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### Translation Control of Swarming Proficiency in *Bacillus subtilis* by 5-amino-pentanoylated Elongation Factor P

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## Translation Control of Swarming Proficiency in *Bacillus subtilis* by 5-amino-pentanolylated Elongation Factor P

### Comments

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# Translation Control of Swarming Proficiency in *Bacillus subtilis* by 5-Amino-pentanolylated Elongation Factor P<sup>\*S</sup>

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Elongation factor P (EF-P) accelerates diprolyl synthesis and requires a posttranslational modification 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 that 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-aminopentanol moiety attached to Lys<sup>32</sup> 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 posttranslational modification 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)<sup>3</sup> remedies proline-induced pausing by binding to the ribosome near the peptidyl transfer site and makes synthesis of oligoproline entropically favorable (5). The presence of EF-P alone is not sufficient to maintain efficient translation of oligoproline because the active protein also requires posttranslational modification (6–9).

Although EF-P is conserved in all bacteria, EF-P posttranslational modification systems are seemingly diverse (6, 9, 10). In Gammaproteobacteria, the genes *yjeK*, *yjeA*, and *yjcM* coordinate the attachment of (*R*)- $\beta$ -lysine onto Lys<sup>34</sup> of EF-P (*Salmonella enterica* numbering), followed by a hydroxylation of Lys- $\beta$ -Lys<sup>34</sup> (7, 11). In contrast, both Gamma- and Betaproteobacteria harbor the gene *earP*, which posttranslationally glycosylates Arg<sup>32</sup> 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 because of an abundance of functionally diverse poly-proline-containing proteins (12, 13), prompting us to determine whether 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 (13).

To ascertain the posttranslational modification state of *B. subtilis* EF-P, we characterized a missense Lys<sup>32</sup>-to-Ala mutant, the residue analogous to the modification site in Gamma- and Betaproteobacteria. Consistent with a role important for the function of EF-P, swarming motility was impaired in *efp*<sup>K32A</sup> to a similar extent as observed in  $\Delta efp$  mutants, whereas sporulation was unaffected in either mutant. Furthermore, use of a chromosomally inserted reporter system determined that  $\Delta efp$  and *efp*<sup>K32A</sup> 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-as-

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<sup>S</sup> This article contains supplemental Figures 1 and 2 and Tables 1–6.

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<sup>3</sup> The abbreviations used are: EF-P, elongation factor P; LB, Luria-Bertani; IPTG, isopropyl 1-thio- $\beta$ -D-galactopyranoside; ETD, electron transfer dissociation; HCD, higher-energy collision-induced dissociation; mls, macrolide-lincosamide-streptogramin B; ICR, ion cyclotron resonance.

sociated 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-aminopentanol moiety covalently linked to Lys<sup>32</sup>. Taken together, the data indicate *B. subtilis* requires EF-P to be post-translationally modified 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, *B. subtilis* and *Escherichia coli* strains were grown in Luria-Bertani (LB) broth (0.5% NaCl, 0.5% yeast extract, and 1% Tryptone) or LB agar plates fortified with 1.5% Bacto agar. When appropriate, antibiotics were included at the following concentrations: 5  $\mu\text{g/ml}$  kanamycin, 100  $\mu\text{g/ml}$  spectinomycin, 100  $\mu\text{g/ml}$  ampicillin, or 1  $\mu\text{g/ml}$  erythromycin plus 25  $\mu\text{g/ml}$  lincomycin.

For swarm assays, strains were grown to mid-log phase at 37 °C in 3 ml of LB medium, and 1 ml was harvested by centrifugation. Cells were resuspended to an  $A_{600}$  of 10 in PBS buffer containing 0.5% India ink. 0.7% agar LB plates were dried for 10 min in a laminar flow hood, inoculated with 10  $\mu\text{l}$  of cell resuspension, dried for an additional 10 min, and incubated at 37 °C. The swarm radius was recorded every 30 min for 6 h along the same axis for consistency.

