BRIEF COMMUNICATION
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Lys34 of translation elongation factor EF-P is hydroxylated by YfcM

Lauri Peil[1,4,5], Agata L Starosta[2,5], Kai Virumäe[3], Gemma C Atkinson[1], Tanel Tenson[1], Jaanus Remme[3]* & Daniel N Wilson[2]*

Lys34 of the conserved translation elongation factor P (EF-P) is post-translationally lysinylated by YjeK and YjeA—a modification that is critical for bacterial virulence. Here we show that the currently accepted *Escherichia coli* EF-P modification pathway is incomplete and lacks a final hydroxylation step mediated by YfcM, an enzyme distinct from deoxyhypusine hydroxylase that catalyzes the final maturation step of eukaryotic initiation factor 5A, the eukaryotic EF-P homolog.

EF-P binds ribosomes and stimulates peptide bond formation[1–4]. EF-P is conserved in all bacteria[5] and is orthologous to eukaryotic initiation factor 5A (eIF-5A)[6]. eIF-5A bears a unique modification, hypusine[7], which is added post-translationally by the successive action of deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH)[7]. Lys34 of *E. coli* EF-P is also modified[8]; however, the genes encoding DHS and DOHH are absent from the *E. coli* genome. Instead, two unrelated enzymes, YjeK and YjeA, participate in the EF-P modification pathway[5,9–12]. YjeK is a lysine 2,3-aminomutase that converts (*S*)-α-lysine to (*R*)-β-lysine[13], whereas YjeA has homology to class II lysine-tRNA synthetases and transfers the (*R*)-β-lysine to Lys34 of EF-P[9–11]. Lysinylation of EF-P is critical for the stimulatory effect of EF-P on peptide-bond formation[12], and deletion of *efp*, *yjeA* or *yjeK* results in growth defects, antibiotic sensitivity and attenuation of virulence[9,10,14–18].

MS analyses indicate that lysinylation of Lys34 of EF-P by YjeK and YjeA results in a +128-Da modification[9–11], whereas endogenous EF-P bears a +144-Da modification[8,12]. To address this discrepancy, we used an *E. coli* lysate–based immunoprecipitation approach, coupled with high-resolution MS and stable isotope labeling, to characterize the chemical nature of the *E. coli* EF-P modification and delineate the modification pathway *in vivo* (**Supplementary Methods**). In agreement with previous studies[8,12], the mass (monoisotopic, unless otherwise stated) of chromatographically purified endogenous full-length EF-P was determined to be 20,591.6 Da, which is 144.2 ± 0.1 Da larger than the expected mass of unmodified EF-P (20,447.4 Da, with N-terminal methionine cleaved off; **Fig. 1a**). Proteolysis of immunoprecipitated endogenous EF-P using the endopeptidase LysC, which preferentially cleaves on the carboxyl side of unmodified lysine, produced a peptide ($K._{32}$PGK*GQAFARVK$_{42}$.L, where periods indicate sites of cleavage and the asterisk indicates the modified lysine that is not cleaved) with a mass of 1,301.76 Da, an increase of 144.09 Da relative to the theoretical size of 1,157.67 Da (**Fig. 1b**). An identical mass increase was also observed using chymotrypsin (**Supplementary Results**, **Supplementary Fig. 1**). Peptides with mass increases corresponding to a partially modified (+128 Da) state were also detected, but only at ~1–2% of the level of the fully modified form (**Supplementary Fig. 2**).

