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Characterization of the Detachable Rho-Dependent Transcription Terminator of the *fimE* Gene in *Escherichia coli* K-12

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The *fim* genetic switch in the chromosome of *Escherichia coli* K-12 is an invertible DNA element that harbors the promoter for transcription of the downstream *fim* structural genes and a transcription terminator that acts on the upstream *fimE* regulatory gene. Switches oriented appropriately for structural gene transcription also allow *fimE* mRNA to read through, whereas those in the opposite orientation terminate the *fimE* message. We show here that termination is Rho dependent and is suppressed in a *rho* mutant or by bicyclomycin treatment when *fimE* mRNA is expressed by the *fimE* gene, either from a multicopy recombinant plasmid or in its native chromosomal location. Two *cis*-acting elements within the central portion of the 314-bp invertible DNA switch were identified as contributors to Rho-dependent termination and dissected. These *fim* sequence elements show similarities to well-characterized Rho utilization (*rut*) sites and consist of a *boxA* motif and a C-rich and G-poor region of approximately 40 bp. Deletion of the *boxA* motif alone had only a subtle negative effect on Rho function. However, when this element was deleted in combination with the C-rich, G-poor region, Rho function was considerably decreased. Altering the C-to-G ratio in favor of G in this portion of the switch also strongly attenuated transcription termination. The implications of the existence of a *fimE*-specific Rho-dependent terminator within the invertible switch are discussed in the context of the *fim* regulatory circuit.

Escherichia coli and other bacteria end the process of transcript elongation by employing one of the following two types of transcription terminator: intrinsic terminators and factor-dependent terminators (36, 40, 43, 58). Intrinsic terminators typically consist of an approximately 20-bp stretch of DNA that is composed of a G+C region of hyphenated dyad symmetry followed by a run of T residues. This specifies in the RNA transcript a stable stem-loop structure that is followed by up to eight U residues (8, 31, 50). These U residues base pair weakly with the corresponding A residues in the template DNA strand, and in combination with the formation of the RNA stem-loop structure, lead to destabilization of the RNA polymerase-template-transcript elongation complex and the consequent termination event (43, 61).

Factor-dependent terminators utilize the hexameric ring-shaped Rho protein to facilitate transcript release from regions of the template that are not characterized by intrinsic RNA-DNA hybrid helix instability (5, 7, 37, 40, 47). Rho-dependent terminators lack the obvious structural features that are associated with intrinsic terminators (7). In general, they consist of a bipartite structure that extends for up to 150 bp of DNA and have a high proportion of C relative to G residues (2, 14, 20, 33, 39, 44, 60, 62). The Rho utilization (*rut*) site of a Rho-dependent terminator is a site on the RNA transcript which generally has little secondary structure and is recognized by the RNA-binding Rho factor, while the transcription stop point (*tsp*) is the region within which Rho enhances transcription termination of paused RNA polymerase complexes (6, 17, 35, 45).

The Rho monomer is composed of two domains, both of which bind RNA. The N-terminal domain binds to a ribosome-free region of the nascent transcript at a *rut* site, and the C-terminal domain uses energy from the hydrolysis of ATP to translocate towards the elongating RNA polymerase (11, 15, 33, 34, 44, 53, 56, 59). Following engagement with the polymerase, Rho acts as a helicase, using ATP binding and hydrolysis to unwind the RNA-DNA hybrid, and presumably pulls the transcript out of the RNA polymerase RNA exit channel, thereby terminating transcription (39, 41, 42, 52). Unlike intrinsic terminators, for which the termination points can be localized to 1 to 3 bp, termination sites within Rho-dependent terminators can be distributed over as much as 100 bp of DNA (28, 43).

Both factor-dependent and intrinsic terminators of transcription are normally associated permanently with the transcripts that they regulate. In contrast, this study describes the dissection of a Rho-dependent terminator in the *fim* gene cluster of *E. coli* K-12 that is alternately connected to and disconnected from the gene that it regulates by site-specific DNA inversion.

The *fim* genes encode type 1 fimbriae, which are stiff, rod-like, proteinaceous appendages that protrude from the bacterial cell surface and are composed mainly of the FimA subunit protein (24), with a fibrillum at the tip that is made up of the FimH adhesin and the FimF and FimG adaptor proteins (19, 22). The FimH protein mediates lectin-like binding to D-mannosides (27), contributing to attachment of the bacteria to host surfaces during colonization. The promoter used for transcription of the *fimA* gene is located within a 314-bp invertible DNA sequence known as the *fim* switch, or *fimS* (1, 10). Site-specific recombination between 9-bp inverted repeats that flank *fimS* inverts the switch. The result is a process of phase variation in

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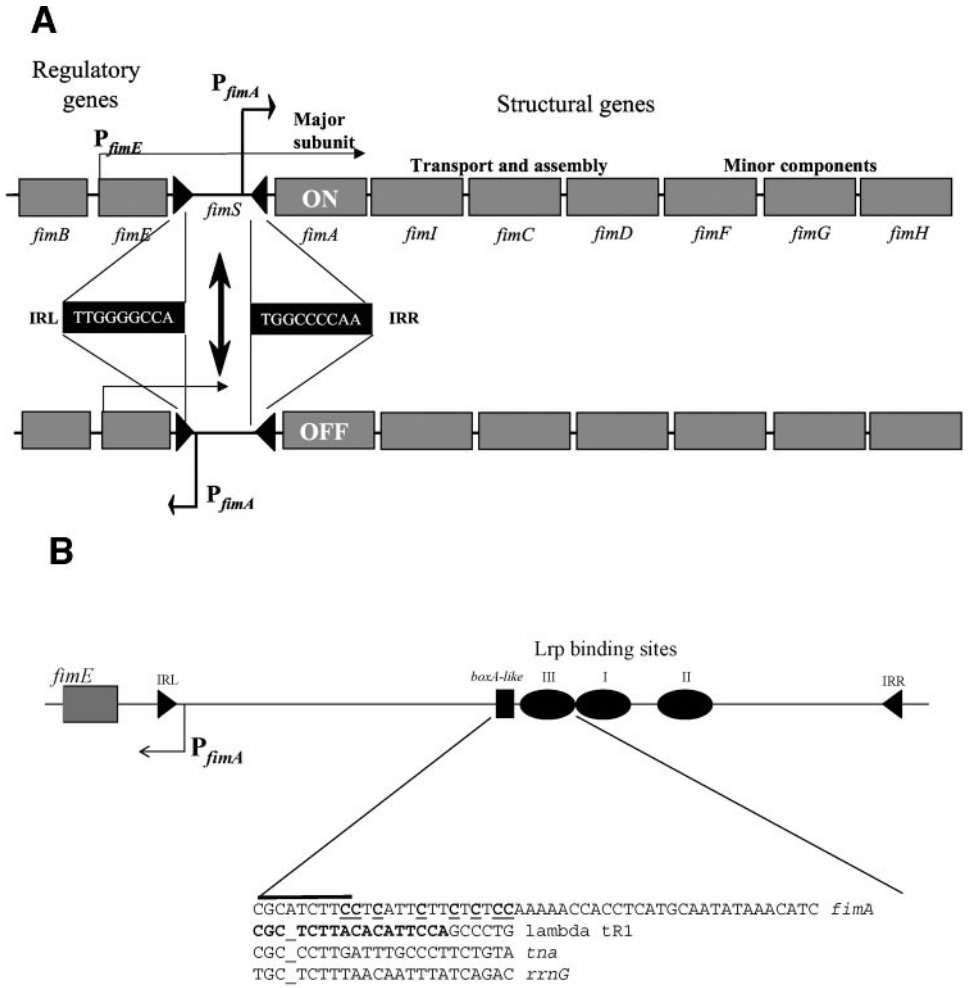