Sporulation efficiency was determined as described previously (15). Overnight cultures were used to inoculate 3 ml of growth medium (0.01% casein hydrolysate, 25 mM L-glutamate, 15 mM L-alanine, 10 mM L-asparagine, 1 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NH}_4\text{Cl}_2$ , 0.77 mM  $\text{Na}_2\text{SO}_4$ , 1.2 mM  $\text{NH}_4\text{NO}_3$ , 3.7  $\mu\text{M}$   $\text{FeCl}_3$ , 0.4 mM  $\text{MgSO}_4$ , 0.002%  $\text{CaCl}_2$ , 10  $\mu\text{M}$   $\text{MnSO}_4$ , and 1  $\mu\text{M}$  L-tryptophan) and grown to mid-log phase at 37 °C. Cultures were harvested by centrifugation, resuspended in 3 ml of resuspension medium (3  $\mu\text{M}$   $\text{FeCl}_3$ , 37  $\mu\text{M}$   $\text{MgCl}_2$ , 90 mM  $\text{MnCl}_2$ , 5 mM  $\text{NH}_4\text{Cl}_2$ , 0.67 mM  $\text{Na}_2\text{SO}_4$ , 0.45 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{NH}_4\text{NO}_3$ , 0.002% L-glutamate, 1 mM  $\text{CaCl}_2$ , and 40 mM  $\text{MgSO}_4$ ) and incubated overnight at 37 °C. The following morning, cultures were diluted in  $\text{H}_2\text{O}$  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 of LB to an  $A_{600}$  of 0.01. Cultures were incubated at 37 °C with shaking at 175 rpm, and  $A_{600}$  was monitored every 30 min for 7 h.

For analysis of colony morphology on LB agar, strains were grown to mid-log phase at 37 °C, diluted into  $\text{H}_2\text{O}$ , 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  $A_{600}$  of 10 in LBGM (0.5% NaCl, 0.5% yeast extract, 1% Tryptone, 0.5% glycerol, and 0.05 mM  $\text{MnSO}_4$ ). A 10- $\mu\text{l}$  cell resuspension was used to inoculate 10 ml of 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  $\Delta\text{efp}$ , the upstream flanking region was amplified with primer pair 4031/4032, and the downstream flanking region was amplified with primer pair 4033/4034. A 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 at 37 °C. Plasmid eviction was subsequently induced by incubating 3 ml of 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 *efp*<sup>K32A</sup> 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. A Gibson assembly was used to ligate the flanking regions into the SmaI site of pMiniMAD, and the resulting plasmid was transformed and evicted as described for  $\Delta\text{efp}$  construction. *Mls*-sensitive colonies were analyzed for the retention of the *efp*<sup>K32A</sup> allele through sequencing.

*B. subtilis* 168 strains deficient in spermidine biosynthesis were purchased from the Bacillus Genetic Stock Center (Ohio State University). Mutant strains were grown in minimal salt medium as described previously (19) and lysed in 25 mM Tris (pH 8) with 100  $\mu\text{g/ml}$  of lysozyme for 30 min at 37 °C, followed by addition of 3 units of DNase and incubation for another 30 min at 37 °C. The lysate was clarified on a tabletop centrifuge spun at 20,000  $\times$  g for 15 min, decanted, and flash-frozen to be stored at –80 °C for further analysis.

**EF-P-FLAG Mutant Construction**—An IPTG-inducible *efp* construct was constructed at the *amyE* locus by amplification of the *efp* gene with primer pair 3575/3576. The resulting fragment was digested with NheI and SphI restriction enzymes and ligated into the corresponding restriction sites of pDRIII (a gift from David Rudner, Harvard Medical School). pDRIII contains the  $P_{\text{hyspank}}$  promoter, the *lacI* lactose repressor, and a spectinomycin resistance cassette. The resulting plasmid was used to transform DS2569. Genomic DNA harvested from a spectinomycin-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 a Gibson assembly and transformed into DK2050. The *amyE* locus of spectinomycin-resistant isolates was sequenced to verify the introduction of the FLAG tag. The *efp*<sup>K32A</sup> allele was introduced into this construct by amplifying the *amyE* locus of DK755 with primer pairs 3177/4250, 4251/4038, and 4039/3180. The resulting fragments

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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>PPP</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>PPP</sub> plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). Otherwise, a Gibson assembly was used to generate GFP<sub>PPN</sub> with primer pairs 7516/7515 and 7514/7517.

The fusion constructs *flhP* and *flip* were amplified from *B. subtilis* 3610 genomic DNA and cloned into pDR111 by Gibson assembly using 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 that 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 pair 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.

***spoIIIIE::kan* Construction**—To construct the *spoIIIIE::kan* allele, upstream and downstream flanking regions of *spoIIIIE* 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 a Gibson assembly. The resulting fragment was transformed into DS2569, and kanamycin-resistant isolates were selected. The *spoIIIIE::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<sub>PPX</sub> reporter constructs were inoculated into fresh LB broth and grown to mid-log phase. When mid-log was reached, cells were induced with 1 mM IPTG for 1 h. After 1 h of induction, 1 ml of cells was collected, washed three times with 1× phosphate-buffered saline (22), and an A<sub>600</sub> was taken for purposes of normalization. Wild-type *B. subtilis* transformed with pDR111 was 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 medium. After 16 h of growth, cells were induced for 7 h with 1 mM IPTG. Cells were diluted 5-fold, and an A<sub>600</sub> was taken prior to GFP fluorescence measurements.

