Divergent Protein Motifs Direct Elongation Factor P-Mediated Translational Regulation in *Salmonella enterica* and *Escherichia coli*

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**ABSTRACT** Elongation factor P (EF-P) is a universally conserved bacterial translation factor homologous to eukaryotic/archaeal initiation factor 5A. In *Salmonella*, deletion of the *efp* gene results in pleiotropic phenotypes, including increased susceptibility to numerous cellular stressors. Only a limited number of proteins are affected by the loss of EF-P, and it has recently been determined that EF-P plays a critical role in rescuing ribosomes stalled at PPP and PPG peptide sequences. Here we present an unbiased *in vivo* investigation of the specific targets of EF-P by employing stable isotope labeling of amino acids in cell culture (SILAC) to compare the proteomes of wild-type and *efp* mutant *Salmonella*. We found that metabolic and motility genes are prominent among the subset of proteins with decreased production in the *Δefp* mutant. Furthermore, particular tripeptide motifs are statistically overrepresented among the proteins downregulated in *efp* mutant strains. These include both PPP and PPG but also additional motifs, such as APP and YIRYIR, which were confirmed to induce EF-P dependence by a translational fusion assay. Notably, we found that many proteins containing polyproline motifs are not misregulated in an EF-P-deficient background, suggesting that the factors that govern EF-P-mediated regulation are complex. Finally, we analyzed the specific region of the PoxB protein that is modulated by EF-P and found that mutation of any residue within a specific GSCGPG sequence eliminates the requirement for EF-P. This work expands the known repertoire of EF-P target motifs and implicates factors beyond polyproline motifs that are required for EF-P-mediated regulation.

**IMPORTANCE** Bacterial cells regulate gene expression at several points during and after transcription. During protein synthesis, for example, factors can interact with the ribosome to influence the production of specific proteins. Bacterial elongation factor P (EF-P) is a protein that facilitates the synthesis of proteins that contain polyproline motifs by preventing the ribosome from stalling. Bacterial cells that lack EF-P are viable but are sensitive to a large number of stress conditions. In this study, a global analysis of protein synthesis revealed that EF-P regulates many more proteins in the cell than predicted based solely on the prevalence of polyproline motifs. Several new EF-P-regulated motifs were uncovered, thereby providing a more complete picture of how this critical factor influences the cell’s response to stress at the level of protein synthesis.
been shown that relative to findings for the wild-type strain (10–13). It has further
sors, as well as motility defects and impaired nutrient utilization
types, including increased susceptibility to a range of cellular stres-
s.

| SILAC Expression Ratio (WN1269 / WN1308) | Proteins Identified in SILAC | Proteins with SigB < 0.01 |
|----------------------------------------|-----------------------------|-------------------------|
| Ratio > 2                               | 275                         | 49                      |
| Ratio < 2                                | 69                          | 38                      |
| Total                                   | 1517                        | 98                      |

FIG 1 A subset of proteins is significantly misregulated in Δefp Salmonella. The histogram outlines the
distribution of protein synthesis ratios identified in SILAC. Columns indicate the number of proteins
with an average synthesis ratio between two neighboring x axis values. Underlined values in the x axis
indicate a change in scale. The inset table shows the number of SILAC hits demonstrating a greater than
2-fold difference in protein level between the efp+ (WN1269) and Δefp (WN1308) strains. The second
column further indicates the proteins with a significance B value of less than 0.01 in at least one trial.
“Total” indicates the number of proteins identified in at least one replicate regardless of expression ratio,
and the second column of this row includes proteins with any expression ratio that had a significance B
value of less than 0.01 in at least one trial. Synthesis ratios shown are the average normalized heavy/light
ratios of three biological replicates.

translation initiation factor 5A (eIF5A), wherein a unique β-lysine
residue is added to a conserved lysyl residue by the combined
activities of PoxA and YjeK (the PYE pathway). The EF-P protein
has a shape similar to that of a tRNA and interacts with the ribo-
some in a unique position contacting the E site of the 30S subunit
and the P site on the ribosome. The β-lysylated residue of EF-P is
oriented in the ribosome such that it projects into the peptidyl
transferase center (PTC), presumably to modulate peptide bond
formation. We and other laboratories have noted that deletion of
any of the PYE genes in Salmonella results in pleiotropic pheno-
types, including increased susceptibility to a range of cellular stres-
sors, as well as motility defects and impaired nutrient utilization
relative to findings for the wild-type strain (10–13). It has further
been shown that β-lysylated EF-P also undergoes hydroxylation by YfcM, but this additional modification does not appear to be
critical for EF-P function based on phenotypic and in vivo analyses
(14, 15).

