Under conditions of tight coupling between translation and transcription, the ribosome enables synthesis of full-length mRNAs by preventing both formation of intrinsic terminator hairpins and loading of the transcription termination factor Rho. While previous studies have focused on transcription factors, we investigated the role of Escherichia coli elongation factor P (EF-P), an elongation factor required for efficient translation of mRNAs containing consecutive proline codons, in maintaining coupled translation and transcription. In the absence of EF-P, the presence of Rho utilization (rut) sites led to an ~30-fold decrease in translation of polyproline-encoding mRNAs. Coexpression of the Rho inhibitor Psu fully restored translation. EF-P was also shown to inhibit premature termination during synthesis and translation of mRNAs encoding intrinsic terminators. The effects of EF-P loss on expression of polyproline mRNAs were augmented by a substitution in RNA polymerase that accelerates transcription. Analyses of previously reported ribosome profiling and global proteomic data identified several candidate gene clusters where EF-P could act to prevent premature transcription termination. In vivo probing allowed detection of some predicted premature termination products in the absence of EF-P. Our findings support a model in which EF-P maintains coupling of translation and transcription by decreasing ribosome stalling at polyproline motifs. Other regulators that facilitate ribosome translocation through roadblocks to prevent premature transcription termination upon uncoupling remain to be identified.

Bacterial mRNA and protein syntheses are often tightly coupled, with ribosomes binding newly synthesized Shine-Dalgarno sequences and then translating nascent mRNAs as they emerge from RNA polymerase. While previous studies have mainly focused on the roles of transcription factors, here we investigated whether translation factors can also play a role in maintaining coupling and preventing premature transcription termination. Using the polyproline synthesis enhancer elongation factor P, we found that rapid translation through potential stalling motifs is required to provide efficient coupling between ribosomes and RNA polymerase. These findings show that translation enhancers can play an important role in gene expression by preventing premature termination of transcription.

In Bacteria, mRNA translation can initiate as soon as the ribosome-binding site (RBS) emerges from RNA polymerase (RNAP), and the translating ribosome closely follows the elongating RNAP during the first round of translation (1). Coupling between transcription and translation is maintained in part by ribosomal protein S10, which may interact with RNAP (1) and NusG (2, 3). Interplay between transcription and translation plays a central role in controlling gene expression; for example, trilling ribosomes can prevent formation of nascent terminator hairpins (4) or occlude binding of the transcription termination factor Rho (5). Close coupling of transcription and translation also ensures continual synthesis of cellular mRNAs—the trailing ribosome blocks RNAP backtracking that leads to arrest, and in so doing inhibits premature transcription termination (1, 6, 7). Conversely, when translation slows and is uncoupled from transcription, nascent mRNAs become susceptible to Rho-mediated release (transcriptional polarity [8]). Rho-dependent terminators are found in leader regions, within coding sequences, and at the ends of transcriptional units (9) and account for at least 20% of the identified transcription terminators in Escherichia coli (10).

Variations in translation elongation rates have been implicated in gene regulation (11), mRNA decay (12, 13), codon bias (14), and protein folding (15). The mechanism of addition is the same for all amino acids during protein synthesis; however, the speed of peptidyl transfer is not uniform, and ribosomes elongate at different rates (16). Among all genetic code amino acids, proline displays by far the lowest rate of peptidyl transfer on the ribosome (17, 18). Proline is the only natural cyclic amino acid, and its pyrrolidine ring imposes structural constraints on the positioning of the amino acid in the peptidyl transferase center, resulting in slow peptide bond formation and occasional ribosome stalling (19). To overcome this potential bottleneck, robust synthesis of proteins with consecutive prolines requires elongation factor P (EF-P) (20–24). EF-P and its eukaryotic homolog elF5A prevent
ribosome pausing and stalling during polyproline synthesis by increasing the rate of peptide bond formation entropically via positioning and stabilization of peptidyl-Pro-tRNAPro (20, 25–27). Ribosome stalling caused by certain peptides may lead to mRNA cleavage around the stop codon (28, 29) or at sense codons when translation elongation is prevented (30). Stalling translation elongation can also have deleterious effects on transcription, including premature termination and RNAP backtracking (Fig. 1).

