Translation control of swarming proficiency in *Bacillus subtilis* by 5-amino-pentanolylated elongation factor P

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ABSTRACT

Elongation factor P (EF-P) accelerates diprolyl synthesis and requires a posttranslational modification (PTM) to maintain proteostasis. Two phylogenetically distinct EF-P modification pathways have been described and are encoded in the majority of Gram-negative bacteria, but neither is present in Gram-positive bacteria. Prior work suggested the EF-P encoding gene (*efp*) primarily supports *Bacillus subtilis* swarming differentiation, whereas EF-P in Gram-negative bacteria has a more global housekeeping role, prompting our investigation to determine whether EF-P is modified and how it impacts gene expression in motile cells. We identified a 5-amino-pentanol moiety attached to Lys32 of *B. subtilis* EF-P that is required for swarming motility. A fluorescent *in vivo* *B. subtilis* reporter system identified peptide motifs whose efficient synthesis was most dependent on 5-aminopentanol EF-P. Examination of the *B. subtilis* genome sequence showed that these EF-P dependent peptide motifs were represented in flagellar genes. Taken together these data show that in *B. subtilis* a previously uncharacterized PTM of EF-P can modulate the synthesis of specific diprolyl motifs present in proteins required for swarming motility.

Ribosomes along with a set of translation factors, translate genetic information by polymerizing amino acids into proteins. Of the 20 amino acids common to all organisms, proline is the slowest to form a peptide bond, especially when consecutive proline residues are polymerized (1,2). If not resolved, the slow synthesis of poly-proline sequences can lead to translational pausing (3,4). Bacterial translation elongation factor P (EF-P) remedies proline-induced pausing by binding to the ribosome near the peptidyl-transfer site, and makes synthesis of oligoprolines entropically favorable (5). The presence of EF-P alone is not sufficient to maintain efficient translation of oligoprolines, as the active protein also requires posttranslational modification (6-9).

While EF-P is conserved in all bacteria, EF-P posttranslational modification systems (EPMS) are seemingly diverse (6,9,10). In gamma-proteobacteria, the genes *yjeK, yjeA* and *yfcM* coordinate the attachment of (R)-β-lysine onto Lys34 of EF-P (*Salmonella enterica* numbering), followed by a hydroxylation of Lys-
β-Lys34 (7,11). In contrast, both gamma- and beta- proteobacteria harbor the gene earP that posttranslationally glycosylates Arg32 of EF-P with L-rhamnose, which is produced by rmlABCD encoded proteins (6,9). Thus far, EF-P function and modification systems have only been studied in detail for a narrow set of Gram-negative bacteria. Gram-negative efp mutants exhibit severe pleiotropy due to an abundance of functionally diverse poly-proline containing proteins (12,13), prompting us to determine if EF-P is similarly important in Gram-positive bacteria. A previous study carried out insertional mutagenesis and identified efp as necessary for swarming motility in the Gram-positive bacterium Bacillus subtilis, whereas vegetative growth and swimming motility were unimpaired (14). Another study reported defects in sporulation in B. subtilis efp mutants (Ohashi et al., 2003).

To ascertain the posttranslational modification state of B. subtilis EF-P we characterized a missense Lys32 to Ala mutant, the residue analogous to the modification site in Bacillus subtilis, whereas vegetative growth and swimming motility were unimpaired (14). A previous study carried out insertional mutagenesis and identified efp as necessary for swarming motility in the Gram-positive bacterium Bacillus subtilis, whereas vegetative growth and swimming motility were unimpaired (14). Another study reported defects in sporulation in B. subtilis efp mutants (Ohashi et al., 2003).

To ascertain the posttranslational modification state of B. subtilis EF-P, we characterized a missense Lys32 to Ala mutant, the residue analogous to the modification site in gamma- and beta-proteobacteria. Consistent with a role important for the function of EF-P, swarming motility was impaired in efpK32A to a similar extent observed in efp mutants, while sporulation was unaffected in either mutant. Furthermore, use of a chromosomally inserted reporter system determined Δefp and efpK32A strains were both unable to efficiently translate the canonical EF-P dependent sequence of three consecutive proline residues. Bioinformatic analysis of the B. subtilis genome identified several swarming motility associated genes with diprolyl motifs that were shown to be EF-P dependent, as indicated by the in vivo reporter system. Finally, structural investigation by mass spectrometry elucidated a 5-amino-pentanol moiety covalently linked to Lys32. Taken together, the data indicate B. subtilis requires EF-P to be posttranslationally modified in order to control the synthesis of a subset of proteins containing specific diprolyl motifs in the swarming motility regulon.

**EXPERIMENTAL PROCEDURES**

**Strains and growth conditions** - Unless otherwise noted, Bacillus subtilis and Escherichia coli strains were grown in Luria-Bertani (LB) broth (0.5% NaCl, 0.5% yeast extract, 1% Tryptone) or LB agar plates fortified with 1.5% Bacto agar. When appropriate, antibiotics were included at the following concentrations: 5 µg/mL kanamycin, 100 µg/mL spectinomycin, 100 µg/mL ampicillin, or 1 µg/mL erythromycin plus 25 µg/mL lincomycin (mls).