Proteomic analysis of Salmonella poxA and efp mutants and
Agrobacterium efp (chvH) mutants suggested that EF-P acts in a
targeted manner rather than as a global translation factor (10, 11,
13, 16). Recent work has demonstrated that EF-P rescues the
translation of elongating polypeptides that are stalled at particular
tripetide motifs, in particular PPP or PPG (9, 17, 18). EF-P was
recently shown to not have an effect at other types of stalling
motifs, such as the C-terminal motifs that inhibit release factors
(9). The discovery of EF-P target motifs lends insight into why
specific proteins are subject to EF-P-mediated regulation. Fur-
thermore, the pleiotropic phenotypes exhibited by bacteria lack-
ing EF-P could likely be explained by the presence of polyproline
stretches in factors essential for the pheno-
type. For instance, a number of flagellar
genes contain such motifs, and their
misregulation likely contributes to the
motility defect observed in PYE mutants
(10, 13). However, previous work has fo-
cused on in vitro study of particular EF-P
targets and has not thoroughly evaluated
the role of EF-P in vivo.

Here we present an unbiased in vivo
comprehensive analysis of the Salmonella
proteins affected by EF-P, using stable-isotope labeling (SILAC) (19, 20). While we confirmed that several proteins con-
taining polyproline motifs are subject
to EF-P-mediated regulation, we have
found that the presence of polyproline
tracts is not sufficient to predict EF-P de-
pendence, since many proteins containing
such motifs are unaffected by the
absence of EF-P. We also identified alter-
nate target sequences in addition to PPP
and PPG that render peptide synthesis
dependent on EF-P. Finally, we investi-
gated the determinants of EF-P depen-
dence for pyruvate oxidase (PoxB), which
was previously shown to be downregu-
lated approximately 8-fold in E. coli
strains lacking PoxA despite the absence
of a polyproline sequence (21).

RESULTS

Identification of EF-P-regulated proteins by SILAC. We previ-
ously examined the proteome of the Salmonella enterica poxA
mutant using two-dimensional difference gel electrophoresis (2D-
DIGE). In that study, total cellular proteins from the wild type and
poxA mutants were labeled with fluorescent dyes, mixed in equal
amounts, and then separated by 2D-PAGE prior to analysis (11).
Using this technique, we determined that a relatively small subset
of proteins were affected by perturbations in the PYE pathway—a
finding in agreement with earlier work on the efp mutant of Agro-
bacterium (16). However, we were able to unambiguously identify
only a small number of these proteins by mass spectrometry due to
crowding on the 2D gel.

To gain a more comprehensive view of the effect of EF-P on
protein levels, we employed stable isotope labeling of amino acids
in cell culture (SILAC) in conjunction with quantitative mass
spectrometry-based proteomics to examine the proteome of an
efp mutant strain of Salmonella enterica serovar Typhimurium
strain SL1344 (strain WN1308). We examined the profiles of three
biological replicates and in total were able to detect, quantify, and
identify a total of 1,517 proteins, or approximately 34% of the
strain SL1344 (strain WN1308). We examined the profiles of three
biological replicates and in total were able to detect, quantify, and
identify a total of 1,517 proteins, or approximately 34% of the
4,514 proteins predicted to be encoded in the
Salmonella
strain SL1344 genome (Fig. 1). To identify proteins showing altered
levels, we first selected candidates that showed a significant
difference between the wild-type and efp mutant strains in at least
one of the three biological replicates with a significance B (an
internal significance score as described in Materials and Methods)
cutoff of 0.01. By this criterion, 87 proteins showed changes of
2-fold or greater and 28 displayed a change of greater than 10-fold.
Of the 87 significantly misregulated proteins, 49 showed decreased steady-state levels in the *efp* mutant strain and are more likely to be direct targets of EF-P owing to its characterized stimulatory effect on translation (17, 18).