**Western Blotting**—2 mg of purified EF-P was sent to Cocalico Biologicals for polyclonal antiserum generation in a rabbit host. Anti-SigA antiserum was a gift from Masaya Fujita (University of Houston). 1 ml of mid-log cultures grown at 37 °C was harvested by centrifugation. Pellets were resuspended to an A<sub>600</sub> 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, and 1 mM PMSF) and incubated at 37 °C for 30 min. The appropriate volume of 6× SDS loading dye was added, and samples were incubated at 95 °C for 5 min. 12-μl samples were separated on a 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 (Thermo Fisher 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). Isoelectric focusing gels required refrigeration during the run and were prefocused for 5 min at 100 V. Prior to loading the samples, the wells were rinsed with cathode buffer (50 mM NaOH) to remove unreacted ammonium persulfate. Gels were focused for 1 h at 200 V, 1 h at 300 V, and 30 min at 500 V. Isoelectric focusing gels were then incubated in Towbin buffer for 5 min and transferred onto a Hybond<sup>TM</sup>-C Extra nitrocellulose membrane (GE Healthcare) according to protocols established previously (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. Student's *t* test was performed for comparisons of growth rate doubling times as well for fluorescence ratios comparing wild-type/*efp*<sup>K32A</sup> and wild type/ $\Delta$ *efp*. The data for PPN were not normally distributed, and fluorescence ratios were instead compared using a Wilcoxon signed-rank test.

**Purification of Recombinant and Native *B. subtilis* EF-P**—Native *B. subtilis* EF-P purified for top-down mass spectrometry was adapted from a prior paper with the following changes (25). Wild-type *B. subtilis* 3610 cells were grown in 40 liters of LB medium. Cells were harvested by centrifugation at 7500 rpm for 10 min (JLA 16.250 rotor) and resuspended in 100 ml of buffer A (25 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol) and supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals). Cell lysis was performed on a French press with three passages, and the lysate was clarified by centrifuging at 75,000 × *g* for 90 min at 4 °C (JA 25.50 rotor). *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 being loaded onto a Hiprep XK26 Sepharose Q column (65 ml) using an AKTA Prime FPLC machine. EF-P-containing fractions, as

determined by immunoblot analysis, were collected and concentrated to 200  $\mu$ l using an Amicon Ultra centrifugal filter (molecular weight cut-off 10 kDa). A concentrated sample containing EF-P was injected onto a Hiloal 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  $\mu$ l and dialyzed against buffer A.

Purification of native *B. subtilis* EF-P by multistep chromatography proved to be inefficient for further analysis. Therefore, FLAG-tagged *B. subtilis* EF-P was chromosomally integrated into a  $\Delta$ *efp* *B. subtilis* strain and purified with anti-FLAG M2 magnetic beads (Sigma-Aldrich) following the instructions of the manufacturer. Recombinant intein-tagged *B. subtilis* EF-P was expressed in XJB BL21(DE3) cells in an autoinduction medium 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 overnight 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 *B. subtilis* EF-P was initially analyzed as described before by tandem LC-MS<sup>+</sup> using a triple-quadrupole mass spectrometer (API III+, Applied Biosystems) prior to being introduced to 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/Fourier transform-ICR mass spectrometer (7T, LTQ FT Ultra, Thermo Scientific) operated with a standard (up to *m/z* 2000) or extended mass range (up to *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**—Tandem LC-MS<sup>2</sup> and tandem LC-MS<sup>3</sup> analyses 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 (Pico-Tip<sup>TM</sup> emitter, 75  $\mu$ m internal diameter, New Objective) packed in-house with 40 cm of Magic C18 AQ 100-Å reverse-phase media (Michrom Bioresources Inc.). Native FLAG-tagged and recombinant EF-P were 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/ $\mu$ l, and 2  $\mu$ l 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 3–50% 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 *m/z* range of 350–1200, with target *m/z* values of 380.5495 and 414.2393. The mass resolution was set to 120,000, and the mass tolerance was set to 25 ppm. For each cycle, up to four ions with target *m/z* values from the precursor scan were selected for MS<sup>2</sup> and MS<sup>3</sup> analysis. If the precursor *m/z* was 380.5495, then *m/z* 477.2566 was selected for MS<sup>3</sup> analysis, and if the precursor *m/z* was 414.2393, then *m/z* 578.3403 was selected

for MS<sup>3</sup>. The MS<sup>2</sup> analysis was performed using electron transfer dissociation (ETD) with charge-dependent ETD parameters and analyzed with a Orbitrap mass analyzer with the resolution set to 30,000. The MS<sup>3</sup> analysis was performed using higher-energy collision-induced dissociation (HCD) with 40% normalized collision energy and analyzed with the Orbitrap with the mass resolution set to 30,000. The isolation window for both MS<sup>2</sup> and MS<sup>3</sup> was 1.6 *m/z*.