For swarm assays, strains were grown to mid-log phase at 37°C in 3 mL LB media, and 1 mL was harvested by centrifugation. Cells were resuspended to an OD600 of 10 in PBS buffer containing 0.5% India Ink. 0.7% agar LB plates were dried for 10 minutes in a laminar flow hood, inoculated with 10 µL cell resuspension, dried an additional 10 minutes, and incubated at 37°C. Swarm radius was recorded every 30 minutes for 6 hours along the same axis for consistency.

Sporulation efficiency was determined as described previously (15). Overnight cultures were used to inoculate 3 mL growth medium (0.01% Casein Hydrolysate, 25 mM L-glutamate, 15 mM L-alanine, 10 mM L-asparagine, 1 mM KH2PO4, 25 mM NH4Cl, 0.77 mM Na2SO4, 1.2 mM NH4NO3, 3.7 µM FeCl3, 0.4 mM MgSO4, 0.002% CaCl2, 10 µM MnSO4, 1 µM L-tryptophan) and grown to mid-log phase at 37°C. Cultures were harvested by centrifugation, resuspended in 3 mL resuspension medium (3 µM FeCl3, 37 µM MgCl2, 90 mM MnCl2, 5 mM NH4Cl, 0.67 mM Na2SO4, 0.45 mM KH2PO4, 1 mM NH4NO3, 0.002% L-glutamate, 1 mM CaCl2, 40 mM MgSO4) and incubated overnight at 37°C. The following morning, cultures were diluted in H2O and plated onto LB plates. Dilutions were subsequently incubated at 80°C for 20 min to heat kill vegetative cells and replated onto LB plates. Colony forming units were counted following overnight incubation at 37°C. Growth curves were performed by subculturing overnight cultures into 25 mL LB to an OD600 of 0.01. Cultures were incubated at 37°C shaking at 175 rpm and OD600 monitored every 30 min for 7 hours.

For analysis of colony morphology on LB agar, strains were grown to mid-log phase 37°C, diluted into H2O and plated onto LB agar plates. After incubation at 37°C overnight, colonies were imaged using a Leica EZ4D microscope. Pellicle assays were performed by growing strains to mid-log phase at 37°C and harvesting 1 mL by centrifugation. Cells were resuspended to an OD600 of 10 in LBGM (0.5% NaCl, 0.5% yeast extract, 1% tryptone, 0.5% glycerol, 0.05 mM MnSO4). A 10 µL cell resuspension was used to inoculate 10
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mL LBGM in 6-well plates (Corning). Following incubation at 22°C for 2 days, pellicles were imaged against a black background with a tripod mounted Canon PowerShot A620 camera. Colony architecture analysis was performed by stamping colonies grown overnight at 37°C onto 1.5% agar LBGM plates. Following incubation at 22°C for 5 days, colonies were imaged as described for the pellicle assay.

Strain construction – All strain descriptions are detailed in supplemental tables 1 and 2, with primers used for the generation of plasmids and strains found in supplemental table 2. To generate ∆efp, the upstream flanking region was amplified with primer pair 4031/4032 and the downstream flanking region was amplified with primer pair 4033/4034. Gibson assembly was used to ligate the flanking regions into the SmaI restriction site of pMiniMAD, which contains a temperature sensitive origin and mls resistance cassette (16-18). The resulting construct was transformed into DK1042 and mls resistance was selected for at 37°C. Plasmid evication was subsequently induced by incubating 3 mL LB cultures at room temperature overnight. Resulting mls sensitive colonies were analyzed by PCR to ascertain whether eviction resulted in reversion to the wild type efp allele or deletion.

To generate efpK32A at the native site, primer pair 4031/4039 was used to amplify the upstream flanking region and primer pair 4034/4038 was used to amplify the downstream flanking region. Primers 4038 and 4039 are complementary to one another and encode the K32A mutation. Gibson assembly was used to ligate the flanking regions into the Smal restriction site of pMiniMAD, which contains a temperature sensitive origin and mls resistance cassette. The resulting plasmid was used to transform DS2569. Genomic DNA harvested from a spec-resistant transformant (DK755) was used to amplify the amyE locus with primer pairs 3177/4250 and 3180/4251. Primers 4250 and 4251 are complementary and introduce a FLAG epitope to the C-terminus of EF-P. The two fragments were ligated with Gibson assembly and transformed into DK2050. The amyE locus of spec-resistant isolates were sequenced to verify the introduction of the FLAG tag. The efpK32A allele was introduced into this construct by amplifying the amyE locus of DK755 with primer pairs 3177/4250, 4251/4038/4039/3180. The resulting fragments were ligated by Gibson assembly, transformed into DK2050 and sequence verified.