The backtracked RNAP is incapable of nucleotide addition but is stably bound to the DNA, blocking the progression of the replication fork and introducing single-strand and double-strand DNA breaks (DSBs), leading to increased mutation rates and genome instability (31).

While loss of coupling during translation of a defective mRNA represents an important quality control surveillance mechanism, transient ribosome stalling on functional messages must be minimized to ensure uninterrupted mRNA synthesis. In this work, we investigated the impact of the translation anti-pausing factor EF-P on the coupling of transcription to translation across an EF-P-dependent motif. Utilizing a reporter with a polyproline motif upstream of a termination site, it was shown that the absence of EF-P promoted uncoupling, thereby allowing premature RNA release. These findings show that EF-P plays an important role in gene expression by minimizing reductions in translation elongation rates that would otherwise lead to uncoupling from RNAP.

RESULTS

Efficient polyproline synthesis decreases Rho-dependent transcription termination. To investigate whether ribosomal stalling in a Δeft E. coli strain can uncouple translation from transcription, a reporter was constructed with a transcriptional fusion of sfGFP (superfolder green fluorescent protein [sfGFP]) and mCherry, each with a Shine-Dalgarno sequence (Fig. 2A). GN, PPPPPP, and PPG motifs were inserted at the beginning of sfGFP; PPPPPP and PPG motifs induce ribosome stalling that can be alleviated by EF-P (22). Rho is an ATP-dependent RNA translocase and helicase that binds to a Rho utilization (rut) site on the nascent mRNA, translocates along the RNA toward the RNAP, and finally triggers the dissociation of the transcription elongation complex (32). In the classic model, Rho tracks along the RNA until it reaches the elongating RNAP and pulls out the RNA from the enzyme by means of its helicase activity (33). In an “allosteric” model, Rho associates with RNAP throughout transcription and, following the capture of rut, pulls the nascent RNA through, progressively shortening it and accumulating topological stress that inactivates the elongation complex by destabilizing the RNA-DNA hybrid (34). In either model, a closely coupled ribosome would inhibit Rho-mediated termination.

To assess uncoupling, a (TC)_15 rut site was placed downstream of the peptide motif. This rut site was previously shown to lead to Rho-dependent termination in the absence of protein synthesis (32). To explore the extent to which the observed effect is attributable to Rho, which is essential in E. coli (35), Psu, a bacteriophage P4 protein (36) that inhibits Rho with high efficiency and specificity (37, 38), was expressed from a compatible plasmid. If ribosomal stalling at PPX motifs uncouples transcription and translation, the rut site would be expected to decrease synthesis of
superfolder GFP (sfGFP) and mCherry in Δefp, but not wild-type (WT), E. coli strains. In the absence of Psu or in the presence of the inactive E56K mutant Psu (which lacks the ability to bind Rho [38, 39]), a >30-fold decrease in mCherry fluorescence was observed from reporters encoding PPX motifs in the Δefp strain, compared to only a 5-fold decrease in the WT strain (Fig. 2B, shown on gray background); similar changes in GFP fluorescence were observed (see Fig. S1A in the supplemental material). In contrast, the GN reporter showed similar levels of expression in the presence and absence of EF-P. Notably, in the WT strain, all reporters produced the fluorescent proteins similarly and showed ~5-fold improved mCherry fluorescence when Rho was inhibited by Psu (Fig. 2B, shown on gray background). This apparent Rho-mediated polarity may indicate the background, stalling-independent, level of uncoupling, for example due to ribosomes that fail to engage mRNAs early during their synthesis.

To confirm that the observed effects on fluorescent protein production were due to changes in mRNA abundance, mCherry transcript levels were quantified (Fig. 2C). Two hybridization products were observed, one of higher weight representing the full-length gfp-mCherry transcript (~2,000 nucleotides [nt]) and a second transcript (~1,200 nt). The smaller product is consistent with termination at a stem-loop structure predicted by ARNold (40, 41), (AGUGACUAAAGCGCCGCGUUGCGU GGGA; free energy of ~8.40 kcal/mol [42]). Transcript levels for gfp-mCherry produced from the PPPPPP reporter in the WT strain were ~2-fold higher in the presence of Psu, consistent with residual polarity noted above. In the Δefp mutant, the transcript level for gfp-mCherry was ~7-fold higher when Rho was inhibited by Psu production (Fig. 2C). Together, these observations support the proposed role of EF-P in preventing uncoupling.