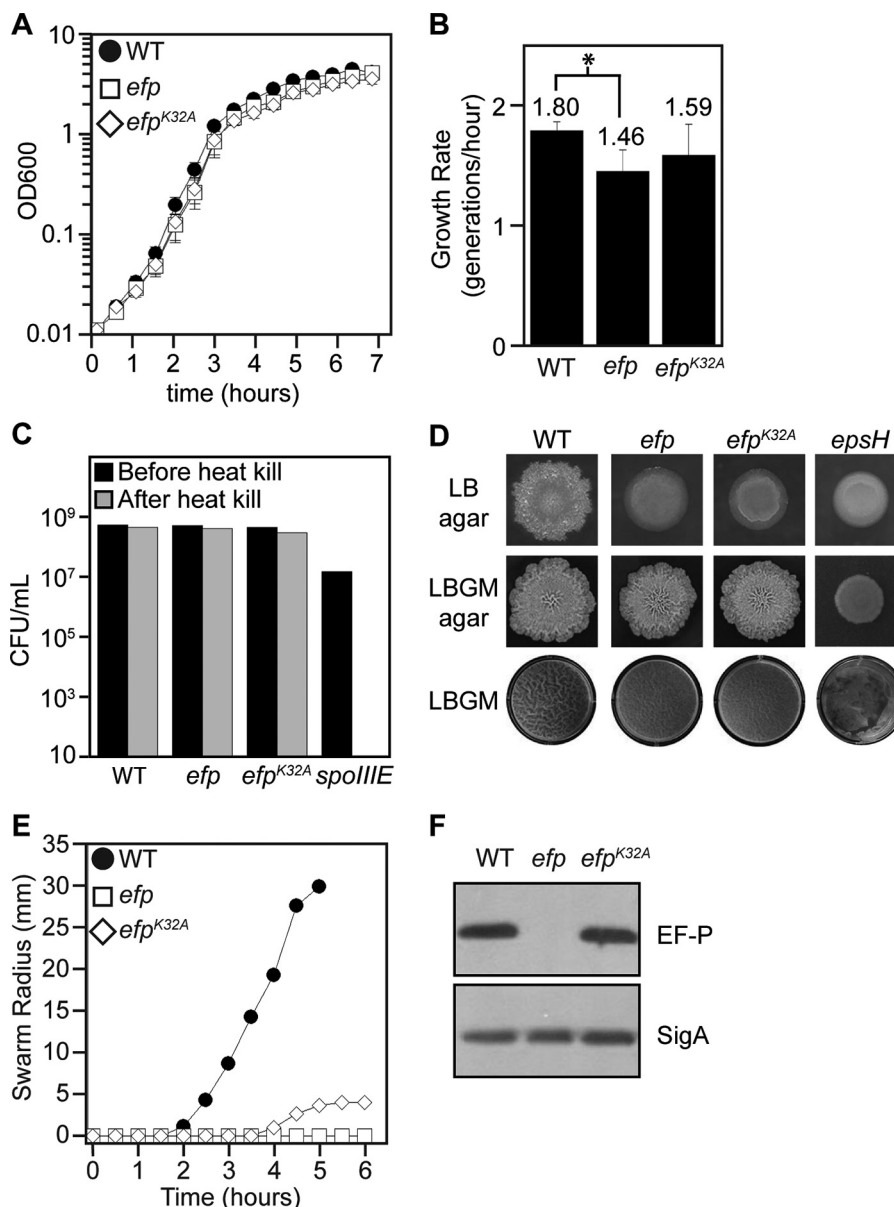
## Results

**Lys<sup>32</sup> of EF-P Is Critical for Function**—In the majority of Gammaproteobacteria, EF-P is posttranslationally modified on a conserved lysine residue, whereas most Betaproteobacteria and a few Gammaproteobacteria 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 Lys<sup>32</sup>, and to determine its biological relevance, it was replaced with alanine at the native chromosomal locus (*efp*<sup>K32A</sup>). Only a slight reduction in growth rate compared with the wild type was observed in the *B. subtilis*  $\Delta$ *efp* or *efp*<sup>K32A</sup> missense mutation strains, in sharp contrast to the significant reductions in growth rates observed for *efp* mutants in other bacteria (Fig. 1, A and B) (6, 7). Furthermore, a sporulation defect was reported previously in a *B. subtilis* *efp* mutant in laboratory strains (13), but no sporulation defect was observed in the  $\Delta$ *efp* or *efp*<sup>K32A</sup> strains when compared with the 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 what has been reported for Gram-negative bacteria.