Construction of GFP <sub>PPX</sub>, fliP-GFP, flhP-GFP, and recombinant EF-P expression plasmids Using the pDR111 plasmid as described above, IPTG inducible GFP<sub>PPX</sub> constructs were generated by modifying templates used in prior studies (4,20). GFP and GFP<sub>NN</sub> were amplified with the restriction sites NheI and SphI and cloned into pDR111 using primer pairs 4832/4834 and 6449/4834, respectively. XL1 Blue cells were transformed with the resulting plasmid and screened by colony PCR for the presence of a correct insert. To generate the PPX motifs of interest site-directed mutagenesis was performed using primer pairs 4293/4294 (PPG), 4067/4068 (PPV), 4069/4070 (PPW), 1373/1374 (PPL), and 7825/7826 (PPR) on the GFP<sub>NN</sub> plasmid using QuikChange Site-Directed Mutagenesis kit (Stratagene). Otherwise Gibson assembly was used to generate GFP<sub>NN</sub> with primer pairs 7516/7515 and 7514/7517.

Fusion constructs fliP and flip were amplified from B. subtilis 3610 genomic DNA and cloned into pDR111 by Gibson assembly using...
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primer pairs 7340/7341 and 7343/5272. The linker region, GSGGG, was inserted between *gfp* and the fusion proteins using the primer pair 3933/7342. All reporter constructs were sequenced to confirm the correct clone was obtained. Each reporter construct was transformed into strains DS2569, DK3235, and DK2050 and assessed for the presence of GFP fluorescence. Chromosomal integration into the correct location was obtained with primer pairs 7122/7123 flanking the AmyE locus and LacI gene. Recombinant expression of *B. subtilis* EF-P was obtained by amplifying *B. subtilis* EF-P from genomic DNA of strain 3610 with primer pairs 2712/1961. The amplified efp fragment was digested with SapI and XhoI, cloned into the intein expression vector pTYB11 and transformed into XJB BL21(DE3) for expression.

*spoIIIE::kan* construction - To construct the *spoIIIE::kan* allele, upstream and downstream flanking regions of *spoIIIE* were amplified with primer pairs 3393/3394 and 3395/3396, respectively. The kanamycin resistance cassette from pDG780 was amplified with primer pair 3250/3251 (3) and ligated between the flanking regions with Gibson assembly. The resulting fragment was transformed into DS2569 and kan resistant isolates selected. The *spoIIIE::kan* allele was transduced into 3610 using the general transducing phage SPP1 (21).

Assessing PPX motif dependence in vivo - Overnight cultures of the various GFP*PPX* reporter constructs were inoculated into fresh LB media and grown to mid-log. Once mid-log was reached cells were induced with 1 mM IPTG for 1 hour. After 1 hour of induction, 1 mL of cells were collected and washed 3 times with 1X phosphate buffered saline (22) and an OD$_{600}$ was taken for purposes of normalization. Wild type *B. subtilis* transformed with pRD111 were used to account for background fluorescence. Fluorescent readings for GFP were measured on a Fluorolog-3 as described previously (4,20).

In the case of the fusion constructs, colonies from plates were directly inoculated into minimal salt media. After 16 hours of growth cells were induced for 7 hours with 1 mM IPTG. Cells were diluted 5-fold and an OD$_{600}$ was taken prior to GFP fluorescence measurements.

Western blotting - Two mg purified EF-P was sent to Cocalico Biologicals for polyclonal antiserum generation in a rabbit host. Anti-SigA antiserum was obtained as a gift from Masaya Fujita, University of Houston. One mL mid-log cultures grown at 37°C were harvested by centrifugation. Pellets were resuspended to an OD$_{600}$ of 10 in lysis buffer (20 mM Tris pH 7.0, 10 mM EDTA, 1 mg/ml lysozyme, 10 µg/ml DNase I, 100 µg/ml RNase I, 1 mM PMSF) and incubated at 37°C for 30 min. The appropriate volume of 6X SDS loading dye was added and samples were incubated at 95°C for 5 min. Twelve uL samples were separated on an 12% SDS polyacrylamide gel and transferred onto nitrocellulose. Nitrocellulose blots were probed with 1:40,000 dilution anti-EF-P or 1:80,000 anti-SigA polyclonal antiserum and subsequently probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Blots were developed with Pierce ECL substrate (ThermoFisher scientific).

Isoelectric focusing - Isoelectric focusing gels were adapted from previous studies with minor alterations (8,23). In brief, proteins were resolved on a native isoelectric focusing gel with a pH gradient range of 4.5–5.4. (Pharmalyte 4.5–5.4 GE-Healthcare). IEF gels required refrigeration during the run and were prefocused for 5 minutes at 100 volts. Prior to loading the samples, wells were rinsed with cathode buffer (50 mM NaOH) to remove unreacted ammonium persulfate. Gels were focused for 1 hour at 200 volts, 1 hour at 300 volts and 30 minutes at 500 volts. IEF gels were then incubated in Towbin buffer for 5 minutes and transferred onto Hybond™-C Extra nitrocellulose membrane (GE Healthcare) according to previously established protocols (8).