**EF-P effect is potentiated by accelerating transcription.** Coupling of transcription and translation implies that RNAP and the ribosome move at identical rates (43), yet the speed of either ma-
chinery can be modulated by nucleic acid signals and accessory factors in the cell. The above results indicate that EF-P can maintain coupling by alleviating ribosome stalling at polyproline motifs. However, coupling could also be broken if RNAP is moving too fast, for example, upon modification by a transcription anti-termination factor (44). We hypothesized that the effect of efp deletion will be exacerbated by a mutation in RNAP that increases termination (44). We noted that the effects of accelerated transcription on the reporter paralleled due to ribosome stalling (in the absence of EF-P) and RNAP acceleration (by H526Y substitution) (45). Together, these results indicate that EF-P can compensate for defects in protein production under conditions when coupling between RNAP and the ribosome is broken by disparate changes in their rates.

Efficient polyproline synthesis maintains transcription through intrinsic terminators. The above results establish the role of EF-P in preventing premature Rho-mediated termination. To investigate the effects of ribosome stalling on Rho-independent termination, a reporter was constructed in which a stall site (PPPPPP) was placed upstream of the intrinsic terminator hairpin (GN motif with no hairpin) from E. coli rpoB. RpoB2 enzyme has been shown to decrease termination via uncoupling, while on the other hand, RpoB2 directly inhibits termination. However, its role of EF-P in preventing premature Rho-mediated termination (46). Also inhibition of Rho by Psu led to smaller increases in expression by the rpoB2 RNAP (~1.6-fold) compared to the rpoB RNAP (5-fold [highlighted in grey in Fig. 3]). These observations are consistent with a faster transcription elongation rate in rpoB2 (39), which makes it more resilient to Rho (48, 49) and thus less susceptible to inhibition by Psu.

In contrast, in the Δefp strain insertion of the PPPPPP motif led to a large decrease in mCherry production, particularly when Rho was active (more than 10-fold with either RNAP). When Rho was inhibited by Psu, the observed PPPPPP effect was greater with the B2 RNAP (4-fold, 3E+06 to 7.6E+05) than with the rpoB RNAP (1.5-fold, 7E+06 to 4.8E+06), consistent with the idea that a fast RNAP would exacerbate uncoupling triggered by ribosome stalling. With both reporters, expression of Psu stimulated mCherry production by either RNAP, but to a different extent. A comparable increase was also detected in mRNA levels (see Fig. S1B in the supplemental material). In the case of the rpoB RNAP, the level of mCherry production was increased 7-fold but increased only 3-fold with the rpoB2 enzyme (Fig. 3, highlighted in black). The greater defect observed with the rpoB2 RNAP reveals additive effects of reducing coupling by ribosome stalling (in the absence of EF-P) and RNAP acceleration (by H526Y substitution). Together, these results indicate that EF-P can compensate for defects in protein production under conditions when coupling between RNAP and the ribosome is broken by disparate changes in their rates.
In the absence of the stalling motif (GN motif), the pyrL hairpin terminator reduced mCherry expression by B2 RNAP only 6-fold in both the WT and ΔrpoB strains harboring pBAD30HAPYT plasmid with a PPPPPP or GN insert. For each strain, two rpoB2 chromosomal backgrounds were tested, rpoB2 (WT) and rpoB2. The values shown on a gray background are the fold changes in mCherry fluorescence in the presence of hairpin. The means for at least three biological replicates are shown on the graph, and error bars indicate 1 standard deviation.