FIG. 1. Structure of the *fim* operon and location of the features associated with Rho-dependent termination of *fimE* transcription. (A) The segment of the horizontal line that is bracketed by filled triangles represents the invertible *fim* switch (*fimS*). The angled arrow at *fimE* represents the promoter (P_{fimE}) of that gene and shows the direction of transcription. In the ON phase, the transcript extends through the *fim* switch, whereas in the OFF phase it terminates in *fimS*. The angled arrow within *fimS* represents the *fimA* promoter (P_{fimA}). In the ON phase (upper diagram), this arrow is directed towards *fimA*, and it is directed away in the OFF phase (lower diagram). Switching between the ON and OFF phases involves site-specific recombination between the inverted repeats IRL and IRR (filled triangles). The sequences of the 9-bp inverted repeats are shown in the filled rectangles. (B) Expanded view of *fimS* in the OFF orientation. The 3′ end of the *fimE* gene is shown on the left side of the diagram, and the positions of the three binding sites for Lrp (filled ovals with Roman numerals) and the *boxA* sequence (filled box) are indicated. The 52-nucleotide sequence associated with the Rho-dependent transcription termination *rut* site is given at the bottom of the figure. The overlined portion is the 8-bp *fimS* sequence that shows the most identity to other *boxA* elements. C residues that were converted to G by SDM are shown in bold and underlined. The identified *fimS* Rho-dependent terminator (accession number X03923) (24) is aligned with the corresponding parts of the Rho-dependent terminator from bacteriophage lambda (λ tR1; the *rutA* element is shown in bold; accession number J02460) (38), the *boxA* motif and part of the *rut* site of the *E. coli tna* operon leader region (accession number M11990) (57), and the *boxA* sequence of the *E. coli* ribosomal operon *rrnG* (accession number V00350) (51).

which the *fimA* gene is alternately connected to (phase ON) and disconnected from (phase OFF) its promoter. DNA inversion is catalyzed by the tyrosine integrase site-specific recombinases FimB and FimE (9, 25). These highly homologous proteins have distinct DNA inversion activities at *fimS*. FimB inverts the switch in the ON-to-OFF and OFF-to-ON directions with approximately equal efficiencies, whereas FimE shows a strong bias for inverting *fimS* in the ON-to-OFF direction (13, 32, 54). When FimB and FimE are coexpressed, the ON-to-OFF bias imposed by FimE predominates. However, the orientation of the switch feeds back into the DNA inversion mechanism. Called orientational control (55), this process includes a role for phase-variable termination of the *fimE* mRNA (23). With *fimS* in the ON orientation, *fimE* transcription traverses the switch to produce a stable transcript of ~1,800 bases. When *fimS* is in the OFF orientation, *fimE* transcription terminates within the switch, resulting in an unstable truncated transcript of only ~900 bases (Fig. 1). The instability of the truncated *fimE* transcript is responsible for a strong reduction in FimE recombinase levels, allowing FimB to reset *fimS* to the ON orientation (23). Therefore, *fimS* is a bifunctional invertible genetic element: it is responsible for phase-variable transcription initiation at the *fimA* structural gene and for phase-variable transcription termination at the

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant details	Reference or source
E. coli K-12 strains		
AAEC178	Δ*fim*	23
AAEC198A	MG1655 Δ*lacZYA* Φ(*fimA-lacZYA*)	4
CJD3000	AAEC178 *rho-15*	23
MM1	VL386 *fimB*::*kan*	S. G. J. Smith
VL386	ϕ(*fimA-lacZ*)λpL(209) *fimE*::IS*l*	1
W3110	Wild type	C. Yanofsky
Plasmids		
PBAD24	Expression vector containing the P_{BAD} promoter and the *araC* gene	18
PSAJ109	*fimE* and *fimS* in pBAD24, *fimE* under P_{BAD} control	23
PSAJ112	*fimE* and *fimS* (ON orientation) in pBAD24, *fimS* locked by deletion of IRL, −10 *fimA* promoter inactivated by SDM	23
pPH100	pSAJ109 with *fimS* locked OFF, IRL sequence changed using SDM	This study
pPH101	pPH100 with 8-bp *boxA*-like feature deleted by inverse PCR	This study
pPH102	pPH101 with 22 bp of the *fimS* terminator deleted by inverse PCR	This study
pPH103	pPH102 with a further 22 bp of the *fimS* terminator deleted by inverse PCR	This study
pPH104	pPH100 with *boxA* sequence scrambled by SDM	This study
pPH105	pSAJ112 with *boxA* sequence from *fimS* ON replaced with *fimS* OFF *boxA* by SDM	This study
pPH106	pPH100 with C-rich region 1 changed by SDM	This study
pPH107	pPH106 with C-rich region 2 changed by SDM	This study

fimE regulatory gene. We investigated the structure of the *fimS* terminator to discover the basis of its phase-variable transcription termination activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains used in this study were derivatives of *E. coli* K-12 and are described in Table 1, together with details of the plasmids used in this work. Bacteria were cultured routinely at 37°C in Luria-Bertani (LB) broth or on LB agar plates. The growth medium was supplemented where required with the following antibiotics: carbenicillin (100 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), and kanamycin (50 μg ml^{-1}). Bicyclomycin was a generous gift from Fujisawa Pharmaceutical (Osaka, Japan) and was used at a final concentration of 50 μM.

DNA manipulations and plasmid construction. Standard procedures were used for DNA manipulations (49). Plasmid pSAJ109 contains the *fimE-fimS* portion of the *fim* operon cloned in the expression plasmid pBAD24 (Table 1). In this plasmid, the arabinose-inducible P_{BAD} promoter drives *fimE* transcription (23). This plasmid was modified to lock the *fim* switch permanently in the OFF orientation so that the effect of Rho on *fimE* transcription termination could be studied in a population of exclusively OFF-phase *fimS* elements. This was achieved by altering the left-hand inverted repeat (IRL) from 5′-TTGGGGCCA A-3′ to 5′-TTGGGGGGTT-3′ by site-directed mutagenesis (SDM) to produce plasmid pPH100 (Table 1).

The *fimS boxA* element (5′-CGCATCTT-3′) at the 5′ end of the *fimS rut* region (Fig. 1B) shares a high degree of sequence homology (7/8 bp match) with the *boxA* sequence at the 5′ end of the *rutA* element of the lambda tR1 terminator and with the *boxA* sequence of the Rho-dependent *tna* operon leader region terminator (6/8 bp; Fig. 1B). This element was deleted from the *fimS* locked-OFF plasmid pPH100 by inverse PCR using primers INV_FOR_BOXA and INV_REV (Table 2), generating plasmid pPH101 (Table 1). Next, 22 bases of the C-rich, G-poor *rut* region located immediately 3′ of the *boxA* deletion in pPH101 were removed (30 base pairs deleted from pPH100 in total) to create plasmid pPH102 (Table 1). This was also performed by inverse PCR, and the reaction was primed with oligonucleotides INV_FOR_B+22 and IN_REV (Table 2). A further 22 bases located immediately 3′ of the deletion in pPH102 were removed by inverse PCR (52 base pairs deleted from pPH100 in total) to produce plasmid pPH103 (Table 1). The oligonucleotide primers used were INV_FOR_B+44 and INV_REV (Table 2). The eight bases of the *boxA* element (5′-CGCATCTT-3′) of plasmid pPH100 were also mutated such that each purine was replaced by a pyrimidine and vice versa. The resulting plasmid was named pPH104 (Table 1), and the base changes were made by SDM using primers BOXA_SCR_TOP and BOXA_SCR_BTM (Table 2).