Bioinformatics and statistical methods - The fasta file for the genome of *Bacillus subtilis* 168 was obtained from ftp://ftp.ncbi.nih.gov/genomes/Bacteria/. In house scripts were written with biopython to search PPX motifs for each gene in *B. subtilis* and return the identity of the motif as well the gene name and total instances of PPX motifs (24). Microsoft office software was used to view the data. A student’s t-test was performed for comparisons of growth rate doubling times, as well for fluorescence ratios comparing wild type/efp$^{K32A}$ and wild type/Δefp. Data for PPN was not normally distributed, and fluorescence ratios were instead compared using a Wilcoxon signed-rank test.
Purification of recombinant and native \textit{B. subtilis} EF-P - Native \textit{B. subtilis} EF-P purified for Top-Down mass spectrometry was adapted from a prior paper with the following changes (25). Wild type \textit{B. subtilis} 3610 cells were grown in 40 liters of LB media. Cells were harvested by centrifugation at 7,500 rpm for 10 minutes (JLA 16.250 rotor) and resuspended in 100 mLs of Buffer A (25 mM Tris-Cl pH 8, 150 mM NaCl, 10% glycerol, 2 mM ßME) and supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals). Cell lysis was performed on a French press with 3 passages and lysate was clarified by centrifuging at 75,000 x g for 90 minutes at 4°C (JA 25.50 rotor). \textit{B. subtilis} EF-P was precipitated between 40-60% of ammonium sulfate, as determined by immunoblotting precipitated fractions. Fractions containing EF-P were pooled and dialyzed in Buffer A without glycerol before loaded onto a Hiprep XK26 Sepharose Q column (65 mL) using an AKTA Prime FPLC machine. EF-P containing fractions, as determined by immunoblotting analysis, were collected and concentrated to 200 uL using an Amicon Ultra centrifugal filter (MWCO 10 kDa). Concentrated sample containing EF-P was injected onto a Hiload 26/600 Superdex 200 prep grade column (330 ml) and eluted off in Buffer A without glycerol. Fractions containing EF-P were concentrated to 100 uL and dialyzed against Buffer A.

Purification of native \textit{B. subtilis} EF-P by multistep chromatography proved to be inefficient for further analysis, therefore FLAG-tag \textit{B. subtilis} EF-P was chromosomally integrated into a \textit{Δ}efp \textit{B. subtilis} strain and purified with Anti-FLAG M2 magnetic beads (Sigma Aldrich) following the manufactures instructions. Recombinant intein-tagged \textit{B. subtilis} EF-P was expressed in XJB BL21(DE3) cells with auto-induction media as described before with slight modifications (25). Lysate expressing recombinant EF-P was applied to an in-house packed intein column (New England Biolabs), and allowed to sit over night at room temperature to increase cleaving efficiency. Protein was eluted in 50 mM DTT and dialyzed in Buffer A.

High Resolution Top-Down Mass Spectrometry - Individual native and recombinant \textit{B. subtilis} EF-P were initially analyzed as described before, by tandem LC-MS+ using a triple quadrupole mass spectrometer (API III+; Applied Biosystems), prior to being introduced to the Fourier-transform-ICR by direct infusion nanospray (6,26,27). Envelopes of multiply charged ions were measured for each sample on a hybrid linear ion-trap/FT-ICR mass spectrometer (7T, LTQ FT Ultra, Thermo Scientific) operated with standard (up to \textit{m/z} 2000) or extended mass range (up to \textit{m/z} 4000). Spectra were derived from an average of 500 transient signals and mass resolution was set to 100,000. Data were analyzed exactly as before using ProSight PC 2.0 software (Thermo Fisher) (6).

Bottom-up proteomics and structural investigation - LC-MS$^2$ and LC-MS$^3$ analysis were performed with an Easy-nLC 1000 (Thermo Scientific) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific). The LC system consisted of a fused-silica nanospray needle (PicoTip™ emitter, 75 μm ID, New Objective) packed in-house with 40 cm of Magic C18 AQ 10 Å reverse-phase media (Michrom Biosources Inc.). Native FLAG-tagged and recombinant EF-P was separated by SDS-PAGE and in-gel digested with Chymotrypsin (Promega) as described in a previous study (28). Peptide samples were resuspended in 0.1% formic acid with 2% acetonitrile at a concentration of 400 ng/μL and 2 μL was loaded onto the column and separated using a two-mobile-phase system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The chromatographic separation was achieved over a 38-min gradient from 3% to 50% B (3–30% B for 30 min, 30–50% B for 5 min, and 50% B for 3 min) at a flow rate of 300 nL/min. The mass spectrometer was operated in a data-dependent MS/MS mode over the \textit{m/z} range of 350–1200 with target \textit{m/z} values of 380.5495 and 414.2393. The mass resolution was set to 120,000, and mass tolerance was set to 25 ppm. For each cycle, up to 4 ions with the target \textit{m/z} values from the precursor scan were selected for MS$^2$ and MS$^3$ analysis. If the precursor \textit{m/z} was 380.5495 then \textit{m/z} 578.3403 was selected for MS$^3$ analysis and if the precursor \textit{m/z} was 414.2393 then \textit{m/z} 587.3043 was selected for MS$^3$. The MS$^2$ analysis was performed using ETD (Electron Transfer Dissociation) with charge dependent ETD parameters and analyzed with the Orbitrap mass analyzer with resolution set to 30,000. The MS$^3$
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RESULTS