**EF-P suppresses Rho-dependent transcription termination in vivo.** While the major known function of EF-P is to facilitate the robust synthesis of polyproline motifs, this may not provide a complete picture of the role of EF-P in gene expression. The proteomic data from stable isotope labeling with amino acids in cell culture (SILAC) experiments performed in both *Salmonella enterica* serovar Typhimurium (22) and *E. coli* (24) indicated that only a minority of proteins containing the PPP motif were downregulated in the ΔrpoB strain. In *E. coli*, ~13% of proteins and ~21% of mRNAs displayed at least 2-fold differences between the WT and ΔrpoB strains, as measured by SILAC (24) and transcriptome sequencing (RNA-seq) (23), respectively. In both data sets, PPX-containing genes are almost equally represented in the upregulated and downregulated genes, suggesting that EF-P may have effects on both transcription and translation, consistent with the data presented above. It is conceivable that ribosomes stalling at polyproline motifs could favor formation of either a terminator or antiterminator RNA structure, thus enhancing or decreasing termination efficiency, respectively.

In our analysis, we utilized artificial constructs in which strong Rho-dependent or intrinsic termination signals were placed between a PPPPPP motif and a reporter gene. While these model terminators enabled us to demonstrate a role for EF-P in transcription-translation coupling, understanding of the physiological effects of EF-P requires identification of its cellular targets. We sought to identify termination signals sensitive to EF-P in vivo. Bioinformatic prediction of Rho-dependent terminators is not feasible, since other than C richness, there are very few other conserved features (51), and the accessibility of RNA is a determining factor in Rho recruitment. Although a few selected strong Rho-dependent terminators have been studied extensively, less is known on a genome-wide scale. Recent work by Peters et al. (52) used two strand-specific, global RNA-profiling techniques to obtain high-resolution maps of Rho-dependent termination in WT
E. coli, which would not directly target detection of any EF-P-dependent effects.

PPX-containing proteins downregulated in the \( \Delta efp \) strain in both RNA-seq and SILAC data sets (Table 1) could represent EF-P targets. For regions downstream of the PPX motif, C/G ratios and secondary structure propensities were determined. There is an observed depletion of G residues from \( rut \) sites (51), which is believed to play an indirect role in Rho binding by making the RNA less likely to form strong secondary structures (10). Among several genes having C-rich regions predicted to be devoid of secondary structures, only \( cadA \) was shown to have a Rho-dependent terminator (52), which starts 40 bp before the stop codon of \( cadA \). The PPG motif in \( cadA \) is 144 bp upstream of this \( rut \) site (Fig. 5A).

To investigate the effects of PPG and EF-P on \( cadA \) termination, we utilized hybridization probes complementary to sequences before and after the PPG motif. The contribution of Rho-dependent termination was assessed by additionally probing for WT and \( \Delta efp \) strains expressing Psu. The dot blot (see Fig. S2A in the supplemental material) indicated no change in the before/after probe ratio for the WT whether Psu was expressed or not. This could be expected for the WT, since Rho cannot load onto a \( rut \) site when transcription and translation are coupled. In the \( \Delta efp \) strain, a 1.6-fold reduction in the before/after probe ratio was detected in the absence of Psu (Fig. 5B), consistent with the notion that ribosomal stalling at PPG motifs in the absence of EF-P can uncouple transcription and translation, thus allowing Rho termination. In contrast, analyses performed for \( ygdH \) and \( hslU \) did not reveal any differences in the \( \Delta efp \) strain (Fig. S3 and S4), consistent with the absence of Rho-dependent signals in these genes (52).

Potential intrinsic termination sites. An intrinsic terminator is an RNA signal composed of a GC-rich stem-loop structure followed by a U-rich region (51). For all the PPX-containing genes with RNA reads differing by more than 2-fold between the WT and \( \Delta efp \) strains, the transcript sequence was analyzed using AR-Nold to identify intrinsic terminators (40–42, 53). From a total of 255 genes, 49 were predicted to contain an intrinsic terminator. Of these 49 genes, only 15 had the predicted intrinsic terminator

![In vivo probing for \( cadBA \).](image)