Plasmid pSAJ112 is derived from pSAJ109 and has the *fimS* element locked permanently in the ON phase (23). The *boxA* element of the *fimS rut* region (5′-CGCATCTT-3′) was inverted, bringing it into the path of the RNA polymerase, which initiated the transcription of *fimE* as it traversed the phase ON switch. This allowed this component of the *fimS* terminator to be tested in isolation for its effects on *fimE* transcription termination. The resulting pSAJ112 derivative was named pPH105 (Table 1) and was constructed by SDM using primers ON_BOXA-ENG_BTM and ON_BOXA_ENG_TOP (Table 2). A portion of the C-rich *fimS rut* region 3′ of the *boxA* component (Fig. 1B) was mutated by converting the C residues to G's by SDM. Using plasmid pPH100 as the template, four C residues were altered to G residues by using primers CMUT1_TOP and CMUT1_BTM (Table 2; C1 modification), generating plasmid pPH106 (Table 1). Plasmid pPH107 (Table 1) was derived from pPH106 such that the next four C residues were mutated to G residues by using primers CMUT2_TOP and CMUT2_BTM (Table 2; C2 modification), resulting in eight C-to-G substitutions in total. None of the genetic manipulations described in this section resulted in a detectable change in plasmid copy number.

The integrity of each new construct was confirmed by DNA sequencing. The orientation of the *fimS* switch was confirmed by a PCR-based assay in which the oligonucleotide primers OL4 and OL20 (Table 2) were used to amplify the switch DNA and its ON/OFF status was then assessed by restriction digestion analysis (54).

RNA manipulations. Cultures of strain AAEC178 harboring pBAD24 derivative plasmids pPH100 to pPH107 were grown in LB at 37°C in the absence of arabinose. At the mid-exponential phase of growth (optical density at 600 nm, ~0.5), transcription from the P_{BAD} promoter was induced by the addition of arabinose to a final concentration of 0.2%. Cells were harvested after another 60 min of growth, washed in phosphate-buffered saline, and lysed for RNA extraction (see below). In experiments where Rho was inhibited by bicyclomycin treatment, the antibiotic and arabinose inducer were added to the culture at the same time. A similar protocol was used for experiments with the temperature-sensitive *rho* mutant CJD3000 (Table 1), except that the bacteria were grown at 30°C until the mid-exponential phase of growth and were then shifted to 42°C for 60 min following the addition of arabinose. This heat shock procedure inhibits Rho factor production. Growth at 42°C does not influence *fimE* transcription termination or message stability in a wild-type strain (23). RNAs were extracted using the TRI (Sigma) procedure according to the kit maker's instructions. DNAs were removed from RNA samples with an Ambion DNA-free kit. The purified RNAs were subjected to reverse transcription-PCR (RT-PCR) using a QIAGEN OneStep RT-PCR kit in compliance with the manufacturer's directions. RNA quantity and quality were determined by measuring the absorbance of the sample (A_{260}/A_{280}) in triplicate. All RT-PCRs were performed within the linear range of a logarithmic scale, as determined by performing trial RT-PCRs with twofold serial dilutions of DNA-free RNA as the template, with a fixed number of amplification cycles. Under our experimental conditions, 200 ng of purified DNA-free RNA was used as the template in reactions that underwent 18 amplification cycles. The amount of RNA loaded and its integrity were moni-

TABLE 2. Oligonucleotides used for this study

Primer name	Sequence (5′-3′)	Purpose
BOXA_SCR_BTM	AGAAGAATGAGGCCTCGTATTCGAGCCACAGAAACG	Sequence scramble of *boxA* by SDM
BOXA_SCR_TOP	CGTTTCTGTGGCTCGAATACGAGGCCTCATTCTTCT	Sequence scramble of *boxA* by SDM
CMUT1_BTM	GGTTTTTGGAGAGAACAATCACCAAGATGCGTCGAGCC	SDM base pair changes to C-rich region
CMUT1_TOP	GGCTCGACGCATCTTGGTGATTGTTCTCTCCAAAAACC	SDM base pair changes to C-rich region
CMUT2_BTM	GCATGAGGTGGTTTTTCCACACAACAATCACCAAGATG	SDM base pair changes to C-rich region
CMUT2_TOP	CATCTTGGTGATTGTTGTGTGGAAAAACCACCTCATGC	SDM base pair changes to C-rich region
INV_FOR_B+22	CACCTCATGCAATATAAACATC	Deletion of terminator
INV_FOR_B+44	TATAAATAAAGATAACAATAGAATATT	Deletion of terminator
INV_FOR_BOXA	CCTCATTCTTCTCTCCAAAAA	Deletion of 8-bp *boxA*
INV_REV	CTAACGTTTCTGTGGCTCGA	Deletion of terminator
IRLMUT_BTM	GATATGGACAGTAACCCCCCAATTGTCTTG	SDM to lock *fimS* OFF
IRLMUT_TOP	CAAGACAATTGGGGGGTTACTGTCCATATC	SDM to lock *fimS* OFF
OL20	CCGTAACGCAGACTCATCCTC	PCR inversion assay
OL4	GACAGAACAACGATTGCCAG	PCR inversion assay
ON_BOXA_ENG_BTM	TTCTGTGGCTCGAAAGATGCGCCTCATTCTTCTCTCC	SDM insertion of *fimS* OFF *boxA* into *fimS* ON orientation
ON_BOXA_ENG_TOP	GGAGAGAAGAATGAGGCGCATCTTTCGAGCCACAGA	SDM insertion of *fimS* OFF *boxA* into *fimS* ON orientation
RT-a	GATATGGACAGTAACCCCC	RT-PCR of *fimS*
RT-b	CGAGCCACAGAAACGTTAGC	RT-PCR of *fimS*
RT-c	TGGCTTAATATTCTATTG	RT-PCR of *fimS*
RT-d	GGAAAGCATCGCGGACAAAC	RT-PCR of *fimS*
RT-e	GGGGCCATTTTGACTC	RT-PCR of *fimS*
RT-ON a	CTATGAGTCAAAATGGCCCC	RT-PCR of *fimS*
RT-ON b	GCTAACGTTTCTGTGGCTCG	RT-PCR of *fimS*
RT-ON c	CGACAAAAAGCATCTAACTG	RT-PCR of *fimS*
RT-FWD	AGATACTCGTTTAATTCAGG	RT-PCR of *fimS*

tored by agarose gel electrophoresis. In all cases, the amplimer sizes (measured electrophoretically) were as expected. RT-PCR DNA bands were quantified by densitometry using AlphaEaseRC software, version 3.2.1 (Alpha Innotech). Bands were recognized and quantified automatically from unsaturated images using the analysis tool suite. The number given for the total area of each band was converted to a percentage relative to the area of a control band (usually the corresponding band obtained from the wild type). To ensure that there were no variations in priming efficiency among the different reverse oligonucleotides, control reactions were done with the forward primer (*RT-fwd*) in combination with each of the reverse primers (*RT-a*, *RT-b*, *RT-c*, *RT-d*, and *RT-e*), using a fixed quantity of DNA as the template. The experiments were performed at least three times, and typical data from one experiment are shown.