Lys32 of EF-P is critical for function

In the majority of gamma-proteobacteria, EF-P is posttranslationally modified on a conserved lysine residue, while most beta-proteobacteria and a few gamma-proteobacteria modify EF-P on an analogous arginine residue. Mutation of efp at its posttranslational modification site in these organisms has highly pleiotropic effects similar to a null mutant including a significant reduction in growth rate (29, 30). The homologous residue in B. subtilis EF-P is Lys32, and to determine its biological relevance, it was replaced with alanine at the native chromosomal locus (efp\(^{K32A}\)). Only a slight reduction in growth rate compared to wild type was observed in the B. subtilis Δefp or efp\(^{K32A}\) missense mutation strains, in sharp contrast to the significant reductions in growth rates observed for efp mutants in other bacteria (Fig. 1A and B)(6,31). Furthermore, a sporulation defect was previously reported in a B. subtilis efp mutant in laboratory strains (32), but no sporulation defect was observed in either Δefp or efp\(^{K32A}\) strains when compared to wild type in our strains (Fig. 1C). Colonies of the efp mutant had a smooth morphology on LB medium, often indicative of a defect in biofilm formation (Fig. 1D). However, no defect in either complex colony morphology or floating pellicles was observed in solid or liquid biofilm-promoting media, respectively (Fig. 1D). We conclude that the phenotype of an efp mutant is less pleiotropic in B. subtilis than has been reported for Gram-negative bacteria.

EF-P has previously been shown to be required for a flagellar-mediated form of surface migration called swarming motility (14). Consistent with a role important for the function of EF-P in B. subtilis, the efp\(^{K32A}\) mutant displayed an impaired ability to swarm, although not to the extent observed in an efp deletion mutant (Fig. 1E). Importantly, the EF-P\(^{K32A}\) protein was synthesized to the same level as wild type when assayed by western blotting, indicating that the mutation affected EF-P function rather than protein stability (Fig. 1F). These data suggest a physiological role of EF-P in swarming motility.

Motility related genes code EF-P dependent motifs

The absence of EF-P in B. subtilis is associated with a swarming defect, whereas vegetative growth is only slightly impaired, suggesting EF-P may play a role in the synthesis of a subset of motility related proteins. A bioinformatic search for the canonical pause sequence PPP in the ancestral strain B. subtilis 3610 identified 34 genes, with none linked to motility or swarming. When expanded to all diprolyl motifs the search revealed 927 genes encoded proteins with PPX motifs, of which 59 were essential and 14 were associated with motility (Supplementary Table 3). From the essential genes, 19 out of the 20 possible PPX motifs were represented with PPR being absent and PPG the most abundant (Supplemental Table. 3). The motility genes contained 12 unique PPX motifs: PPD, PPN, PPI, PPT, PPR, PPK, PPV, PPG, PPF, and PPA, of which PPV had the highest number of occurrences (Supplemental Table. 3). Based on comparison to ribosome profiling studies of EF-P dependent motifs, we selected from the above motifs those previously shown to produce a strong pause in Escherichia coli Δefp strains (PPW, PPN, PPG, PPF) and motifs less likely to create a pause (PPV, PPR, PPL) (3,4) to test using an in vivo reporter.

A single copy, IPTG inducible, in vivo reporter to measure pause strength of PPX motifs was engineered to form a chromosomal insertion at the native amyE locus in B. subtilis. The reporter consisted of the P\(_{\text{amyE}}\) IPTG inducible promoter, a variable representative poly-proline motif inserted in-frame after the fourth codon with the remainder of gfp fused translationally downstream. This allowed the use of GFP florescence to quantify the efficiency of synthesis of a particular PPX motif. The average ratio of GFP\(_{\text{PPX}}\) fluorescence produced in wild type compared to the Δefp mutant strain decreased in the following order: PPW (8.87±2.46), PPP (4.41±1.36), PPG (4.40±1.29), PPN (3.15±0.15), PPR (2.97±1.06), PPV (2.24±0.06) and PPL (2.15±0.48) (Fig. 2). We conclude that B. subtilis EF-P improves translation efficiency of poly-prolyl motifs at a level comparable to that reported for Gram-negative bacteria. Contribution of the
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putative modification site with respect to the in vivo function of EF-P was also assessed using the native efpK32A mutant, revealing no statistical difference when compared to the corresponding wild type/Δefp motif except in the case of PPV (p < 0.05): PPW (11.85±3.61), PPG (4.59±0.42), PPR (3.91±1.68), PPN (3.70±0.76), PPP (3.35±0.49), PPL (1.73±0.35) and PPV (1.49±0.18) (Fig. 2).