Fragmentation of both the chymotrypsin (**Supplementary Fig. 1**) and LysC peptide revealed the modification site to be Lys34 (**Fig. 1b**), as expected[5,8–10,12]. In the case of the LysC peptide, fragmentation of the doubly charged peptide precursor gave rise to four intense doubly charged peaks corresponding to the modified peptide, but with nominal mass losses of 86 Da, 104 Da, 128 Da and 145 Da (**Fig. 1c**; peaks labeled X1–X4, respectively, in **Fig. 1b**). Similar peaks were also observed when a triply charged peptide precursor was fragmented through MS (**Fig. 2a**,**b** and **Supplementary Table 1**). These mass peaks are consistent with the loss of a series of fragments from the modified Lys34 but not from the C-terminal Lys42, as determined by ^{18}O labeling (**Supplementary Table 1** and **Supplementary Fig. 3**); X4 has a mass loss of 145 Da, which would result if the C6-N6 bond of the lysinylated Lys34 was fragmented (F4 in **Fig. 1c**), whereas the mass loss of 128 Da (X3) is consistent with fragmentation at the amide bond between the ε-amino group of Lys34 and the carbonyl carbon of the added lysine (F3 in **Fig. 1c**). These fragmentation peaks therefore support the attachment of the activated lysine to the ε-amino group of Lys34. In contrast, mass losses of 86 Da and 104 Da (X1 and X2, respectively) would arise when the linkage between the C2(α) and C3(β) positions of the added lysine are fragmented, with the additional loss of water (18 Da) from the peptide in X2 (F1 and F2 in **Fig. 1c**). Notably, the loss of the 86-Da mass is explainable only if β-lysine, rather than α-lysine, is attached to Lys34. Similar proteolysis and MS analyses of endogenous EF-P immunoprecipitated from *E. coli* AT713 strains lacking *yjeA* (Δ*yjeA*) or *yjeK* (Δ*yjeK*) revealed a complete loss of the EF-P modification (**Supplementary Fig. 4**), consistent with YjeK and YjeA being critical for modification of EF-P[9–11].

Given the discrepancy of 16 Da between the modification mass of the endogenous EF-P (+144 Da; **Fig. 1**)[8,12] and *in vitro* lysinylated EF-P (+128 Da)[9,10], we reasoned that a third enzyme should exist in the EF-P modification pathway. We identified an *E. coli* gene, *yfcM*, as a potential candidate on the basis of its genomic co-occurrence in many bacterial genomes with *yjeA*, *yjeK* and *efp* (**Supplementary Table 2**). To investigate the potential involvement of YfcM in the modification of EF-P *in vivo*, we immunoprecipitated endogenous EF-P from the *E. coli* AT713 strain lacking the *yfcM* gene (Δ*yfcM*). The Lys34-containing fragment from LysC proteolysis had a mass of 1,285.76 Da (**Fig. 2a**), a mass increase of 128.09 Da relative to the expected mass (930.53 Da) of the unmodified fragment. When YfcM was exogenously overexpressed from a plasmid (pYfcM) in the Δ*yfcM* strain, the mass of the LysC fragment increased to 1,301.76 Da (**Fig. 2b**), which is 144.09 Da larger than the mass of the

[1]Institute of Technology, University of Tartu, Tartu, Estonia. [2]Gene Center and Department for Biochemistry, Center for integrated Protein Science Munich, University of Munich, Munich, Germany. [3]Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia. [4]Present address: Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, UK. [5]These authors contributed equally to this work. *e-mail: wilson@lmb.uni-muenchen.de or jremme@ebc.ee