RESULTS

Identification of a Rho-dependent terminator in *fimS*. Previous work had shown that *fimS* contains a transcription terminator that terminates *fimE* transcription when *fimS* is in the OFF, but not the ON, orientation (23). RNA polymerase reads through *fimS* in the OFF orientation in a *rho-15* mutant background, indicating that the *fimS* terminator requires the Rho protein for function. Measuring the length of the *fimE* transcript by Northern blot analysis with *fimS* in the OFF orientation allowed us to estimate that the terminator was located at approximately the midpoint of the switch (23). Precise identification of a potential Rho terminator by sequence analysis alone is difficult because of the absence of readily identifiable features such as inverted repeats. We decided to determine the likely location of the terminator using an approach based on RT-PCR. For this assay, oligonucleotides were designed to prime reverse transcription of the *fimE* transcript from different positions within OFF-phase *fimS* (Fig. 2). It was anticipated that the terminator would strongly reduce the level of transcript that would be available for use as a template for RT-PCR for positions lying 3′ of the terminator. It was also expected that the presence of the *rho-15* mutation or the presence of bicyclomycin (an inhibitor of Rho) in the culture would overcome this reduction, confirming that a Rho-dependent termination event was responsible. Due to the low level of *fimE* transcription in its native chromosomal location (23), we utilized pBAD24-derived plasmids, by which the termination of *fimE* transcripts initiating at an arabinose-inducible promoter could be monitored in the bacterial strain AAEE178 (Table 1), from whose chromosome the entire *fim* gene cluster has been deleted.

The *fim* switch is rich in regulatory features concerned with the efficient operation of the DNA inversion mechanism. These include three binding sites for the leucine-responsive regulatory protein (Lrp), which is required for normal inversion of *fimS* (12, 48). Based on our measurements of the length of the truncated *fimE* transcript that is associated with OFF-phase *fimS* and our knowledge of the *fimE* transcription initiation site, we estimated that the most likely location for the DNA sequence specifying the terminator would be at or near Lrp binding site III (23). With this in mind, a forward primer (*RT-fwd*) was designed to prime DNA synthesis from a cDNA template encompassing *fimE-fimS* that was generated from *fimE* mRNA by reverse transcription primed with one of a series of oligonucleotides that primed from different positions within the *fimS* element, on either side of the Lrp-III site (*RT-a*, *RT-b*, *RT-c*, *RT-d*, and *RT-e*) (Fig. 2).

Transcripts crossing the wild-type phase OFF switch were detected readily using primer *RT-a* or *RT-b* with the wild-type strain, whereas amplimers generated using any of the primers (*RT-c*, *RT-d*, and *RT-e*) that bound to the 3′ side of the Lrp-III site were approximately sixfold less abundant (Fig. 2A, per-

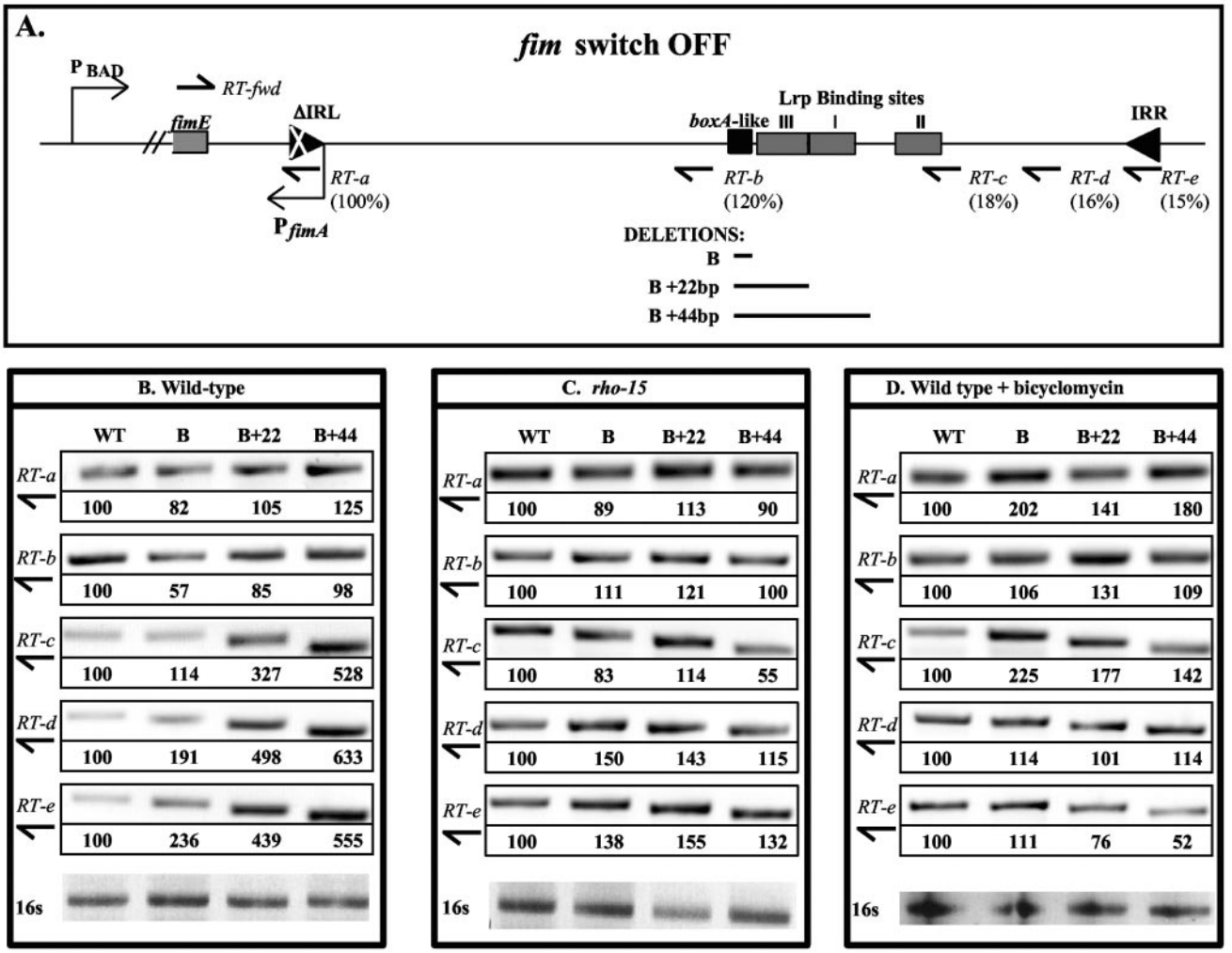

FIG. 2. Effect of a *rho* mutation and bicyclomycin on *fimE* readthrough of the locked OFF-phase *fim* switch. The RT-PCR strategy used to detect the *fimE* transcript at different positions along the switch is summarized at the top of panel A. Reverse primers *RT-a* and *RT-b* detected the transcript at locations 5′ of the putative Rho-dependent terminator, while detection at 3′ positions was performed using primers *RT-c*, *RT-d*, and *RT-e*. The level of transcript detected with each primer from constructs harboring wild-type *fimS* in untreated wild-type bacteria was normalized to the value for *RT-a* (100%) and is shown in parentheses below each primer name. Termination was also assayed in three mutant derivatives of the locked OFF switch. These had just the 8-bp *boxA* sequence deleted (B) or were deleted for the *boxA* sequence together with the following 22 bases (B + 22) or the following 44 bases (B + 44). In the lower three panels (B, C, and D), RT-PCR data are shown for the locked OFF switch (WT) and its three deletion mutant derivatives in wild-type *E. coli* strain AAEC178 (B), a *rho-15* mutant (C), and strain AAEC178 treated with the Rho-inhibiting antibiotic bicyclomycin (D). In all cases, expression of the *fimE* transcript was from the P_{BAD} promoter induced with arabinose. The name of the reverse primer used for the RT-PCR experiment is given to the left of each set of four RT-PCR products. The intensity of each product band was quantified by densitometry and expressed as a percentage of that obtained with the undeleted construct (WT; 100%). The ethidium bromide-stained 16S rRNA shown at the bottom of panels B, C, and D indicates that equivalent amounts of total RNA were used in the PCRs.