**Identification of amino acid motifs enriched in EF-P-regulated proteins.** To determine if misregulation of specific proteins in *efp* mutants can be attributed to certain motifs, we analyzed the SILAC data for the prevalence of specific peptide sequences in proteins showing a strong decrease or increase in the *Salmonella efp* mutant. Since tripeptide motifs (PPP and PPG) had previously demonstrated EF-P-dependent translation, we searched for the frequency of each possible tripeptide motif in the 10% of proteins most strongly affected (either up or down) by EF-P. We compared the actual frequency of each motif to its expected frequency and the frequency observed in the remaining 90% of proteins identified in SILAC. An enrichment score was calculated based on the ratio between observed occurrences of a tripeptide within this group, normalized against the actual-over-expected occurrences in all retrieved SILAC hits (see Materials and Methods). Using this method, we identified a number of tripeptide motifs that were enriched in the 10% of proteins that were most downregulated in the *efp* mutant (Table 1). The two top-scoring tripeptide sequences identified were PPP and PPG, supporting the recent findings regarding EF-P regulation and also acting as a proof of principle for our analysis. Additionally, a number of previously uncharacterized sequence motifs were also identified, including the third-highest scoring sequence, APP, implicating them as potential EF-P-dependent motifs. While this article was in preparation, Woolstenhulme et al. identified the APP-containing motifs MRAPP and WAPP as sequences capable of stalling ribosomes (9).

**Alternative non-proline-containing motifs can render protein synthesis dependent on EF-P.** Results from the motif analysis lend support to the previously characterized EF-P-dependent sequences but also propose novel motifs that may require EF-P for efficient translation. To assess the possibility that EF-P plays a role in the translation of motifs other than proline, we constructed a number of translational fusion plasmids wherein putative target sequences were inserted in-frame at the fourth codon of *gfp*. Green fluorescent protein (GFP) fluorescence was significantly decreased in an *efp* mutant relative to that in wild-type *E. coli* when any of the PPP, PPG, or APP motifs were inserted into GFP (Table 2). Interestingly, insertion of a YIR-YIR motif also caused production of the GFP reporter to be dependent on EF-P. This is the first EF-P target motif identified that lacks a proline residue. In contrast, none of the other predicted motifs—including an unrepeated YIR sequence—yielded a significant difference in synthesis. We further analyzed the proteins containing non-EF-P-dependent motifs to determine the reasons they were enriched in our bioinformatic analysis. Of the 10% of proteins that were most downregulated in the *efp* mutant, 16 of the 29 proteins containing an RME, PFF, or YIR motif also have a PPP, PPG, or APP motif, which may explain their EF-P dependence. For the 13 proteins that contain an RME, PFF, or YIR motif but lack a PPP, PPG, or APP motif, we aligned the residues upstream of the putative regulatory motif but did not identify any conserved sequences (data not shown). The underlying cause of the EF-P dependence of these proteins remains to be investigated.

**The presence of a PPP, PPG, or APP motif is not sufficient to confer dependence on EF-P.** To investigate whether the charac-

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**TABLE 1** Bioinformatic analysis predicts EF-P-regulated tripeptide motifs

| Motif | Occ	extsubscript{high} | Exp	extsubscript{high} | Occ	extsubscript{mid} | Exp	extsubscript{mid} | Occ	extsubscript{low} | Exp	extsubscript{low} | Enrich	extsubscript{high} | Enrich	extsubscript{low} | P value* |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------|
| PPP   | 18              | 4.96            | 15              | 29.1            | 0               | 3.16            | 4.09            | 0.00            | 2.1E-10   |
| PPG   | 28              | 8.48            | 26              | 49.7            | 1               | 5.40            | 3.82            | 0.21            | 5.6E-14   |
| APP   | 18              | 11.1            | 22              | 65.1            | 0               | 7.07            | 3.38            | 0.00            | 7.6E-10   |
| RME   | 12              | 5.30            | 16              | 31.1            | 2               | 3.38            | 3.00            | 0.79            | 1.3E-04   |
| YIR   | 10              | 4.64            | 14              | 27.2            | 1               | 2.96            | 3.00            | 0.47            | 4.7E-04   |
| TQM   | 11              | 3.64            | 17              | 21.4            | 0               | 2.32            | 2.95            | 0.00            | 1.4E-04   |
| PFF   | 10              | 2.84            | 15              | 16.6            | 2               | 1.81            | 2.78            | 0.87            | 7.4E-05   |
| DPP   | 5               | 6.73            | 8               | 39.4            | 1               | 4.29            | 2.68            | 0.84            | 2.0E-07   |
| FFL   | 5               | 6.15            | 8               | 36.1            | 1               | 3.92            | 2.68            | 0.84            | 1.4E-06   |
| QNA   | 22              | 10.0            | 43              | 58.8            | 5               | 6.39            | 2.36            | 0.84            | 3.0E-05   |