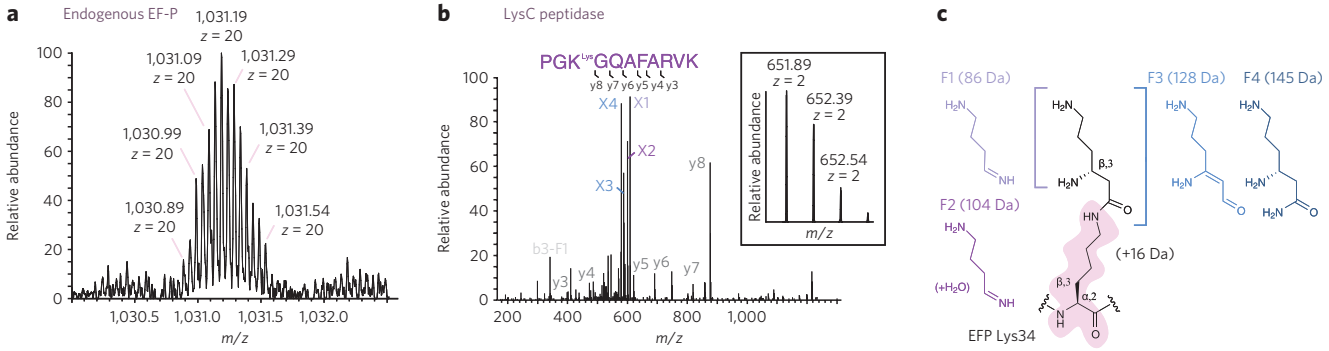

Figure 1 | Lys34 of endogenous EF-P is modified by 144.09 Da. (**a**) MS spectrum of full-length endogenous EF-P isolated chromatographically from wild-type *E. coli* strain MRE600. (**b**) MS/MS spectra of LysC fragment from immunoprecipitated EF-P with a mass of 1,301.76 Da, a mass increase of 144.09 Da compared to the theoretical mass; shown are y and b fragments as well as peaks X1–X4. The b and y ions correspond to N- and C-terminal charge-carrying fragments of peptide, respectively, where peptide backbone cleavage has taken place at CO-NH bonds. Under the conditions used, y ions are preferentially produced. b3-F1 represents an ion that corresponds to the b3 peptide fragment with the F1 fragment removed during fragmentation in the MS. (**c**) Chemical structure of lysinylated Lys34 with predicted chemical structures of mass loss fragments for peaks X1–X4 (structures F1–F4, respectively), indicated with their respective nominal molecular weights.

unmodified fragment and identical to that observed for endogenous EF-P (**Fig. 1b**). Identical mass changes were also observed using chymotrypsin proteolysis (**Supplementary Fig. 5**). These findings indicate that YfcM is required for the addition of exactly 15.995 Da of the 144 Da that comprises the EF-P modification. The precision of the mass difference is indicative of one atom of molecular oxygen, which has a monoisotopic mass of 15.995 Da, thus revealing YfcM as a potential hydroxylase (mono-oxygenase). The hydroxylation activity of YfcM was validated with an *in vitro* assay using recombinant purified YfcM and EF-P (bearing +128-Da modification) proteins, supplemented with protein-free filtrate of an *E. coli* Δ*yfcM* strain plus a cofactor cocktail (**Fig. 2c** and **Supplementary Fig. 6**). In the absence of YfcM, no hydroxylation of EF-P was observed even after 16 h of incubation at 30 °C (**Fig. 2c**), whereas 20–30% hydroxylation was observed upon incubation of EF-P with YfcM for 2 h at 30 °C (**Fig. 2c**). Remarkably, although addition of NAD or NADH to the cofactor cocktail had no influence on the extent of hydroxylation (**Fig. 2c**, lanes 3 and 4), NADP effectively abolished hydroxylation (<2%), and NADPH increased hydroxylation substantially (~60%) (**Fig. 2c**).

To determine the site of hydroxylation by YfcM on Lys34 of EF-P, we used differentially deuterated lysine isoforms, namely 3,3,4,4,5,5,6,6-D8-L-lysine (D8-Lys) and 4,4,5,5-D4-L-lysine (D4-Lys), where the hydrogen atoms (1.008 Da) of the C3-C6 and C4-C5 positions of L-lysine, respectively, are substituted with deuterium (2.014 Da). The lysine (and arginine) auxotrophic *E. coli* wild-type AT713 and AT713Δ*yfcM* strains were grown in minimal medium supplemented with D4- or D8-Lys, rather than unlabeled D0-lysine. The endogenous EF-P was immunoprecipitated from the lysates as before and then was digested with LysC. The masses of the Lys34-containing LysC fragment of EF-P isolated from the Δ*yfcM* strain were determined to be 1,297.84 Da when grown in D4-Lys (**Fig. 3a**) and 1,309.91 Da in D8-Lys (**Supplementary Fig. 7a**). In contrast, the same LysC fragments isolated from the wild-type strain had masses of 1,312.83 Da in D4-Lys (**Fig. 3b**) and 1,324.90 Da in D8-Lys (**Supplementary Fig. 7b**). Thus, when compared with the masses from LysC fragments isolated from the Δ*yfcM* strain, the masses of both of the LysC fragments from the wild-type YfcM-containing strain are 14.993 Da larger, instead of the expected 15.995 Da. This is consistent with a 14.988-Da increase expected from the mass loss of a deuterium atom (−2.014 Da) and the addition of a hydroxyl OH group (+17.003 Da (= 15.995 Da + 1.008 Da)). Moreover, because the 1-Da loss is observed in both the D4- and D8-labeled fragments, this indicates that YfcM hydroxylates either the C4(γ) or C5(δ) position but not the C3(β) or C6(ε) of Lys34 (**Fig. 3c** and **Supplementary Fig. 7c**). Amino acid analysis and periodate treatment confirmed the presence of 5-hydroxylysine within *in vivo* expressed and hydroxylated EF-P but not within nonhydroxylated EF-P (**Supplementary Fig. 8**), suggesting that YfcM hydroxylates