centages shown in parentheses, and B, wild-type column). These data were consistent with termination of *fimE* transcription at a location between those bound by primers *RT-b* and *RT-e*. When the experiment was repeated with a *rho-15* strain, the *fimE* transcript was readily detected using all five reverse primers (*RT-a*, *RT-b*, *RT-c*, *RT-d*, and *RT-e*) (Fig. 2C, wild-type column). Similarly, when the experiment was performed with the wild-type strain in the presence of the Rho-inhibiting antibiotic bicyclomycin, all five reverse primers readily amplified the *fimE* transcript (Fig. 2D, wild-type column). Moreover, Northern blot analysis showed that the inactivation of Rho function by bicyclomycin treatment resulted in a full-length (~1,800-nucleotide) stable *fimE* message (data not shown), a finding that was consistent with data obtained previously with the *rho-15* mutant (23). Taken together, these results support the hypothesis that a Rho-dependent terminator is located within *fimS* and suggest that the majority of termination stop points occur within the region between the sites targeted by reverse primers *RT-b* and *RT-e*. Moreover, these results validated the use of the RT-PCR-based assay as an approach suitable for identifying the *cis*-acting elements required for the *fimE* mRNA Rho-dependent termination event.

Identification of sequences required for Rho-dependent termination. An inspection of the *fimS* sequence (OFF orienta-

tion) in the region suggested by RT-PCR analysis to contain the Rho-dependent terminator led to the identification of a section capable of specifying a *rut* region. This 52-bp portion of the *fimE* transcript was abnormally rich in pyrimidine residues, with 19 C and 15 T residues out of 52 total (Fig. 1B). The same sequence contained only two G residues. The presence of such a high concentration of C relative to G residues is a feature of several well-characterized *rut* sites, as Rho interacts preferentially with C-rich, G-poor RNA (2, 14, 20, 33, 39, 46, 60, 62). Moreover, while Rho-dependent terminators may have poor sequence homology with one another, it was evident that this region of *fimS* aligned with other *rut* elements, in particular with the *boxA* and *rutA* regions of lambda tR1 (13/16 bp identity; Fig. 1B) (16, 17), the *boxA* sequence of the *rrnG* operon (6/8 bp identity) (51), and the *boxA* element from the leader region of the *tna* operon (6/8 bp identity; Fig. 1B) (26).

To assess the importance of the *fimS rut* region, derivatives of the switch were constructed from which appropriate *rut* DNA elements were deleted. The deletions removed just the 8 bases of the *boxA* element (5′-CGCATCTT-3′) (removing 3 C nucleotides), *boxA* plus 22 bases of the C-rich region (removing 12 C residues in total), or *boxA* plus 44 bases of the C-rich region (removing 19 C residues in total). The ability of the mutant switches to terminate *fimE* transcription was monitored in wild-type cells, *rho-15* mutant cells, and wild-type cells treated with the Rho-inhibiting antibiotic bicyclomycin. The results showed that the 8-bp *boxA* deletion alone had a subtle but detectable effect on the wild type. Up to twofold more *fimE* transcript was detectable using reverse primer *RT-c*, *RT-d*, or *RT-e*, which primes cDNA production from sites that are distal to the putative terminator stop points, than with primer *RT-a* or *RT-b*, which primes from positions proximal to the terminator (Fig. 2B). However, the removal of 22 or 44 bases of the C-rich sequence in addition to the *boxA* element resulted in a progressive increase in detection of the *fimE* transcript by the reverse primers *RT-c*, *RT-d*, and *RT-e*. In the case of the 44-bp deletion (52 bp deleted in total, including 19 C residues), increases of up to sixfold were detected with *RT-e* compared with the level detected with *RT-a*. This was consistent with a significant weakening of the effect of the Rho-dependent terminator, presumably due to the loss of the *rut* site. No increase in *fimE* transcript level was detected using the *RT-c*, *RT-d*, or *RT-e* primer with any of the constructs when mRNA was assayed in the *rho-15* mutant or the wild-type strain treated with bicyclomycin (Fig. 2C and D). This was consistent with the Rho protein requiring the *fimS rut* site for function. These data showed that the Rho-dependent terminator within *fimS* is located in the region of Lrp site III and consists of the *boxA*-like element and the C-rich, G-poor sequences located immediately 3′ of it (Fig. 1B).

The *fimS boxA* element alone is not essential for termination. The deletion analysis data suggested that removal of the *boxA* element alone resulted in only a modest effect on *fimE* transcription termination. In order to investigate this further, we altered the residues of *fimS boxA* (5′-CGCATCTT-3′) by site-directed mutagenesis such that each purine was replaced by a pyrimidine and vice versa (Fig. 3). This removed any similarity to the *fimS boxA* element (or the λ tR1 or *tna boxA* elements) at the level of the nucleotide sequence without changing the length of the *fimE* transcript. In this case, the altered *fimS* gene still terminated *fimE* transcription, as measured by the RT-PCR assay (Fig. 3).

We next took the native *fimS boxA* element and inverted it in the switch. No other sequence was altered. This modification placed the *fimS* OFF *boxA* element within the *fimE* transcript when the switch was in the ON orientation (Fig. 4). RT-PCR analysis of *fimE* transcription across the ON-phase switch showed that the presence of the *boxA* element had no negative effect on readthrough (Fig. 4). These data confirmed that the presence in the *fimE* transcript of this sequence motif (in either the ON or OFF orientation) did not in itself lead to a strong effect on termination, consistent with the deletion analysis presented in Fig. 2.

The C-rich sequence motif is essential for Rho-dependent transcription termination. Our deletion analysis indicated that within the *fimS rut* region, the C-rich, G-poor sequences located 3′ of the *boxA* element were important for Rho-mediated transcription termination (Fig. 2). We decided to characterize this region further by making a series of targeted mutations changing C residues to G residues without altering the length of the transcript. The nucleotide sequence of the 5′ portion of the putative *rut* site in *fimS* is given in Fig. 1B, where the C residues targeted for substitution are shown in bold and are underlined. The four C residues located immediately 3′ of the *boxA* element were altered to G by SDM (construct C1; Fig. 5A). The next four C residues were also altered to G (construct C2; Fig. 5A). The latter alteration reduced the total number of C residues in the 44 bases located 3′ of the *boxA* element from 16 to 8. The effect of the base substitutions on Rho-dependent termination was measured by the RT-PCR assay. The results showed that the *fimE* transcript now read through the region in the OFF-phase *fim* switch beyond the point where the majority of the transcript had previously terminated. Moreover, the efficiency of readthrough correlated with the number of C-to-G substitutions (Fig. 5B, panel *RT-e*). In a control experiment where cultures harboring the wild-type terminator or its C1 or C2 derivative were treated with bicyclomycin, readthrough measured by all primer sets occurred to similar levels (Fig. 5C). This confirmed that the *rut* sequence rich in C residues was needed for efficient Rho-dependent termination, a finding that was in keeping with descriptions of other Rho-dependent terminators (2, 14, 17, 20, 26, 33, 39, 46, 60, 62).