**FIG 5** In vivo probing for \( cadBA \). (A) Representation of the \( cadBA \) operon with the location of the PPG motif, a predicted terminator (TERM), and the probes (C1 and C2 probe) indicated. (B) Quantification of the dot blot for samples examined with probes C1 and C2. The means for at least three biological replicates are shown, and error bars indicate 1 standard deviation.
FIG 6  In vivo probing for rsxC. (A) The rsxArsCDE-nth operon (regulonDB) with representation of the location of the polyproline motif, predicted terminator, and the probes for R1 (1) and R2 (2). (B) Termination assay to test the functionality of the predicted intrinsic terminator (ITR) for rsxC. NusA, which is essential for termination at some sites, was added to 500 nM where indicated. The means for at least three biological replicates are shown, and error bars indicate 1 standard deviation. (C) Quantification of dot blot probed with probes R1 and R2 for WT and Δefp strains. The means for at least three biological replicates are shown, and error bars indicate 1 standard deviation.

within 500 bp downstream of a PPX motif (see Table S1 in the supplemental material). Five hundred base pairs was selected as an arbitrary cutoff, since transcription and translation are tightly coupled. Two potential terminators, narY and rsxC, were chosen for further analysis.

To test whether the signals in narY and rsxC induced termination in vitro, as would be expected from an intrinsic terminator, we cloned them downstream from a strong rP promoter (Fig. 6A and 7A) and carried out single-round elongation assays with E. coli RNAP. We found that the rsxC signal induced termination only in the presence of NusA (Fig. 6B), an abundant and essential transcription elongation factor that increases pausing and termination at a subset of cellular signals in E. coli and other bacteria (54). The terminator in rsxC is only 16 nt downstream of a PPE motif (Fig. 6A). In vivo probing of rsxC mRNA with probes complementary to sequences before and after the PPE motif detected a 1.8-fold reduction of longer transcript in the Δefp strain compared to the WT (Fig. 6C; see Fig. S2B in the supplemental material). For narY, while the predicted terminator functioned in vitro (Fig. 7B), no significant difference was detected between the WT and Δefp strains in vivo (Fig. 7C and Fig. S2C). The narY hairpin is 323 nt downstream of the PPX motif, and this increased spacing could explain the lack of an EF-P-dependent effect if coupling is interrupted by a different mechanism in the intervening region.

DISCUSSION

In this study, we show that an EF-P-dependent motif upstream of a rut site or an intrinsic terminator can decrease expression of a downstream gene in the absence of EF-P. This indicates that by stalling at polyproline motifs, ribosomes in Δefp cells can uncouple transcription from translation, thereby unmasking downstream transcription termination signals. Taken together, these data reveal that, in addition to its well-documented role in translation, EF-P can affect gene expression at the level of transcription. Transcription termination is a common regulatory checkpoint in bacteria with a range of molecular mechanisms modulating the termination activity of RNAP. RNAP responds to two different termination signals: Rho-dependent terminators or hairpin-dependent intrinsic terminators (50). Rho utilization (rut) sites are characterized by high-C/low-G content, and relatively little secondary structure (9, 33). In this work, we utilized an artificial rut site composed of 15 recombinant U recombinant C (rUrC) repeats. This simple polypyrimidine repeat was reported by Guérin et al. to act as an efficient Rho-dependent terminator in vivo and in vitro (32). They observed termination only in the absence of protein synthesis, when the RNA was available for Rho recruitment. Here, we detected Rho-dependent transcription termination only in Δefp cells that included a polyproline motif upstream of the TC repeats. This indicates that the translational stall caused by a polyproline motif was sufficient to trigger uncoupling from transcription and that EF-P restored coupling (Fig. 2). Similar effects of EF-P on coupling were found with the intrinsic terminator pyrL. Regulation of the pyrimidine biosynthetic operon (pyrBI) is an example of transcription attenuation in E. coli (4) that utilizes coupling of translation to transcription of the leader peptide encoded by pyrL. Tight coupling across the leader peptide is necessary to prevent formation of the terminator (4). With our reporter, we detected a significant increase in termination efficiency in Δefp cells that included a polyproline motif upstream of pyrL compared to a proline-free motif. This increase in termination efficiency was not detected in WT cells (Fig. 4). Transcription and translation are coupled in both Bacteria and Archaea, raising...
the possibility that the archaeal EF-P homolog, aIF5A, may play a similar role. Although coupling is absent in Eukarya, it is possible that eIF5A has a role in the no-go decay (NGD) mechanism, which recognizes mRNAs with translation elongation stalls and targets them for endonucleolytic cleavage (12).