*Prevalence among the SILAC hits of all possible tripeptide combinations of the 20 common amino acids. Occ, motif occurrences in the indicated subgroup of the SILAC data; Exp, expected motif occurrences in the indicated subgroup based on amino acid prevalence; Enrich, calculated motif enrichment index in the indicated subgroup of the SILAC data; high, low, and mid, subgroups of SILAC data encompassing the 10% of proteins with the highest (high) or lowest (low) WN1269/WN1308 ratios; “mid” incorporates the remaining 80%.

**TABLE 2** Verification of predicted EF-P-dependent motifs

| Motif   | GFP fluorescence (WT/efp)* |
|---------|---------------------------|
| Null    | 0.95 ± 0.09               |
| PPPPPP, d | 20.68 ± 0.38             |
| PPPPPP, d | 18.55 ± 0.38             |
| PPPPPP, d | 18.12 ± 0.20             |
| PPPPPP, d | 15.97 ± 0.08             |
| PPP     | 4.72 ± 0.23               |
| PPG     | 10.39 ± 0.66              |
| APP     | 5.88 ± 0.20               |
| RME     | 1.34 ± 0.07               |
| YIR     | 0.96 ± 0.13               |
| YIRYIR  | 7.61 ± 2.54               |
| PFF     | 1.58 ± 0.19               |

*Motifs were assayed for EF-P dependence by insertion into the 4th codon position of GFP.

b Values at 21 h postinduction were normalized to cotranscriptionally expressed mCherry and are shown as a ratio of wild-type and *efp* mutant strains expressing the same construct. All values are the averages ± standard deviations for three biological replicates.

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b No motif inserted into GFP.

d Sequence of six optimal (CCG; 0) or random (1 to 3) proline codons.
terized target motifs are sufficient for EF-P regulation in vivo, we compared the existence of a PPP, PPG, or APP motif in proteins with their SILAC ratios. Of the 422 proteins that contain one of the three target motifs, 100 were conclusively identified in SILAC (Fig. 2A). Only 20 of these were found to be significantly downregulated in the \( \Delta \text{efp} \) strain (ratio > 2) and a significance B score of less than 0.01 in at least one trial. Functional annotation clusters showing significant overrepresentation (P value < 0.05) are shown. Cluster breakdown can be found in Table S4 in the supplemental material. (B) DAVID analysis of SILAC hits showing a decreased protein level in the \( \Delta \text{efp} \) strain (ratio > 2) and a significance B score of less than 0.01 in at least one trial. Functional annotation clusters showing significant overrepresentation (P value < 0.05) are shown. Cluster breakdown can be found in Table S5 in the supplemental material. (C) DAVID analysis showing the most significantly overrepresented clusters among the 422 proteins that contain an EF-P target motif. For clarity, only groups with P values < 0.001 are shown here. For a full list, see Table S6. (D) DAVID analysis showing the only significantly overrepresented cluster (P value < 0.05) among the 20 proteins that fall into all three categories (contain an EF-P target motif and were significantly downregulated in the \( \Delta \text{efp} \) strain in SILAC). Cluster breakdown can be found in Table S6 in the supplemental material.

Taken together, these data suggest that the presence of particular tripeptide motifs may not, in and of itself, be sufficient to cause translational stalling in the absence of EF-P.

To investigate whether the position of a target motif within a protein affected EF-P regulation, we examined the distribution of the proteins where the target motif occurs in the efp mutant strain. A more conservative analysis of proteins with SILAC ratios of less than 2 (without regard for the significance score) found that there were 45 proteins identified in SILAC that were not downregulated in WN1308 yet have a PPP, PPG, or APP motif (12, 22, and 16 proteins, respectively, contain each motif; 5 proteins have two of the three motifs). This demonstrates that a large percentage of proteins that contain a characterized EF-P target motif are not misregulated in the efp deletion strain. Examples include ZipA, SseA, and YtfM, which were identified to have average expression ratios of 1.07, 0.74, and 0.84, respectively, with significance B scores that were far from significant in all three replicates (see Table S2 in the supplemental material). Though they were not misregulated in the efp mutant, Salmonella ZipA has two distinct APP motifs, as well as an APPP motif, SseA has an APPG motif, and YtfM contains a PPP motif.
EF-P disproportionately affects synthesis of signaling and nucleotide-binding/metabolic proteins. To examine whether EF-P affected the synthesis of all classes of proteins similarly or if there was a particular bias toward a specific subset of proteins, the SILAC data were subjected to a functional annotation analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software package (22–24). The program compares a list of genes with functional annotation databases, including GO (gene ontology) terms, KEGG pathways, and SP-PIR (Swiss-Prot and Protein Information Resource) keywords, among others. By comparing the prevalences of proteins belonging to these categories in their respective database and in the input gene list, DAVID generates a P value highlighting significantly overrepresented annotation terms.