The terminator is effective in its native chromosomal location. The experiments described in the preceding sections were performed using recombinant plasmids in which *fimE* was transcribed from the arabinose-inducible P_{BAD} promoter. To assess Rho-dependent *fimE* transcription termination in a more physiologically relevant setting, it was examined in the chromosomally located *fim* gene cluster in three bacterial strains, namely, W3110, AAEC198A, and MM1 (Table 1). Each strain had a wild-type chromosomal copy of the *fimE* gene, from which transcription was expressed from the native promoter. The presence of the FimE recombinase kept the *fim* switch in the OFF phase in these bacteria (data not shown). An RT-PCR assay was designed in which an oligonucleotide primer (*RT-e*) was used to report on *fimE* readthrough of the *fimS*-located Rho-dependent terminator (Fig. 6). Low levels of *fimE* transcript were detected downstream of the terminator only after more than 25 PCR amplification cycles for all three

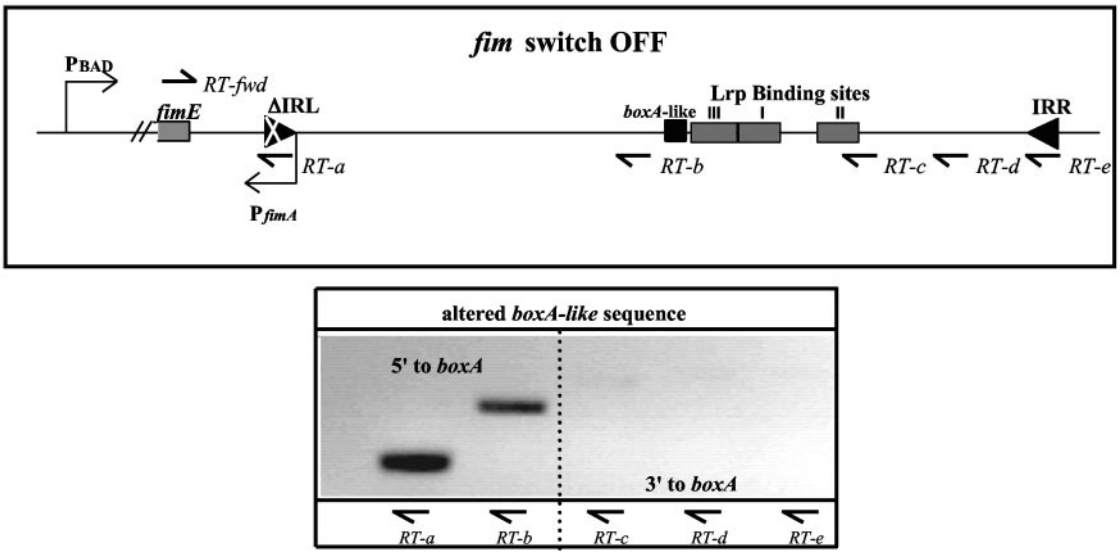

FIG. 3. Altering the 8-bp *boxA* component of the *fimS rut* sequence does not affect transcription termination. The RT-PCR strategy used to detect the *fimE* transcript at different positions along the locked OFF switch is summarized at the top of the figure. Reverse primers *RT-a* and *RT-b* detected the transcript at locations 5′ of the putative Rho-dependent terminator, while detection at 3′ positions was performed using primers *RT-c*, *RT-d*, and *RT-e*. RT-PCR products are shown at the bottom of the figure, labeled with the reverse primers used to detect them. A reduction in transcript levels was recorded at all three positions 3′ of the putative terminator with the altered nucleotide sequence, a pattern that was strongly reminiscent of that seen for the *fim* switch with an unmutated terminator (Fig. 2A).

strains, consistent with the presence of a low-abundance mRNA that was extended to this point (23). However, treatment with the Rho-inactivating antibiotic bicyclomycin allowed transcription readthrough to be detected readily at only 20 cycles using *RT-e* (Fig. 6). These results demonstrated that the Rho-dependent terminator within OFF-phase *fimS* functions when the *fim* operon is in its native location in the chromosome.

DISCUSSION

The 295 bp of the *fimS* element that lie between the IRL and IRR inverted repeats are 70% A+T, which is abnormally high for *E. coli* (3). Moreover, the ON and OFF orientations of the switch potentially attach sequences with different amounts of C to the *fimE* transcript. In the ON orientation, the transcript

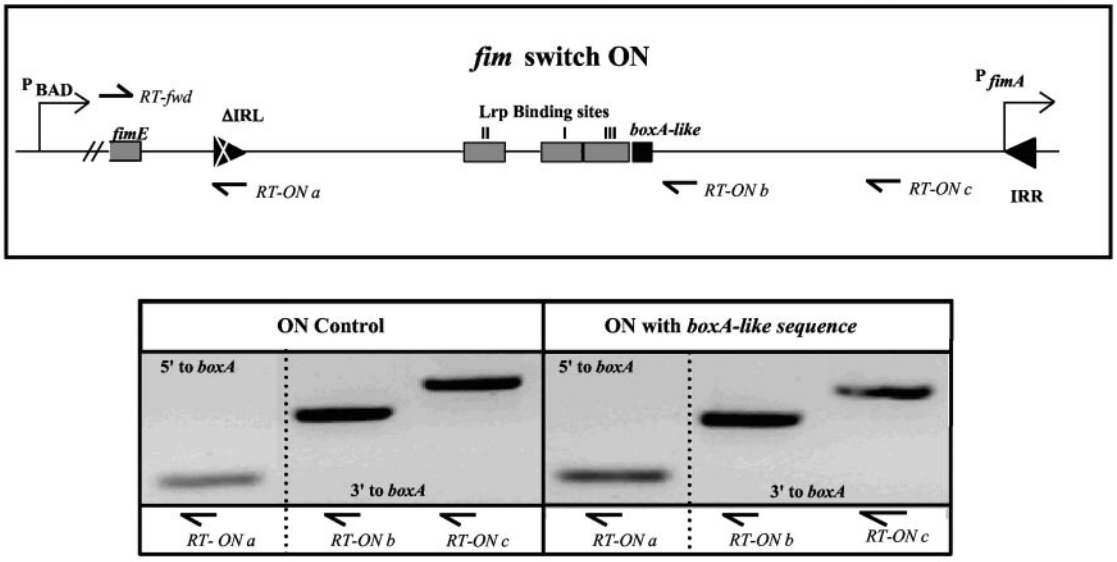

FIG. 4. The 8-bp *boxA* component of the *fimS rut* sequence cannot terminate transcription in the ON phase. The RT-PCR strategy used to detect the *fimE* transcript at different positions along the locked ON switch is summarized at the top of the figure. Reverse primer *RT-ON a* detected the transcript at a location 5′ of the putative Rho-dependent terminator, while detection at 3′ positions was performed using primers *RT-ON b* and *RT-ON c*. RT-PCR products generated from transcripts specified by the phase ON switch without or with the 8-bp *boxA* element are shown at the bottom of the figure, labeled with the reverse primers used to detect them.