A closely coupled ribosome protects the elongating RNAP from a premature attack by Rho and from falling into an arrested state. Accelerating the RNAP or slowing down the ribosome would lead to the loss of coupling, which potentially compromises mRNA and genome integrity (6). While the rate of RNA chain synthesis can be modulated by diverse inputs, it appears contrived that the only cellular factors that increase this rate, those from the universally conserved NusG family, also physically link the RNAP and the ribosome (55), forcing them to move in sync. Similarly, factors that safeguard coupling between transcription and translation by accelerating the ribosome are expected to exist in prokaryotes. Our results implicate a universally conserved EF-P as one of these factors, with others to be discovered.

While we argue that EF-P plays an important role in decreasing transcription termination in translated regions, it is conceivable that EF-P may have an opposite effect in regulatory regions. Many control mechanisms than can either increase or decrease termination efficiency have been reported (50). Ribosomes stalling at polyproline motifs may favor formation of a terminator or anti-terminator RNA structure, thus enhancing or decreasing termination efficiency, respectively. While ribosome stalling typically leads to increased Rho-dependent termination, the opposite occurs in the E. coli tna operon where the stalled ribosome occupies the overlapping rut sequences, preventing Rho binding and increasing transcription of the tna operon (56). Similarly, in the Salmonella mgtA riboswitch, the ribosome stalls during translation of a short proline-rich leader when proline levels are limiting (mgtL). This stall favors the formation of a stem-loop that masks the region where Rho interacts. In contrast, complete translation of mgtL would promote Rho-dependent transcription termination by exposing this region (57). Interestingly, rut sites and PPX-encoding sequences share in common C richness. Since the amino acid proline is coded by CCN, having a PPX guarantees at least 50% C. Furthermore, C-rich mRNA regions as short as 8 nt have been identified as rut sites (57, 58). Thus, it would not be surprising for a rut site itself to include a PPX, which would protect it from being accessed by Rho during translation (59). Nam et al. have recently shown that EF-P is not constitutively expressed in Salmonella enterica serovar Typhimurium (61).

Overall, our findings reveal that the presence of EF-P during translation of polyproline motifs plays a dual role; the first is to support a proper translation elongation rate across this mRNA region, and the second is to maintain coupling between ribosomes and RNAP when potential terminators are transcribed downstream (Fig. 1). Specifically, we found that EF-P maintained coupling of translation and transcription in mRNAs with a rut site or an intrinsic terminator downstream of a polyproline motif. Extensive experimentation guided by bioinformatics is now necessary to identify the degree to which potentially EF-P-altered transcription termination sites contribute globally to gene expression.

**FIG 7** In vivo probing for narY. (A) The narZYW operon (regulonDB) with representation of the polyproline motif, predicted terminator and the site of the probe for narZ (1) and narV (2). (B) Termination assay to test the functionality of the predicted intrinsic terminator for narY. NusA, which is essential for termination at some sites, was added to 500 nM where indicated. The mean for at least three biological replicates is shown and error bars indicate one standard deviation. TE, termination. (C) Quantification of dot blot probed for narZ and narV for WT and Δefp strains. The means for at least three biological replicates are shown, and error bars indicate 1 standard deviation.
MATERIALS AND METHODS