EF-P has previously been shown to be required for a flagellum-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*<sup>K32A</sup> mutant displayed an impaired ability to swarm, although not to the extent observed in an *efp* deletion mutant (Fig. 1E). Importantly, the EF-P<sup>K32A</sup> protein was synthesized to the same level as the 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 that 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 gene-encoded proteins with PPX motifs, of which 59 were essential and 14 were associated with motility (supplemental Table 3). From the essential genes, 19 of the 20 possible PPX motifs were represented, with PPR being absent and PPG the

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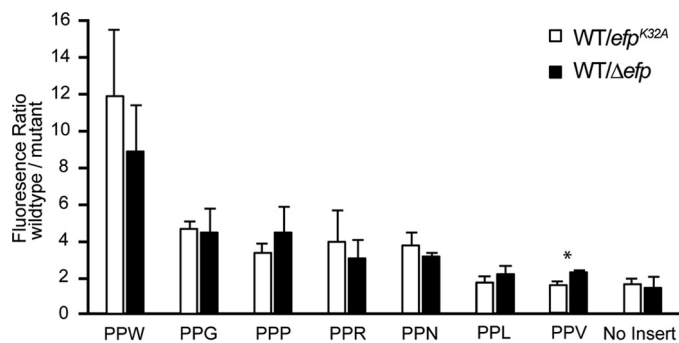


**FIGURE 1. Phenotypic effects of loss of EF-P or mutation of the conserved lysine 32 residue.** *A*, growth curve of strains grown in LB at 37 °C. The symbols represent an average of three replicates, and error bars depict the standard deviation. *B*, exponential growth rate of strains averaged over three replicates. Error bars indicate standard deviation. \*,  $p < 0.05$ . *C*, sporulation assay in which cells were grown in sporulation-promoting medium and colony-forming units determined before and after 20-min incubation at 80 °C. The data represent an average of five replicates. Error bars indicate standard deviation. *D*, colony morphology of strains grown on LB 1.5% agar at 37 °C for 1 day (top row), LBGM 1.5% agar at 22 °C for 5 days (center row), or pellicle formation in LBGM after 2 days of incubation at 22 °C (bottom row). *E*, swarm assays in which symbols represent the average of three 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), *efp*<sup>K32A</sup> (DK3235), *spoIIIE* (DK453), and *epsH* (DS6776).

most abundant (supplemental Table 3). The motility genes contained 12 unique PPX motifs: PPD, PPN, PPI, PPT, PPR, PPK, PPN, PPV, PPW, PPG, PPE, 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 *E. coli*  $\Delta$ *efp* strains (PPW, PPN, PPG, and PPP) and motifs less likely to create a pause (PPV, PPR, and 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  $P_{\text{hyspank}}$  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 fluorescence to quantify the efficiency of synthesis of a particular PPX motif. The average ratio of GFP<sub>PPX</sub> fluorescence produced in the wild type compared with the  $\Delta$ *efp* mutant strain decreased in the following order: PPW ( $8.87 \pm 2.46$ ), PPP ( $4.41 \pm 1.36$ ), PPG ( $4.40 \pm 1.29$ ), PPN ( $3.15 \pm 0.15$ ), PPR ( $2.97 \pm 1.06$ ), PPV ( $2.24 \pm 0.06$ ), and PPL ( $2.15 \pm 0.48$ ) (Fig. 2). We conclude that *B. subtilis* EF-P improves the translation efficiency of poly-prolyl motifs at a level comparable with that reported for Gram-



**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 the *B. subtilis*  $\Delta$ *efp* and *efp*<sup>K32A</sup> strains and compared with the wild type. GFP<sub>PPX</sub> expression was assayed in cells grown in LB in the presence of 1 mM IPTG and harvested during exponential phase. Fluorescence was normalized to *A*<sub>600</sub>, and error bars represent the mean  $\pm$  S.D. from three biological replicates. A Student's *t* test was performed between  $\Delta$ *efp* and *efp*<sup>K32A</sup> strains within the same motif, indicating that PPV was significantly different (\*, *p* = 0.0137).

