM) stabilized EftM, raising the $T_m$ to ~36 °C (Fig. 6B). Although EftM may be modestly stabilized by its co-substrate SAM, this effect would not be sufficient to preserve the enzymatic activity at the elevated temperature. These protein-unfolding experiments thus show that EftM is thermosensitive and becomes nonfunctional at higher temperatures, revealing the molecular basis for the temperature dependence of EftM activity observed in vitro and in vivo.

Discussion

Bacteria possess multiple strategies to adapt to changes in temperature: the heat shock, cold shock, and the low and the high temperature responses (31). These distinct pathways each employ DNA, RNA, or protein molecules as the effectors of the response. In contrast to the heat shock response, which is induced incrementally and is transient, the high temperature response requires a specific elevated temperature and remains active above that point. As such, the high temperature response is an important mechanism by which pathogenic bacteria can detect entry into a mammalian host, leading to induction and continued expression of virulence genes. Temperature-related changes in gene expression are typically regulated at the level of transcription, via changes in DNA structure that expose RNA polymerase, activator or repressor binding sites, or activation of alternative σ factors or through changes in promoter or repressor protein structure or oligomeric state that influence DNA binding. Alternatively, regulation can occur at the level of translation initiation through “RNA thermometer” structures that allow the ribosome access only at the elevated temperature (31). In this study, we have provided evidence for another mechanism of thermoregulation through direct protein structural changes in the P. aeruginosa methyltransferase EftM.

Collectively, our results demonstrate that EftM is a SAM-dependent methyltransferase that rapidly loses activity via irreversible unfolding of its protein structure at ~37 °C. The temperature optimum for growth of P. aeruginosa is ~37 °C (32), but this opportunistic pathogen can grow at temperatures from 22 to 45 °C (33). Our finding that EftM unfolds at 37 °C suggests that the activity of this enzyme is not essential for maximum P. aeruginosa growth but, rather, may play an important role in the transition from environment to host. “Moonlighting roles” for EF-Tu have been described in various bacteria and include chaperone-like properties involved in the bacterial stress response (34) and, through its localization to the bacterial surface, involvement in adherence to numerous proteins and host factors (13, 14, 35–40). A P. aeruginosa EftM mutant that cannot methylate EF-Tu adheres to and invades epithelial cells less well compared with a wild-type strain, and this strain is also less virulent in a murine acute pneumonia model (13). Although we have not excluded the possibility that EftM may methylate other targets, we have shown that trimethylated EF-Tu binds to epithelial cells better than non-modified EF-Tu (13), indicating a direct role of this modification in adherence and presumably virulence. Collectively, these observations and our new findings suggest that the temperature dependence of EftM activity may be one mechanism by which this opportunistic pathogen promotes adherence via lysine 5-trimethylated EF-Tu as it transitions from the environment to the host.

EftM thermoinstability can rapidly halt methyltransferase activity but does not influence the fate of previously methylated EF-Tu. In P. aeruginosa, modified EF-Tu persists for at least 4 h in cells switched from growth at 20 °C to 37 °C (41), providing good evidence that there is no demethylase capable of specifically reversing EF-Tu lysine 5 trimethylation. Thus, the ultimate loss of trimethylated EF-Tu over a time frame of several hours is probably dependent on the rate of EF-Tu, and not EftM,
turnover, implying that the effects of trimethylated EF-Tu will continue to be manifested as *P. aeruginosa* transitions from the environment to the human host during the disease process.

In contrast to the potential influence of lysine 57 modification on *E. coli* growth, we observed no alteration in growth rate in an eftM deletion mutant under standard laboratory conditions (13). Although our MS analysis of *in vivo* modified recombinant EF-Tu suggests that the majority of protein is trimethylated at lysine 5, the fraction of native EF-Tu that is trimethylated in *P. aeruginosa* is not known. If a large proportion of lysine 5-modified protein is observed, as was seen with the recombinant EF-Tu protein, this could point to an influence upon EF-Tu function in protein chain elongation (e.g. altering the speed of translation and/or increasing fidelity). The potential that EF-Tu lysine 5 methylation could impact protein chain elongation is supported by the finding that residues 1–4 of *E. coli* EF-Tu are responsible for properly aligning residues 5–9 to complex with guanine nucleotides and aminoacylated tRNA for efficient protein chain elongation (42). Perhaps the distribution of mono-, di-, and trimethylated EF-Tu impacts these interactions differently, which may provide a previously unappreciated means to control gene expression. If, on the other hand, a relatively small fraction of EF-Tu is methylated, this could suggest that methylation specifically targets EF-Tu to perform functions outside its established role in protein synthesis. Also, although the effect of EF-Tu as an adhesin has been described (14, 39), whether and/or how methylation influences the export of EF-Tu to the bacterial cell surface is currently unknown.

In summary, we have expressed and characterized the thermoregulated SAM-dependent methyltransferase EftM, responsible for methylating lysine 5 of the essential translation factor EF-Tu. EftM binds SAM and SAH with similar affinity and appears to modify EF-Tu in a distributive manner. Most importantly, we have shown that the EftM structure is thermosensitive, a feature that we propose is responsible for driving the observed temperature-dependent methylation phenotype in *P. aeruginosa*. The ability to express and purify functional EftM is a major step necessary for detailed structural and functional analyses of EftM cosubstrate and substrate recognition and enzymatic turnover. Additionally, this work provides a platform to study the potential impacts of EftM methylation of EF-Tu on its canonical role in protein synthesis and other moonlighting functions in *P. aeruginosa*. Methylation of translation factors may be a conserved means of regulating protein chain elongation in bacteria and, as such, has the potential to provide us with deeper understanding of the mechanisms that regulate bacterial protein synthesis.

**Author Contributions—**J. B. G., G. L. C., S. A., and N. T. S. conceived and coordinated the study. J. P. O., E. G. K., S. M. P., J. M., J. J. V., N. Z., E. B. D., and D. M. D. performed the experiments and analyzed the data. J. P. O., E. G. K., J. M., N. T. S., S. A., G. L. C., and J. B. G. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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