The reporter-generated in vivo pause scores suggest that the diprolyl motif PPW has the greatest dependence on EF-P for translation, PPV and PPL have little if any dependence, and PPR, PPN, and PPG are about as dependent as PPP. Using the putative model of the B. subtilis flagellar machinery, the different diprolyl motifs were mapped to 9 of the respective motility genes, revealing the majority of PPX motifs were concentrated in the secretion machinery (Supplemental Figure 1A and B). Other components containing poly-prolines included the stator, basal body, and filament. Mapping the in vivo pause scores to the corresponding flagellar components indicated that motifs with strong and moderate pauses were coded for in the secretion components flhA (PPW), fliP (PPN) and fliL (PPN) (Supplemental Figure 1C). In addition, the stator motB gene and putative rod gene flhP encode the moderate pausing motifs PPR and PPG, respectively (Supplemental Figure 1C). In summary, the flagellar machinery contains a variety of EF-P dependent motifs, with strong pauses found predominantly in the secretion components.

B. subtilis EF-P is post translationally modified at Lys32 with 5-aminopentanol

Posttranslational modifications have previously been shown to be required for the activity of all characterized forms of EF-P and its eukaryotic and archaeal paralogs eIF5A and aIF5A, respectively. To determine whether the B. subtilis protein is also posttranslationally modified, B. subtilis EF-P was purified natively using multistep chromatography, or purified recombinantly from E. coli as an unmodified control, and then analyzed by high-resolution mass spectrometry. Recombinant EF-P yielded a measured monoisotopic mass of 20,455.603 Da (Fig. 4A and Supplemental Table 4) consistent with that calculated from the gene sequence (20,455.374 Da, delta mass 0.229 Da), and native EF-P yielded a measured monoisotopic mass of 20,556.624 Da (Fig. 4D and Supplemental Table 5) that was 101.250 Da heavier. The spectra of both samples revealed adducts that were 113 Da heavier, attributed to TFA adducts formed in the mass spectrometer ion source.

To obtain an elemental composition and determine whether the additional mass localized to a specific residue, bottom-up proteomics was carried out on in-gel chymotrypsin digested native and recombinant FLAG-EF-P peptides. Fragmentation by electron-transfer dissociation (ETD) of the peptide QHKPGKGAFF produced a series of z and c ions, sufficient to assign the additional mass to Lys32 from native EF-P (Fig. 4B). The average mass difference between modified and unmodified fragments (delta mass 101.08 ± 34 Da) was used to assign the mass of 20,556.624 Da (Fig. 4D and Supplemental Table 5) that was 101.250 Da heavier. The spectra of both samples revealed adducts that were 113 Da heavier, attributed to TFA adducts formed in the mass spectrometer ion source.

To add to the limited information available regarding the structure of the modification, ETD/HCD MS^3 was performed to clarify the molecular arrangement of the atoms. The modified and unmodified z ions (m/z 578.339, 477.256) generated during MS^2 ETD, were isolated and fragmented further with higher energy collision induced dissociation (HCD). Based on the resulting spectra, delta masses with respect to the precursor ion and measured ion

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fragments were compared to obtain a set of common and unique mass differences. Common to both modified and unmodified peptides were a series of a, b, and y ions (Figs. 4C and F). From a set of delta masses unique for the modified ion, 16.018 u, 74.057 u, 88.073 u, 132.062 u, 221.089 u, 265.116 u, 279.131 u, and 293.148 u, a charge directed fragmentation pattern of the modification was determined that suggested a 5 carbon chain with a terminal amine group attached to the η amine of lysine (Fig. 4F). However, the fragmentation data was incomplete and did not provide location of the hydroxyl group, which is postulated to be on either the third or fourth carbon, as fragment ions (ε, δ, -α_{4}) measured the addition of an OH on or before the fourth carbon, while ions before the second carbon did not measure the addition of an OH (Fig. 4F).

The structure of the 5-aminopentanol modification bears striking resemblance to hypusine, the essential posttranslational modification of the eukaryotic EF-P paralog eIF5A (Supplemental Figure 2). Hypusine is derived from spermidine, suggesting 5-aminopentanol could also be derived from a polyamine precursor substrate. Furthermore, ETD/HCD MS³ produced an ion (102.092 m/z) matching that of the modification, suggesting the modification can carry a charge, analogous to ß-lysine and hypusine (Fig. 4F). Based on the positive charge gained when EF-P is modified, isoelectric focusing (IEF) followed by western blotting was carried out to assess whether B. subtilis strains deficient in polyamine biosynthesis could modify EF-P. To rapidly test a variety of deletion strains B. subtilis 168 mutants were purchased from the Bacillus Genetic Stock Center (BGSC). B. subtilis EF-P recombinantly purified from E. coli served as an unmodified control, though partial modification with (R)-ß-lysine was observed (Fig. 5). EF-PK32A was also run on the IEF gel and migrated below recombinant B. subtilis EF-P, due to the absence of a modification and the substitution of the lysine residue to an alanine (Fig. 5). EF-P remained modified in mutants disrupted for each step in spermidine biosynthesis, when grown in minimal salt media (Fig. 5). Therefore, it is likely the modification does not originate from the polyamines putrescine or spermidine.