negative bacteria. Contribution of the putative modification site with respect to the *in vivo* function of EF-P was also assessed using the native *efp*<sup>K32A</sup> mutant, revealing no statistical difference compared with the corresponding wild-type/ $\Delta$ *efp* motif, except in the case of PPV (*p* < 0.05): PPW (11.85  $\pm$  3.61), PPG (4.59  $\pm$  0.42), PPR (3.91  $\pm$  1.68), PPN (3.70  $\pm$  0.76), PPP (3.35  $\pm$  0.49), PPL (1.73  $\pm$  0.35), and PPV (1.49  $\pm$  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 nine of the respective motility genes, revealing that the majority of PPX motifs were concentrated in the secretion machinery (supplemental Fig. 1, A 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 Fig. 1C). In addition, the stator *motB* gene and the putative rod gene *flhP* encode the moderate pausing motifs PPR and PPG, respectively (supplemental Fig. 1C). In summary, the flagellar machinery contains a variety of EF-P-dependent motifs, with strong pauses found predominantly in the secretion components. Previous reports indicated that FliP expression is controlled by EF-P in *E. coli*. FliP has also been demonstrated to be required for swarming motility in *B. subtilis*. To validate whether EF-P facilitates the expression of genes involved with swarming, a *fliP-gfp* fusion was generated and expressed in wild-type,  $\Delta$ *efp*, and *efp*<sup>K32A</sup> backgrounds. Significant reductions in GFP fluorescence were measured in the  $\Delta$ *efp* and *efp*<sup>K32A</sup> strains, 1.7- and 3.8-fold compared with the wild type, respectively (Fig. 3A). To determine whether the trend extends to other proteins required for swarming, a *flhP-gfp* construct was produced and found to show similar results to FliP (Fig. 3B). These data suggest that EF-P is required for optimal production of multiple swarming motility-associated proteins.

*B. subtilis* EF-P Is Posttranslationally Modified at Lys<sup>32</sup> with 5-Aminopentanol—Posttranslational modifications have been shown previously 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 were carried out on in-gel chymotrypsin-digested native and recombinant FLAG-EF-P peptides. Fragmentation by ETD of the peptide QHVKPGKGAAF produced a series of z and c ions, sufficient to assign the additional mass to Lys<sup>32</sup> from native EF-P (Fig. 4E). ETD fragmentation of the same peptide, but from recombinant EF-P, achieved complete backbone cleavage without any additional mass on any of the residues (Fig. 4B). The average mass difference between modified and unmodified fragments ( $\Delta$  mass 101.0834 Da) was used to compute the elemental composition of the modification, yielding C<sub>5</sub>H<sub>11</sub>NO with a 6.92-ppm difference between measured and theoretical values (supplemental Table 6).

To add to the limited information available regarding the structure of the modification, ETD/HCD MS<sup>3</sup> was performed to clarify the molecular arrangement of the atoms. The modified and unmodified z5+ ions (*m/z* 578.339, 477.256) generated during MS<sup>2</sup> ETD were isolated and fragmented further with HCD. Based on the resulting spectra,  $\Delta$  masses with respect to the precursor ion and measured ion 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 (Fig. 4, C 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 five-carbon chain with a terminal amine group attached to the  $\eta$  amine of lysine (Fig. 4F). However, the fragmentation data were incomplete and did not provide the location of the hydroxyl group, which is postulated to be on either the third or fourth carbon, because fragment ions ( $\epsilon$ ,  $\delta$ -a<sub>4</sub>) measured the addition of an OH on or before the fourth carbon, whereas ions before the second carbon did not measure the addition of an OH (Fig. 4F).

The structure of the 5-aminopentanol modification bears a striking resemblance to hypusine, the essential posttranslational modification of the eukaryotic EF-P paralog eIF5A (supplemental Fig. 2). Hypusine is derived from spermidine, suggesting that 5-aminopentanol could also be derived from a polyamine precursor substrate. Furthermore, ETD/HCD MS<sup>3</sup>



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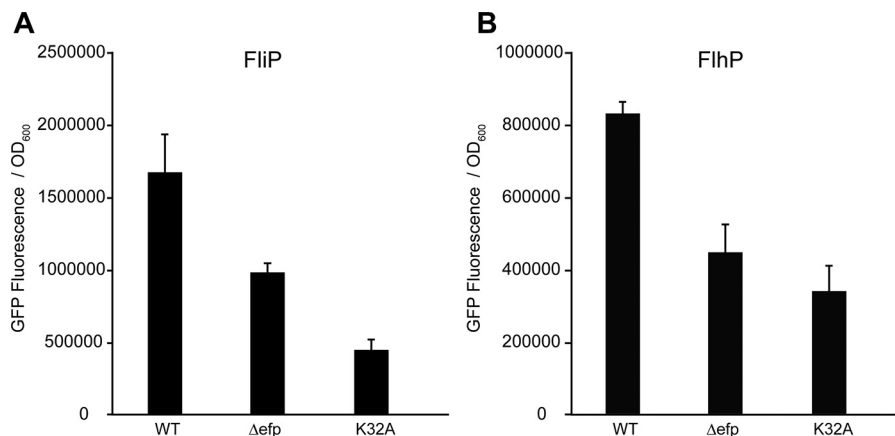