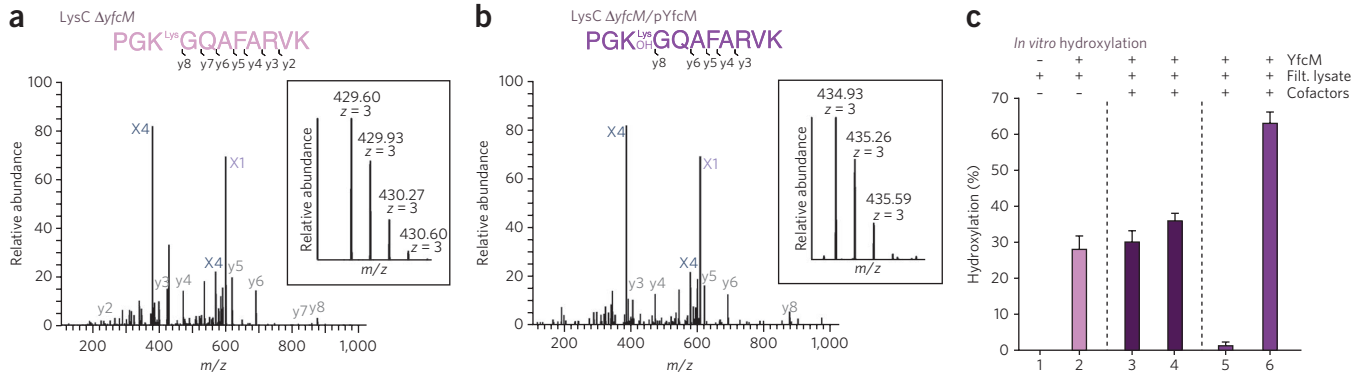

Figure 2 | Fully modified EF-P is dependent on the presence of YfcM. (**a**,**b**) MS/MS spectra of LysC fragment of EF-P from AT713Δ*yfcM* with mass of 1,285.76 Da (**a**) and from AT713Δ*yfcM*/pYfcM with mass of 1,301.76 Da (**b**). (**c**) *In vitro* hydroxylation of EF-P by YfcM, in combination with protein-free filtrate of an *E. coli* Δ*yfcM* strain, with or without cofactor cocktail and additional NAD (lane 3), NADH (lane 4), NADP (lane 5) or NADPH (lane 6). The error bars represent the s.e.m. for independent experiments performed in triplicate.