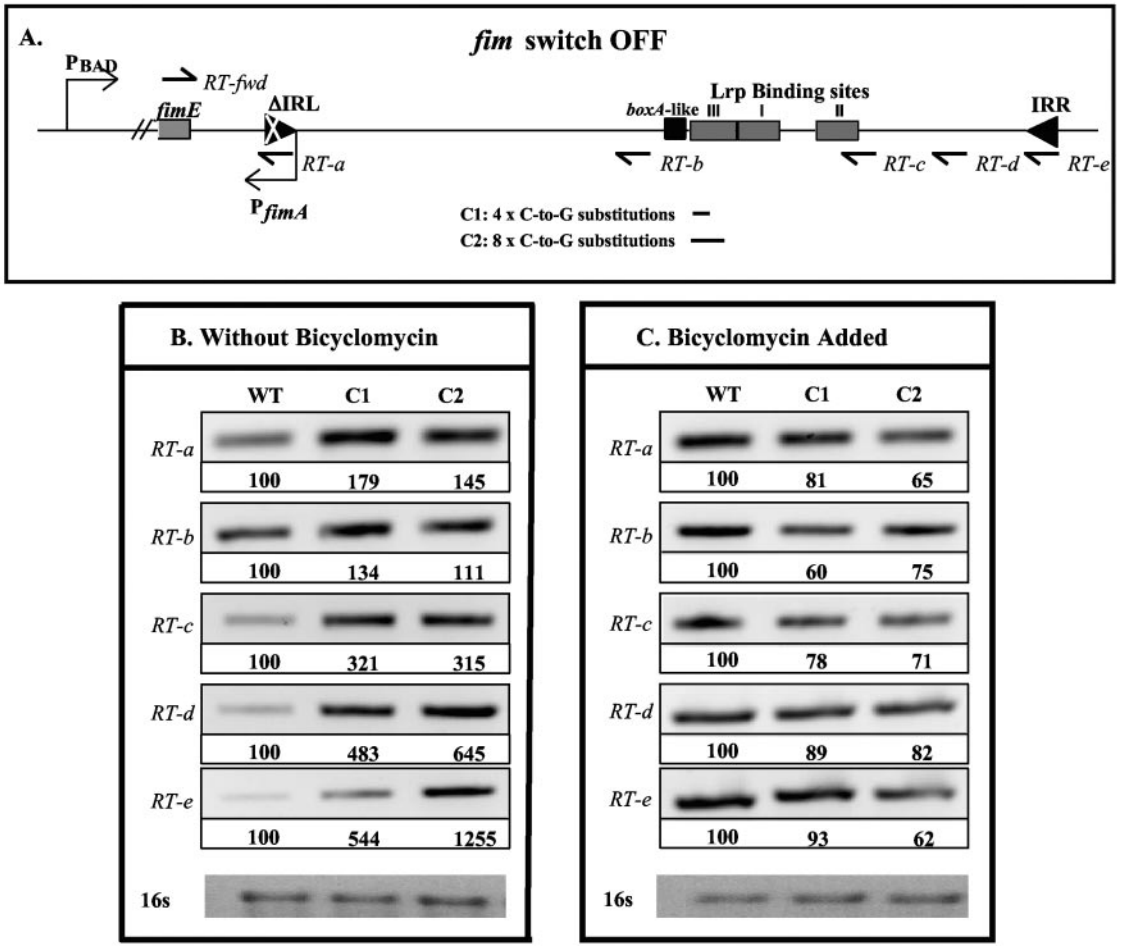

FIG. 5. Converting C residues to G abolishes terminator activity in *fimS*. RT-PCR analysis was used to measure *fimE* transcript levels at positions 5′ and 3′ of the putative Rho-dependent terminator in *fimS* in constructs with a wild-type terminator sequence (WT) or with four C-to-G substitutions (C1) or eight C-to-G substitutions (C2) in the terminator. The RT-PCR strategy used to detect the *fimE* transcript at different positions along the locked OFF switch is summarized in panel A. Reverse primers *RT-a* and *RT-b* detected the transcript at locations 5′ of the putative Rho-dependent terminator, while detection at 3′ positions was performed using primers *RT-c*, *RT-d*, and *RT-e*. The experiment was performed in the absence (B) or presence (C) of the Rho-inhibiting antibiotic bicyclomycin. Band intensities were measured by densitometry and expressed as percentages of the wild-type value (100%) for each reverse primer reaction. The ethidium bromide-stained 16S rRNA shown at the bottom of panels B and C indicates that equivalent amounts of total RNA were used in the PCRs.

specified by the switch region is 10% C, whereas in the OFF orientation it is 20% C. In the OFF orientation, the C residues are not randomly distributed but cluster in a region extending approximately from the center to the IRR portion of the switch (Fig. 1). In the present report, we extend our analysis of the *fimE* mRNA Rho-dependent termination event that occurs specifically when the *fimS* switch is in the OFF orientation by identifying and dissecting the *fimS* Rho utilization (*rut*) element. Consistent with other *rut* sites, such as those of λ tR1 and the *tna* region, the *fimS rut* site consists of a *boxA* element followed by a sequence rich in C residues (Fig. 1B). Using an RT-PCR-based approach, we showed that when the *fimS* switch was in the OFF orientation, transcription from the upstream *fimE* mRNA terminated within the switch (Fig. 2). This termination event required the Rho factor, as the *fimE* transcript extended through the switch in a *rho-15* genetic background or when Rho was inhibited by bicyclomycin treatment (Fig. 2). In order to understand the contribution of the different components of the *fimS rut* site, we made a series of progressive deletions of the *rut* site and tested the effects of these on Rho-dependent *fimE* termination. Our data show that removing just the 8-bp *boxA* feature has only a modest effect on *fimE* termination (Fig. 2). Moreover, scrambling its sequence in the OFF orientation did not decrease Rho-dependent termination events (Fig. 3), and presenting it to RNA polymerase traversing an ON-phase switch, and thereby having it incorporated into the *fimE* ON-phase mRNA transcript, did not induce Rho termination (Fig. 4). However, when *boxA* was deleted in combination with the C-rich, G-poor region of the *fimS rut* site (progressively removing a total of 8 bp [3 C residues], 30 bp [12 C residues], or 52 bp [19 C residues]), up to sixfold more amplimer was returned using primers designed to amplify cDNA from mRNA that extended distal to the terminator (Fig. 2). Additionally, mutating the 52-bp *fimS rut* site (contains 19 C's) such that the first four C residues or the first eight C residues were replaced by G residues caused a

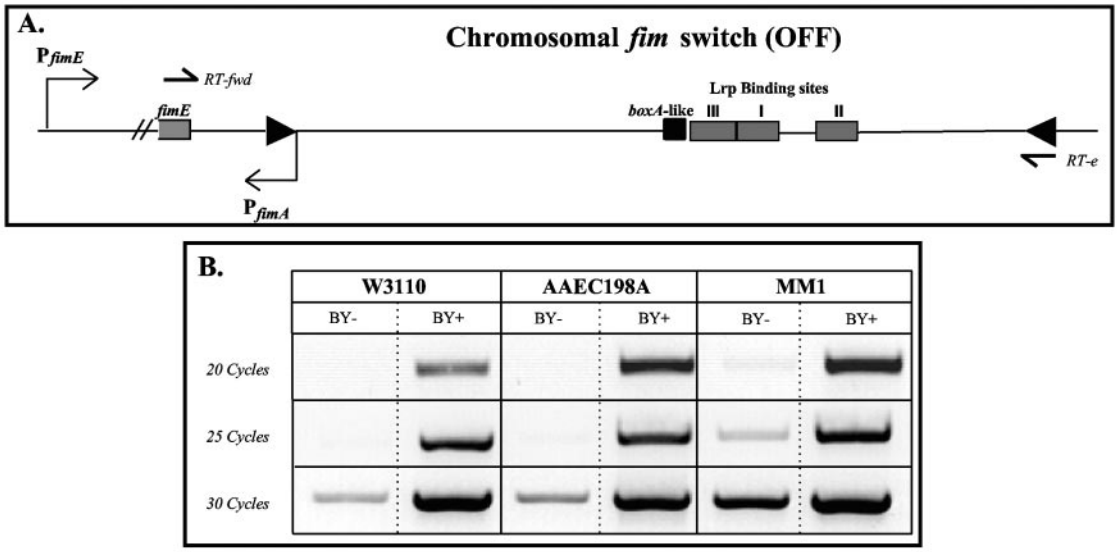

FIG. 6. The *fimE* transcript is subject to Rho-dependent termination within *fimS* on the *E. coli* chromosome. Transcripts expressed from the bacterial chromosome were detected by RT-PCR using reverse primer *RT-e*, specific for transcripts that traverse a position 3′ of the terminator (summarized in panel A), after 20, 25, or 30 PCR cycles in the absence (BY−) or presence (BY+) of the Rho-inhibiting antibiotic bicyclomycin (B). The experiment was performed with three distinct bacterial strains, all of which have *fimS* predominantly in the OFF orientation and one of which (MM1) lacks an active *fimB* gene with which to invert the switch back to the ON phase.

significant (up to 12-fold) decrease in *fimE* mRNA termination within the switch (Fig. 5), confirming that this component of *fimS rut* is crucially important for Rho action. Finally, we showed that the *fimE* gene is subject to Rho-dependent termination within *fimS* in the context of the *E. coli* chromosome when expressed from its native promoter (Fig. 6) as well as in recombinant plasmids when expressed from an ectopic promoter (Fig. 2). Together, these data on the effect of Rho on the *fimS rut* site (23; this work) provide another example of the Rho protein's preference for interacting with RNAs that are low in secondary structure and rich in C residues (2, 14, 16, 20, 33, 39, 62).