FIGURE 3. **EF-P is required for optimal expression of *fliP* and *flhP* swarming motility genes.** Chromosomally inserted *fliP-gfp* and *flhP-gfp* were expressed in the *B. subtilis* wild-type,  $\Delta$ *efp*, and *efp*<sup>K32A</sup> strains. Bacterial cells were grown in minimal salt medium to saturation, followed by a 7-h induction of the fusion protein construct with the addition of 1 mM IPTG. Fluorescence was normalized to A<sub>600</sub> and errors bars represent the mean  $\pm$  S.D. from four biological replicates.

produced an ion (102.092 *m/z*) matching that of the modification, suggesting that the modification can carry a charge, analogous to  $\beta$ -Lys and hypusine (Fig. 4F). Based on the positive charge gained when EF-P is modified, isoelectric focusing 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. *B. subtilis* EF-P recombinantly purified from *E. coli* served as an unmodified control, although partial modification with (*R*)- $\beta$ -lysine was observed (Fig. 5). EF-P<sup>K32A</sup> was also run on the isoelectric focusing gel and migrated below recombinant *B. subtilis* EF-P because of 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 medium (Fig. 5). Therefore, it is likely that the modification does not originate from the polyamines putrescine or spermidine.

### Discussion

The rapid translation of poly-proline residues in Gammaproteobacteria 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 swarming motility abolished and vegetative growth only mildly affected, calling into 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 the  $\Delta$ *efp* and *efp*<sup>K32A</sup> 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*<sup>K32A</sup> strain was shown to produce EF-P at comparable levels to the wild type, thus preventing the possibility of an overexpression artifact, further supporting a prominent role for Lys<sup>32</sup> in the function of EF-P. The *efp*<sup>K32A</sup> mutation resulted in phenotypes similar to the  $\Delta$ *efp* strain, displaying a severe defect in swarming motility compared with the 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 the  $\Delta$ *efp* and *efp*<sup>K32A</sup> 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, whereas PPV and PPL were not dependent on EF-P for translation. Each of these motifs that produced a significant pause in the mutant 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 that their expression depended on EF-P for efficient translation. Although loss of either of these genes abolishes motility (35), the 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 that 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 in sporulation, including the sporulation-essential transcription factor SigE, but 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). The rate of initiation is another factor that was shown recently to influence the dependence on EF-P of ribosomes translating poly-proline-containing proteins (32). Therefore, it is also possible that initiation is the rate-limiting step in translating the majority of *B. subtilis* genes with poly-