Upon analysis of the 49 significantly downregulated proteins identified in SILAC, we find that four clusters demonstrate over-representation, with a cluster P value of less than 0.05 (Fig. 2B; see also Table S4 in the supplemental material). Most prominent among these terms were two-component regulatory systems, with particular emphasis on proteins involved in chemotaxis and motility. Furthermore, metabolic proteins were also abundantly downregulated as annotated by functions in nucleotide binding, oxidative phosphorylation, or proteolysis.

We subsequently examined the S. Typhimurium SL1344 genome for proteins containing EF-P-dependent sequences and found 422 open reading frames (ORFs) encoding a PPP, PPG, or APP motif. These motifs occurred in 112 (PPP), 195 (PPG), and 185 (APP) proteins, with 70 of those proteins containing more than one of these motifs. In contrast, the sequence YIRYIR is not present in any Salmonella protein. To examine the physiological processes involving proteins with EF-P-dependent motifs, we conducted a DAVID cluster analysis and found that the overrepresented functional groups were similar to those identified among proteins that were significantly downregulated in SILAC (Fig. 2C). Many of the identified proteins with a PPP/PPG/APP motif are predicted membrane proteins, a group we previously investigated by comparative gel electrophoresis. We previously identified the outer membrane porin, KdgM, as significantly upregulated in the efp mutant strain.

EF-P-mediated nuclease protection of the 3' end of the transcript was similar between the mutant and wild-type strains as measured by quantitative real-time reverse transcriptase PCR (Fig. S1 in the supplemental material). Interestingly, though similar poxB transcript levels indicate that transcription is not altered, gfp mRNA levels were decreased in strains expressing EF-P-dependent constructs. However, since previous in vitro data have shown EF-P rescuing ribosome stalling in the presence of nucleases (9, 17, 18), we believe that this degradation is not the cause of decreased GFP fluorescence but rather is an effect of ribosome stalling at EF-P-dependent motifs leading to reduced ribosome-mediated nuclease protection of the 3' end of the poxB-gfp transcript.

Decreased fluorescence was observed not only in an efp mutant but also in poxA and yjeK mutants (see Fig. S2 in the supplemental material). In contrast, a strain lacking the YfcM protein did not show any significant difference in synthesis of the PoxB-sgFP fusion construct. These results are consistent with previous data showing that YfcM-mediated hydroxylase of EF-P is not critical for its function (14).

To identify the region essential for EF-P dependence, we systematically generated C-terminal truncations of the poxB sequence fused to sgFP (Fig. 3A). By comparing fluorescence of these constructs in wild-type and efp mutant Salmonella, we nar-
rowed the critical region to a 14-amino-acid stretch between residues 69 and 82 with the sequence VCAGSCGPGNLHLI (Fig. 3B and C). We confirmed that the amino acid (as opposed to the nucleotide) sequence in this region was critical for EF-P dependence by generating a construct with this region shifted to the +1 reading frame and observing an abolition of the requirement for EF-P (see Fig. S3 in the supplemental material).

Residues 69 to 82 of PoxB contain a GPG motif that we hypothesized could, like PPP, mediate the EF-P dependence of the PoxB protein. To address this, we performed site-directed mutagenesis on the GPG motif in the full-length PoxB-sfGFP construct and on additional upstream and downstream residues, converting each residue to a leucine. Leucine was chosen because of its absence from the top-scoring motifs predicted by our analysis of proteins.