Strains and plasmids. The wild-type E. coli strain (BW25113) and the Δsfgfp mutant were from the Keio knockout collection, and kanamycin cassette were removed using FLP recombinase (62, 63). The sequences of the oligonucleotides used in this study are shown in Table S2 in the supplemental material. pBAD30XS (22, 23) was modified by replacing sfgfp with sfgfp while maintaining the Xhol-SpeI cloning site. mCherry was modified by introducing an i-tag upstream (64). sfgfp was N-terminally tagged with (HA)₃ (HA stands for hemagglutinin), generating pBAD30HA (Fig. S4). For the rut reporter pBAD30HATC (Fig. S5), the annealed product of oligonucleotide STC1 and STC2 was inserted in pBAD30HA at the SpeI site in sfgfp. This introduced a linker (SGSGSGSG) followed by the rut site, (TC)₁₅. pBAD30HATC was further double digested with Xhol and SpeI to insert either PIPPPP or PPG by annealed oligonucleotide cloning with oligonucleotide pairs PG1 and PG2 or 6P1 and 6P2, respectively. For the hairpin reporter, a PCR product amplifying only itag-mCherry from pBAD30HA with a forward primer introducing SpeI-Pcil (SPT1) and a reverse primer with XbaI-SpeI was ligated back into pBAD30HA (double digested with SpeI and XbaI) to make pBAD30HAT. The pyrB operon leader peptide was amplified from E. coli with a forward primer introducing SpeI (PF6) and a reverse primer introducing Pcil (PRP). This was inserted in-frame into pBAD30HAT cut with SpeI and Pcil. This resulting plasmid, pBAD30HAPYT2, was further double digested with Xhol and SpeI to insert PIPPPP by annealed oligonucleotide cloning with oligonucleotide pair (6P1 and 6P2) to produce pBAD30HAPYT2. For Psi production from pl2A280PSU, a synthetic cassette (gene block from IDT) was cloned between BglII and HindIII in pET28a, replacing T7P with Prc and Psi. A mutant Psi, E56K, mutant Psu (unable to bind Rho or inhibit Rho-dependent terminiation [38]) was also cloned (pLA280PSU6). The putative intrinsic terminators were cloned downstream of the bacteriophage APR promoter. Synthetic oligonucleotide cassettes (2390 plus 2391; RxsC and 2393 plus 2395; NarY) were ligated into SpeI and BglII sites of pl2A26 (65). Templates for in vitro transcription assays were generated by PCR with oligonucleotides 17 and 231 (Table S1, PDF file, 0.1 MB). Oligonucleotides used in this study are shown in Table S2 in the supplemental material. pBAD30HATC (Fig. S5), the annealed product of oligonucleotide STC1 and STC2 was inserted in pBAD30HA at the SpeI site in sfgfp. This introduced a linker (SGSGSGSG) followed by the rut site, (TC)₁₅. pBAD30HATC was further double digested with Xhol and SpeI to insert PIPPPP by annealed oligonucleotide cloning with oligonucleotide pairs PG1 and PG2 or 6P1 and 6P2, respectively. For the hairpin reporter, a PCR product amplifying only itag-mCherry from pBAD30HA with a forward primer introducing SpeI-Pcil (SPT1) and a reverse primer with XbaI-SpeI was ligated back into pBAD30HA (double digested with SpeI and XbaI) to make pBAD30HAT. The pyrB operon leader peptide was amplified from E. coli with a forward primer introducing SpeI (PF6) and a reverse primer introducing Pcil (PRP). This was inserted in-frame into pBAD30HAT cut with SpeI and Pcil. This resulting plasmid, pBAD30HAPYT2, was further double digested with Xhol and SpeI to insert PIPPPP by annealed oligonucleotide cloning with oligonucleotide pair (6P1 and 6P2) to produce pBAD30HAPYT2. For Psi production from pl2A280PSU, a synthetic cassette (gene block from IDT) was cloned between BglII and HindIII in pET28a, replacing T7P with Prc and Psi. A mutant Psi, E56K, mutant Psu (unable to bind Rho or inhibit Rho-dependent terminiation [38]) was also cloned (pLA280PSU6). The putative intrinsic terminators were cloned downstream of the bacteriophage APR promoter. Synthetic oligonucleotide cassettes (2390 plus 2391; RxsC and 2393 plus 2395; NarY) were ligated into SpeI and BglII sites of pl2A26 (65). Templates for in vitro transcription assays were generated by PCR with oligonucleotides 17 and 1832. E. coli RNA polymerase (66) and NusA (67) were purified as described previously. For the plasmid for intrinsic terminator plAI239, the P₆₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋ شبكة البحث والإنتاج في البكتيريا: الاتصال الجينوميكي للكريسيم و الكريسيم. Curr Opin Microbiol 16:112–117. http://dx.doi.org/10.1016/j.mib.2013.01.010.

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