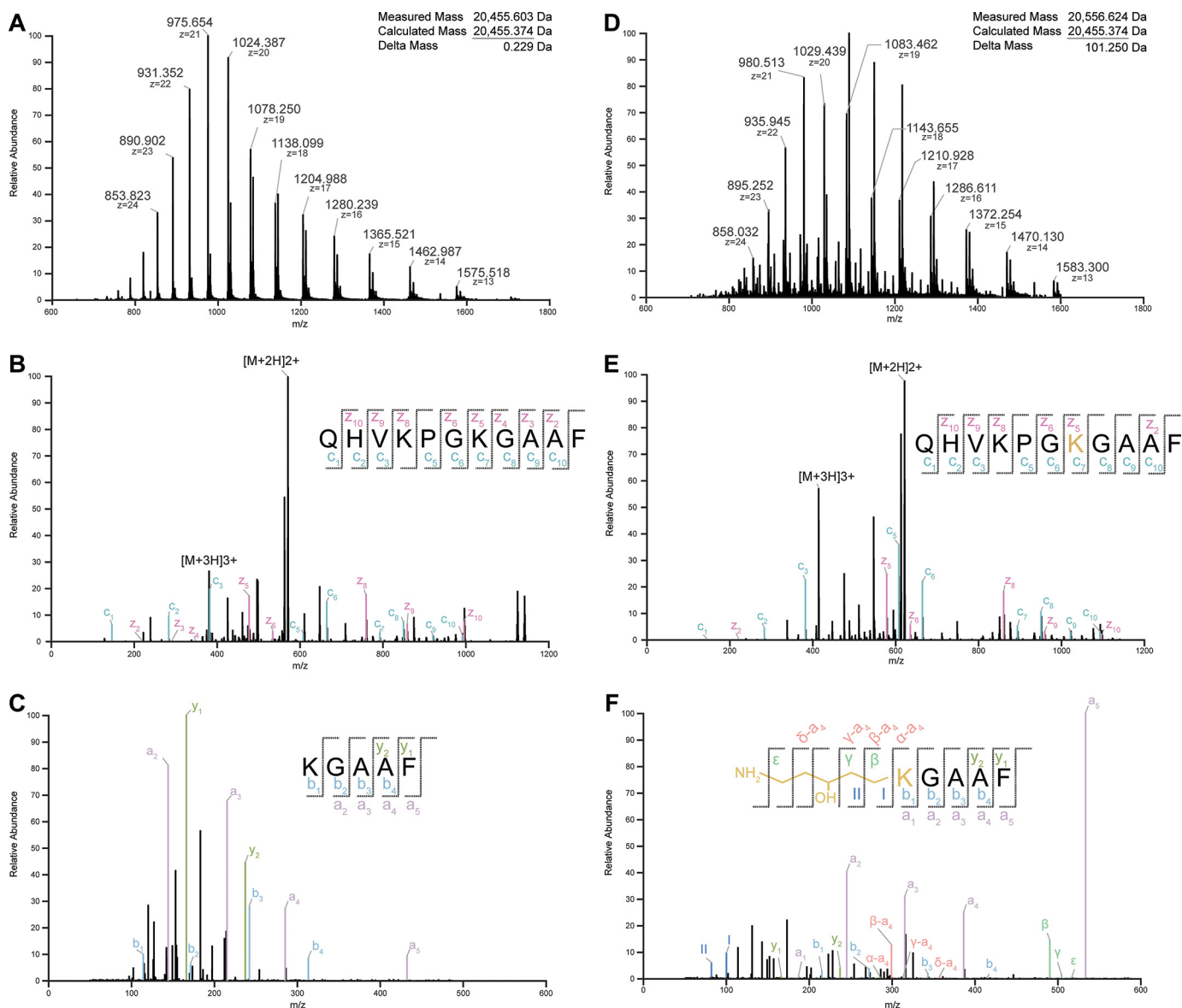


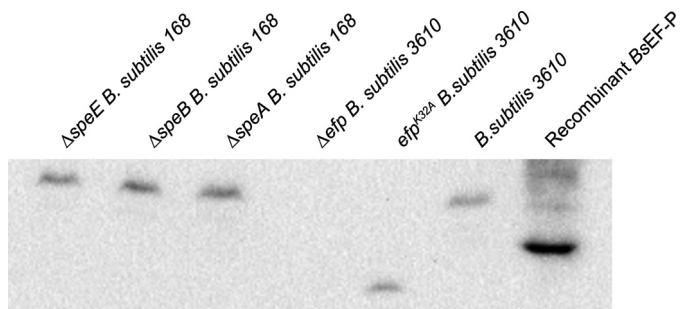
FIGURE 4. **Structural characterization of the *B. subtilis* EF-P posttranslational modification 5-aminopentanol.** A and D, high-resolution mass spectrum of intact recombinant and native B. subtilis EF-P measured on a 7T Fourier transform-ICR. Monoisotopic masses were deconvoluted based on the envelope of multiply charged ions. B and E, chymotrypsin-digested modified and unmodified peptide was sequenced by ETD and revealed Lys<sup>32</sup> to harbor the modification. C and 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.

prolines, whereas 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 because cells may require as many as 30 flagella per cell to swarm (31).

For *B. subtilis* to effectively synthesize certain poly-proline-containing proteins, posttranslational modification of Lys<sup>32</sup> of EF-P is required. Mass spectrometry revealed a novel post-translational modification, 5-aminopentanol, for EF-P from *B. subtilis*, which resembles the previously described modification of eIF5A with hypusine. Although 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 that the hydroxyl moiety is a secondary posttranslational modification, 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 isoelectric 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. Cadaverine 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). To identify the gene(s) responsible for modifying EF-P in

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**FIGURE 5. Isoelectric focusing gel resolves modified and unmodified BseEF-P.** Samples were loaded onto a gel with a sharp pH gradient (4.5–5.4). The deletion strains  $\Delta speE$ ,  $\Delta speB$ , and  $\Delta speA$  were acquired from the Bacillus Genetic Stock Center and have the *B. subtilis* 168 background, whereas  $\Delta efp$ ,  $efp^{K32A}$ , and the 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 toward the top of the gel and negatively charged proteins migrating lower on the gel.

*B. subtilis*, forward genetic screens searching for mutants defective in swarming motility are now necessary. Alternatively, it may be possible to complement a  $\Delta poxA$  mutant with the modification machinery from *B. subtilis* using a genomic library screen because of the resemblance between *B. subtilis* and *E. coli* EF-P. Future endeavors will then be able to determine whether the roles of these modification systems are complementary or whether other non-canonical functions exist.

**Author Contributions**—M. I., A. R., and D. B. K. designed the experiments. A. W., A. R., S. E., P. R. G., and K. R. H. performed the experiments. P. R. G., A. R., A. W., J. P. W., K. F. F., and K. R. H. analyzed the data. A. R., K. R. H., M. I., D. B. K., A. W., and P. R. G. wrote the manuscript. All authors edited and approved the manuscript. Responsibility of data analysis integrity is assumed by all authors. All authors reviewed the results and approved the final version of the manuscript.

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