**Discussion**

The rapid translation of poly-proline residues in gamma-proteobacteria is dependent on a fully modified EF-P, and absence of the modification results in aberrant phenotypes such as impaired swimming motility, growth defects, and compromised membrane integrity (6,33). In contrast, when *efp* is disrupted in *B. subtilis* limited pleiotropy is observed with swimming motility abolished and vegetative growth only mildly effected, calling in to question the broad role of EF-P and its possible modification in *B. subtilis*. Here we investigated the importance of the putative modification site for *efp* in *B. subtilis* by generating a native missense variant, K32A. Sporulation was unaffected in either the ∆*efp* or *efp* K32A strains, contrary to results obtained in a prior study; a discrepancy perhaps attributed to differences in ancestral and laboratory strain backgrounds. (13). Previous studies that replaced modification sites of EF-P with alanine also resulted in phenotypes resembling those where genes involved in modifying EF-P were knocked out (6). However, those mutant *efp* genes were overexpressed in trans from a vector that resulted in dominant-negative phenotypes. The *efp* K32A strain was shown to produce EF-P at comparable levels to wild type, thus preventing the possibility of an overexpression artifact, further supporting a prominent role for Lys32 in the function of EF-P. The *efp* K32A mutation resulted in phenotypes similar to the ∆*efp* strain, displaying a severe defect in swarming motility as compared to wild type. One possible reason for the observed swarming defect is that modified EF-P is required to accelerate the synthesis of diprolyl motifs in proteins required for swarming motility. An assortment of diprolyl motifs encoded in motility related genes were analyzed using an in vivo reporter in both ∆*efp* and *efp* K32A backgrounds. Of the motifs that contributed to pausing in both mutant strains, PPW had the greatest effect PPG, PPP, PPN and PPR all had similar moderate effects, while PPV and PPL were not dependent on EF-P for translation. Each of these motifs produced a significant pause in the mutants strains are found in several motility genes that are essential for swarming (34). For instance, *flIP* and *flhP* encode PPN and PPG motifs, respectively, and *gfp* fusion constructs revealed their expression
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depended on EF-P for efficient translation. While 
loss of either these genes, abolishes motility (35), 
absence of efp could lead to a decrease in the 
levels of flagellar proteins, disrupting secretion 
machinery stoichiometry and overall flagellin 
output (36). The requirement for EF-P during the 
synthesis of particular diprolyl motifs matches the 
trend observed in Gram-negative bacteria, 
confirming the function of EF-P remains the same 
in Gram-positive bacteria, regardless of 
differences in poly-proline abundance (3,4).

The PPP motif has a significant pausing 
effect and 38% of genes encoding PPP are 
involved with sporulation including the 
sporulation essential transcription factor SigE, yet 
no defect in sporulation was observed. By 
investigating the amino acids flanking the triple 
proline in SigE, we observed two leucine residues 
predicted to diminish the reliance on EF-P for 
efficient translation (3,4). Rate of initiation is 
another factor that was recently shown to 
influence the dependence on EF-P of ribosomes 
translating poly-proline containing proteins (37). 
Therefore it is also possible that initiation is the 
rate limiting step in translating the majority of B. 
subtilis genes with poly-prolines, while swarming 
motility may require a substantial increase in the 
production of certain flagellar components 
dependent on modified EF-P to facilitate this 
transition in motile cell behavior. Flagellar 
proteins, particularly structural proteins may be 
needed in abundance as cells may require as many 
as 30 flagella per cell to swarm (Mukherjee and 
Kearns, 2015).

In order for B. subtilis to effectively 
synthesize certain poly-proline containing 
proteins, posttranslational modification of Lys32 
of EF-P is required. Mass spectrometry revealed a 
novel posttranslational modification, 5- 
aminopentanol, for EF-P from B. subtilis, which 
resembles the previously described modification 
of eIF5A with hypusine. While modification of 
B. subtilis EF-P with 5-aminopentanol is 
reminiscent of the addition of positively charged 
molecules to other EF-Ps and IF5As, the 
mechanism by which modifications contribute to 
peptide bond formation remains unclear.