It can be argued that a Rho-dependent terminator is more suitable than an intrinsic terminator for use in controlling transcript traffic through the invertible *fim* switch. This is because the "visibility" of the factor-dependent terminator is altered more radically by DNA inversion. Essentially, the *fimS* terminator becomes invisible to Rho when the switch is in the ON orientation, allowing *fimE* transcription to traverse the switch, with a concomitant and significant positive effect on message stability (23). On the other hand, an intrinsic terminator with its requirement for a stable stem-loop structure that is specified by inverted repeats in the DNA template would display this palindromic feature regardless of the DNA template orientation.

It is interesting that in the extant *fim* switch, there is considerable overlap between the C-rich region of the Rho-dependent terminator and Lrp binding site III (Fig. 1). This may represent an influence for the preservation of both, since any sequence divergence would be expected to alter Lrp binding, and therefore the switching efficiency, and our data show that deletions or C-to-G substitutions have a negative impact on *fimE* termination (Fig. 5), something that is known to feed back into the switch inversion mechanism (23). Bacterial mutants unable to invert the switch at an optimal rate may be at a competitive disadvantage leading to their disappearance from the population. Similar arguments can be advanced concerning conservative influences associated with overlapping regulatory features in other invertible DNA elements. For example, the binding sites for the factor for inversion stimulation in the invertible switch that is responsible for phase-variable expression of flagellar genes in *Salmonella* are located within the coding sequence of the gene that specifies the Hin site-specific recombinase (21). Consequently, bacteria that acquire mutations in the factor for inversion stimulation binding site may also be expected to lose the ability to express the wild-type recombinase, and the associated loss of competitiveness may cause them to be eliminated from the population.

The possibility that Lrp protein binding to Lrp binding site III influences *fimE* transcription termination remains an open question. However, this seems unlikely since under the growth conditions used in our experiments, the Lrp protein is not expected to occupy Lrp site III (48). Lrp may influence the system at other levels. For example, inactivation of the *lrp* gene causes a 50% reduction in *fimE* transcription from its weak native promoter (4). However, in our experiments *fimE* was expressed from the arabinose-inducible and Lrp-independent P_{BAD} promoter. While it is possible that Rho may directly or indirectly influence Lrp expression at some level, the inhibition of Rho function by bicyclomycin treatment does not alter *lrp* gene expression, at least at the level of transcription (our unpublished data).

Differential transcription termination has been described for other systems. The phase-variable expression of capsular polysaccharide in *Neisseria meningitidis* is modulated by premature transcription termination at a cryptic Rho-dependent site in the *siaD* gene coding for polysialyltransferase (29), and the *E. coli clpP-clpX* operon expresses transcripts of different lengths

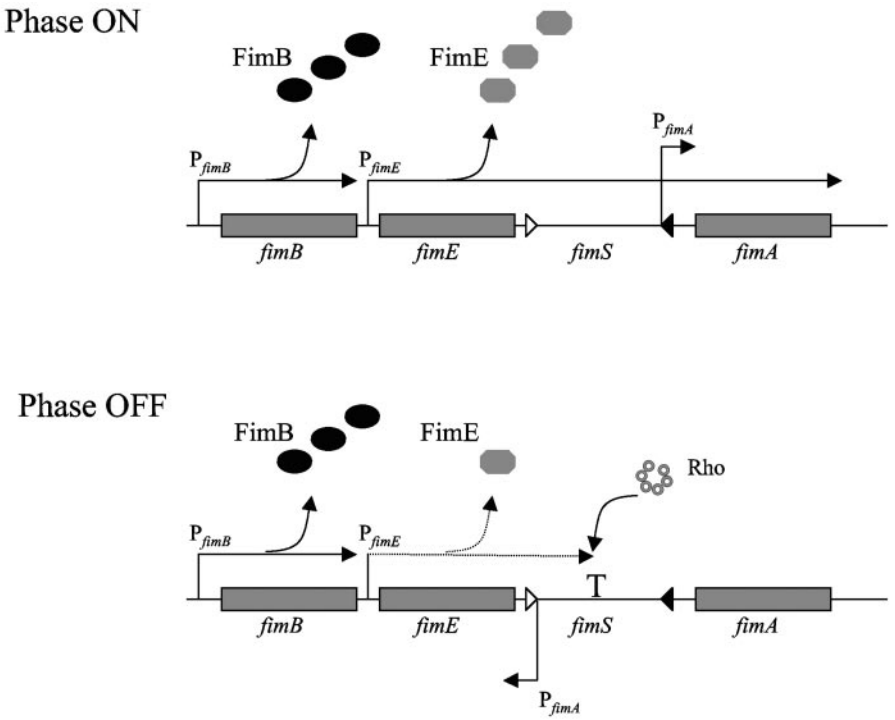

FIG. 7. The orientation of the *fim* switch controls the expression of the FimE recombinase. The *fimB* and *fimE* genes are transcribed from independent promoters. Their transcripts are translated into recombinases that invert the switch without bias (FimB) or with a strong ON-to-OFF bias. The phase ON switch does not terminate *fimE* transcription, and the extended message is stable (23). Bacterial populations coexpressing FimB and FimE have the switch predominantly in an OFF orientation (13). In this case, Rho-dependent termination at the terminator (T) produces a truncated *fimE* message that is unstable (23). This instability (represented by a dotted arrow) results in a strong reduction in FimE protein levels, giving FimB an opportunity to invert the switch back to the ON phase.

when bacteria undergo carbon starvation (30). However, the *E. coli* K-12 *fimS* Rho-dependent terminator differs from other factor-dependent transcription terminators in being specified by an invertible DNA element such that it is alternately attached to and detached from the transcript that it regulates. This provides the *fimE* gene with a valuable element of posttranscriptional control through differential mRNA stability that influences the level of the FimE recombinase in the cell, and thus the orientation of the *fim* switch (23). Thus, *fimS* is a sophisticated genetic element that integrates several distinct biological functions. These include site-specific recombination, in which the *trans*-acting FimB and FimE tyrosine recombinases act on the *cis*-acting inverted repeats to set the orientation of *fimS* in the chromosome. This in turn determines whether or not the P_{fimA} promoter is attached to or detached from the *fimA* gene coding for the major fimbrial subunit protein. Switch orientation also determines whether or not *fimE* transcription is abbreviated, leading to a decline in FimE protein levels (23), or extended such that the level of this OFF-biased recombinase is enhanced (Fig. 7). In this way, the orientation of the switch is self-regulating, and the Rho-dependent *fimE* terminator plays a pivotal role in this homeostatic process.

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