FIG 3 The GSCGPG motif of PoxB renders it dependent on EF-P. (A) Outline of translational fusion constructs expressing full-length (FL) PoxB or C-terminal truncations fused to sfGFP. Construct designations are shown in bold at left as a figure key and indicate the length in codons of each truncated poxB gene. The arrow indicates the transcriptional start site under the control of the PLtet0-1 constitutively active promoter, and “fMet” indicates the ATG start codon of PoxB. Numbers indicate poxB lengths in codons. For clarity, only a selection of constructs is illustrated. (B) Sequence of amino acids 69 to 82 of PoxB. The GSCGPG motif is bold and underlined. (C) Relative GFP fluorescence of PoxB truncations. Values were taken at 10 h postinoculation, were normalized to the optical density at 600 nm (OD600), and are shown relative to those of the wild-type strain expressing the same construct. The same plasmid encoding the first 186 codons of lacZ instead of poxB is included as a control. (D) Relative GFP fluorescence (as in panel C) for single-residue mutations to leucine in the full-length PoxB construct. All values are the averages for at least 3 biological replicates. Each error bar shows 1 standard deviation.
downregulated in the efp mutant (Table 1). We found that mutation of any residue in the GPG motif or in the upstream three residues (sequence GSC) restored GFP fluorescence in the efp mutant (Fig. 3D). In contrast, mutation of residues either downstream or upstream of the GSGCGPG motif had negligible effects on the synthesis of the reporter construct. Notably, the GSGCGPG sequence is found only in PoxB in Salmonella. These data elucidate a novel EF-P-dependent motif that, like the artificially derived YIRYIR, is considerably larger than the previously described tripeptide motifs and can cause a protein to be dependent on EF-P for proper synthesis in vivo.

DISCUSSION

The recent finding that EF-P alleviates stalls caused by polyproline motifs during translational elongation provides a unifying mechanism for the physiological consequences that are triggered by the case of SecM or TnaC, require alternate factors to rescue the stall. Also unresolved is whether EF-P preferentially targets particular subsets of proteins (e.g., nucleotide binding proteins) for the purpose of regulation or if this EF-P-dependent subset of proteins merely requires difficult-to-translate structural features, such as polyprolines, for functional purposes.

MATERIALS AND METHODS

Bacterial strains and plasmids. For SILAC, we employed an arginine and lysine auxotroph (ΔargHΔlysA) derivative of Salmonella enterica serovar Typhimurium strain SL1344 utilized in an earlier study (33). Auxotrophy was confirmed by growth in minimal medium in the absence of lysine or arginine. The Δefp mutation from strain W9134 was transferred into the auxotrophic strain by transduction using the HT105/1 int-201 derivative of phage P22 (34) to generate strain W1308. E. coli strains used for motif verification were derivatives from the Keio knockout collection (35), including Δyfcm, Δyjek, ΔpoxB, and Δefp strains and BW25113 (wild type). Deletion of genes and removal of kanamycin cassettes using FLP recombinase (36) was confirmed by PCR. Plasmids used for motif verification were derivatives of pBAD30 (37) containing a tandem fluorescent fusion cassette composed of green fluorescent protein (GFP) followed immediately by the mCherry Shine-Dalgaro sequence and mCherry, both of which are optimized for synthesis in E. coli (40). This construct is designated pBAD30m700 and served as the PCR template for construction of other reporters (see Table S1 in the supplemental material). Unless otherwise stated, the codons used for motif inserts were optimized according to tRNA abundance in E. coli (41).

Using the red-gam recombinase protocol described above, we generated strain W1405 by deleting the majority of the efp gene (base pairs 145 to 424) from S. Typhimurium strain 14028s such that the new mutation would not influence the promoter of the upstream antiparallel yjek gene. The mutant allele was transduced to a fresh strain background prior to experimentation. Deletions of efp were confirmed by PCR amplification using the primers WNP582 and WNP583 (see Table S1 in the supplemental material). W1405 and the previously used Δefp mutant, W9134 (42), behave identically under all conditions tested (data not shown).

Plasmids used for the PoxB translational fusion assay were generated by PCR amplification of the 5′ UTR (28 bp upstream of the start codon) and full-length (excluding the stop codon) or C-terminally truncated poxB gene using S. Typhimurium strain 14028s genomic DNA as a template. These amplicons were inserted into the NsiI and NheI sites of pXG10sf (38) to generate translational fusions to “superfolder” GFP (sfGFP). The sfGFP is a variant with improved folding and fluorescence in E. coli (39).
is truncated after codon 68 of \( \text{poxB} \) or the location of a specific point mutation in full-length \( \text{poxB} \) (e.g., \( \text{poxB} \) P76L contains a proline-to-leucine mutation at codon 76). LacZ186, included as a control, consists of the first 186 codons of the \( E. coli \) lac2 gene inserted into the NsiI and Nhel sites of the pXG101s plasmid, similar to the PoxB fusions.