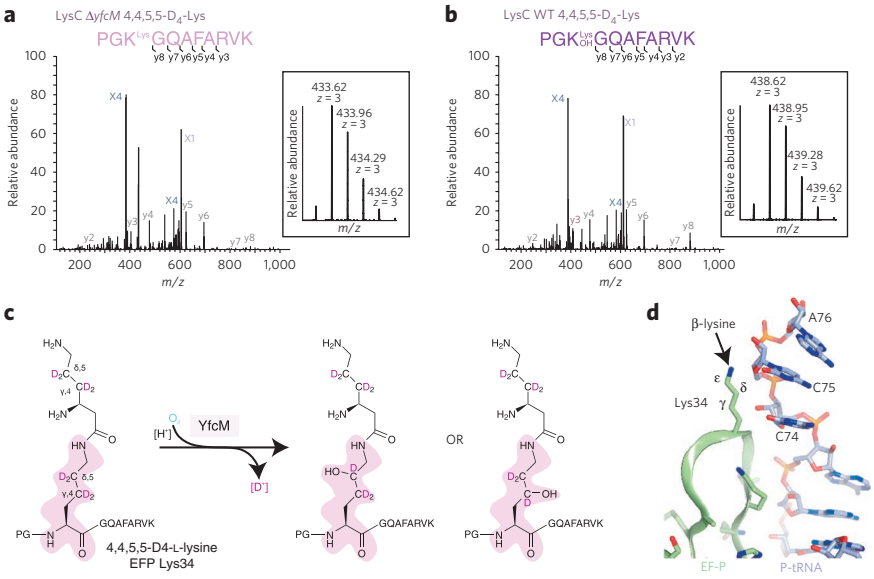

Figure 3 | YfcM hydroxylates the C4 or C5 position of Lys34 of EF-P. (**a**,**b**) MS/MS spectra of LysC fragment of EF-P from AT713Δ*yfcM* (**a**) and wild-type AT713 cells grown in D4-lysine (**b**), with masses of 1,297.84 Da and 1,312.83 Da, respectively. (**c**) Chemical structure of lysinylated Lys34 with potential hydroxyl positions indicated. (**d**) Relative positions of Lys34 of EF-P (green) and the acceptor stem of the P-tRNA (blue) when bound on the ribosome. The γ, δ and ε carbon atoms of Lys34 of EF-P and the nucleotides C74, C75 and A76 of the tRNA are indicated. The model was built by mutation of arginine to lysine (equivalent to Lys34 in *E. coli*) in the *T. thermophilus* EF-P–70S structure[19].

the C5(δ) position of Lys34 in EF-P. We cannot, however, exclude the possibility that hydroxylation of the C4(γ) position also occurs, but such a modification would have to be mutually exclusive with the C5(δ) hydroxylation.

Our data indicate that YfcM is the final enzyme in the EF-P modification pathway: The lack of +16 Da–modified EF-P peptides in any of the Δ*yjeK* or Δ*yjeA* MS spectra (**Supplementary Fig. 4**) suggests that YfcM cannot hydroxylate unmodified EF-P and therefore acts after YjeK and YjeA. Moreover, the +144 Da–modified peptide was never detected in the Δ*yjeK* strain, which most likely indicates that α-lysinylated EF-P is a poor substrate for YfcM and that β-lysinylated EF-P is preferred. This is similar to the eukaryotic situation where deoxyhypusinylation of eIF-5A by DHS is a prerequisite for the subsequent hydroxylation by DOHH[7]. In contrast to data on eIF-5A, which show that the added spermidine moiety is hydroxylated by DOHH, our fragmentation data support the hydroxylation by YfcM of Lys34 of EF-P but not hydroxylation of the added β-lysine (**Fig. 1c**, **Supplementary Table 1** and **Supplementary Fig. 7**). We would therefore propose to rename YjeA, YjeK and YfcM as the EF-P post-translational modification enzymes EpmA, EpmB and EpmC, respectively.

The crystal structure of *Thermus thermophilus* EF-P bound to the 70S ribosome suggests that EF-P stimulates peptide bond formation by interacting with and stabilizing the tRNA at the P site[19]. Modeling *E. coli* EF-P on the ribosome using the *T. thermophilus* structure indicates that Lys34 and the lysinylation moiety would extend toward the peptidyltransferase center and stabilize the terminal 3-CCA end of the tRNA (**Fig. 3d**)[19]. This model suggests that hydroxylation of the C5(δ), but not the C4(γ), position of Lys34 would allow additional potential stabilizing hydrogen-bond interactions with the P-tRNA (**Fig. 3d**). The complete description of the EF-P modification and pathway now opens the way for future studies to address the mechanism of action of EF-P.

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Author contributions

L.P., A.L.S., J.R. and D.N.W. designed research; L.P. performed and analyzed MS data, A.L.S. and K.V. performed biochemistry; G.C.A. performed bioinformatics; and L.P., A.L.S., T.T., J.R. and D.N.W. analyzed data and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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