The modification pathway of EF-P 5- 
aminopentonylation has yet to be completely 
elucidated. Assuming the hydroxyl moiety is a 
secondary PTM, spermidine, which is also an 
intermediate in eIF5A modification, is an 
attractive candidate for a precursor substrate 
during 5-aminopentanol addition. Disruption of 
spermidine biosynthesis did not result in the loss 
of modified B. subtilis EF-P as determined by 
isolectric focusing. Furthermore, spermidine 
would only provide four carbons and require a 
subsequent methylation to match the correct mass 
of the modification. Cadaverine, on the other hand, 
is a polyamine that could provide five carbons and 
match the structure of the modification more 
accurately than spermidine. Cavaderine 
biosynthesis has not been described to date in B. 
subtilis, and as a result there are no obvious 
candidate genes to test for its possible roles in EF- 
P modification (10,19). In order to identify the 
gene(s) responsible for modifying EF-P in B. 
subtilis, forward genetic screens searching for 
mutants defective in swarming motility are now 
necessary. Alternatively, it may be possible to 
complement a ΔpoxA mutant with the modification 
machinery from B. subtilis using a genomic library 
screen, due to the resemblance between B. subtilis 
and E. coli EF-P. Future endeavors will then be 
able to determine if the roles of these modification 
systems are complementary or if other non- 
canonical functions exist.

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**Figure Legends**

**Figure 1. Phenotypic effects of loss EF-P or mutation of the conserved lysine32 residue.** (A) Growth curve of strains grown in LB at 37°C. Symbols represent an average of 3 replicates and error bars depict the standard deviation. (B) Exponential growth rate of strains averaged over 3 replicates. Error bars indicate standard deviation. * p<0.05. (C) Sporulation assay in which cells were grown in sporulation-promoting medium and CFUs determined before and after 20 min incubation at 80°C. Data represent an average of 5 replicates. Error bars indicate standard deviation. (D) Colony morphology of strains grown on LB 1.5% agar at 37°C for 1 day (top), LBGM 1.5% agar at 22°C for 5 days (middle), or pellicle formation in LBGM media after 2 days incubation at 22°C (bottom). (E) Swarm assays in which symbols represent the average of 3 replicates. (F) SDS-PAGE western blot of lysates probed with anti-EF-P or anti-SigA polyclonal antisera. The following strains were used: WT (DK1042), *efp* (DK2050), *efpK32A* (DK3235), *spoIIIE* (DK453) and *epsH* (DS6776).

**Figure 2. In vivo analysis of EF-P dependent PPX motifs found in swarming genes.** Translation efficiency of chromosomally inserted GFP<sub>PPX</sub> reporters were tested in *B. subtilis* ∆*efp* and *efp<sup>K32A</sup>* strains and compared to wild type. GFP<sub>PPX</sub> expression was assayed in cells grown in LB with the presence of 1mM IPTG and harvested during exponential phase. Fluorescence was normalized to OD<sub>600</sub> and error bars represent the mean standard deviation from three biological replicates. A student’s t-test was performed between ∆*efp* and *efp<sup>K32A</sup>* strains within the same motif, indicating PPV was significantly different as denoted by the asterisk (p = 0.0137)

**Figure 3. EF-P is required for optimal expression of *fliP* and *flhP* swarming motility genes.** Chromosomally inserted *fliP-gfp* and *flhP-gfp* were expressed in *B. subtilis* wild type, ∆*efp*, and *efp<sup>K32A</sup>* strains. Bacterial cells were grown in minimal salt media to saturation followed by a 7hr induction of the fusion protein construct with the addition of 1mM IPTG. Fluorescence was normalized to OD<sub>600</sub> and error bars represent that mean standard deviation from four biological replicates.

**Figure 4. Structural characterization of the *B. subtilis* EF-P posttranslational modification 5-aminopentanol.** (A,D) High-resolution mass spectrum of intact recombinant and native *Bs*EF-P measured on a 7T FT-ICR. Monoisotopic masses were deconvoluted based on the envelope of multiply charged ions. (B,E) Chymotrypsin digested modified and unmodified peptide was sequenced by ETD and revealed K32 to harbor the modification. (C,F) ETD-HCD MS<sup>3</sup> of the z<sup>5+</sup> ion and proposed charge directed fragmentation
pattern for the modification. Roman numerals and Greek letters represent ion fragments unique to the modification.

**Figure 5. Isoelectric focusing gel resolves modified and unmodified BsEF-P.** Samples were loaded onto a gel with a sharp pH gradient (4.5-5.4). Deletion strains ΔspeE, ΔspeB and ΔspeA were acquired from the Bacillus Genetic Stock Center and have the *B. subtilis* 168 background, while Δefp, efp<sup>KEA</sup> and wild type were generated in a *B. subtilis* 3610 background strain. The isoelectric focusing gel was probed for EF-P and run with positively charged proteins migrating towards the top of the gel and negatively charged proteins migrating lower on the gel.
Figure 1
Figure 2
Figure 3

Modification of Bacillus subtilis elongation factor P

A

B

Figure 3
Modification of Bacillus subtilis elongation factor P

Figure 4
Modification of *Bacillus subtilis* elongation factor P

Figure 5
Translation control of swarming proficiency in Bacillus subtilis by 5-amino-pentanoylated elongation factor P
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