**SILAC.** For stable isotope labeling of amino acids in cell culture (SILAC), we supplemented morpholinepropanesulfonic acid (MOPS) minimal medium with amino acids at the concentrations previously described (33) and used 0.2% (wt/vol) glycerol as a carbon source. For heavy-isotope-labeled samples, arginine and lysine were replaced with \(^{13}\text{C}_6\)-Arg and \(^{1}\text{H}_4\)-Lys isotopes at the equivalent molar concentration. WN1269 (wild type) was grown in heavy arginine and lysine isotopes, and WN1308 (Δefp) was grown in light isotopes in this medium for 16 h to ensure complete labeling of all proteins. Strains were subsequently subcultured 1:200 in the same media and grown to an optical density (600 nm) of 0.5 (mid-log phase), at which point the cells were harvested by centrifugation. The pellets were subjected to lysis by heating to 99°C for 5 min in fresh lysis buffer (1% deoxycholate [DOC] in 50 mM ammonium bicarbonate [NH\(_4\)][HCO\(_3\)] at pH 8). Cell debris was removed by centrifugation at 13,000 \( \times g \) for 15 min, and the supernatant was frozen at −80°C until used.

**Mass spectrometry and proteomic data analysis.** For analysis of the isotope-labeled lysates, 30 \( \mu \)g (each) of protein from WN1269 (heavy) and WN1308 (light) were combined, fractionated into 12 pieces by gel electrophoresis, and in-gel trypsin digested, following a previously outlined procedure (43). The resulting peptides were subjected to liquid chromatography coupled to tandem mass spectrometry using an Orbitrap XL mass spectrometer (Thermo Scientific) as described previously (44). Data analysis was conducted using the MaxQuant software program (45) to generate an average normalized heavy/light ratio over three biological replicates, and significance B values were calculated using Perseus software (46). To determine significance, we used the cutoff of a significance B score of less than 0.01 in at least one trial. This statistic measures significance within a single trial even for proteins that were identified in only one the three biological replicates. When we assessed significance using the average of significance B scores across all three trials (excluding scores where the protein of interest was not identified in a given trial), we obtained similar percentages of significantly misregulated proteins containing EF-P target motifs. For example, using an average significance B score of less than 0.05, we identified 107 significantly misregulated proteins. Sixty-one of these are greater than 2-fold downregulated in the mutant (WN1405) strains carrying pBAD30 constructs in LB were diluted to an optical density at 600 nm (OD\(_{600}\)) of 0.05 in M9 medium supplemented with 0.2% glyceral. After 2 h or when the OD\(_{600}\) reached 0.1 to 0.15, the culture was supplemented with 0.2% arabinose to induce synthesis of the GFP and mCherry reporter proteins, and fluorescence was assessed using a spectrophotometer (Horiba) at designated time points. Cells were analyzed for GFP using excitation at 481 nm and emission at 507 nm and for mCherry with excitation at 587 nm and emission at 610 nm. The background level with blank medium was subtracted, and the ratio of GFP fluorescence over that of mCherry was calculated. Reported values represent averages and standard deviations determined from three independent experimental replicates.

**PoxB-sfGFP translational fusion assay.** Wild-type (S. Typhimurium strain 14028s) and isogenic Δefp mutant (WN1405) strains carrying plasmids bearing full-length or truncated \( \text{poxB} \) translational fusions to sfGFP were grown in MOPS minimal medium supplemented with 0.2% (wt/vol) glucose and 20 \( \mu \)g/ml chloramphenicol. Growth was conducted for 16 h at 37°C in a Tecan Infinite M200 microplate reader with constant aeration. OD\(_{600}\) and GFP fluorescence readings (excitation and emission wavelengths of 475 nm and 511 nm, respectively) were taken every 15 min. For both OD\(_{600}\) and GFP, background values taken from no-cell controls were subtracted from all readings. For clarity, the wild-type/Δefp fluorescence ratios are displayed as GFP fluorescence per OD\(_{600}\) unit at 10 h of growth. Results obtained when cultures were measured at 6, 8, 12, and 16 h of growth were similar to those at 10 h.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00180-13/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.
Table S1, PDF file, 0.5 MB.
Table S2, XLSX file, 0.2 MB.
Table S3, XLSX file, 0.1 MB.
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