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A large insertion domain in the Rho factor from a low G+C, Gram-negative bacterium is critical for RNA binding and transcription termination activity

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Running title: Termination factor Rho from *Bacteroides fragilis*

ABSTRACT

Rho-dependent termination of transcription (RDTT) is a critical regulatory mechanism specific to bacteria. In a subset of species including most Actinobacteria and Bacteroidetes, the Rho factor contains a large, poorly conserved N-terminal insertion domain (NID) of cryptic function. To date, only two NID-bearing Rho factors from high G+C Actinobacteria have been thoroughly characterized. Both can trigger RDTT at promoter-proximal sites or with structurally constrained transcripts that are unsuitable for the archetypal, NID-less Rho factor of *Escherichia coli* (E_c Rho). Here, we provide the first biochemical characterization of a NID-bearing Rho factor from a low G+C bacterium. We show that *Bacteroides fragilis* Rho (B_f Rho) is a *bona fide* RNA-dependent NTPase motor able to unwind long RNA:DNA duplexes and to disrupt transcription complexes. The large NID (~40% of total mass) strongly increases B_f Rho affinity for RNA, is strictly required for RDTT, but does not promote RDTT at promoter-proximal sites or with a structurally constrained transcript. Furthermore, the NID does not preclude modulation of RDTT by transcription factors NusA and NusG or by the Rho inhibitor bicyclomycin. Although the NID contains a prion-like Q/N-rich motif, it does not spontaneously trigger formation of β -amyloids. Thus, despite its unusually large RNA binding domain, B_f Rho behaves more like the NID-less E_c Rho than NID-bearing counterparts from high G+C Actinobacteria. Our data highlight the evolutionary plasticity of Rho's N-terminal region and illustrate how RDTT is adapted to distinct genomic contents.

Keywords: RNA synthesis; transcription termination; Rho factor; molecular motor; RNA helicase; RNA folding; Bacteroidetes

Abbreviations: NID (N-terminal insertion domain); PBS (Primary binding site); RDTT (Rho-dependent termination of transcription); RNAP (RNA polymerase); Rut (Rho utilization); SBS (Secondary binding site); TEC (transcription elongation complex);

INTRODUCTION

Bacteria are very diverse microorganisms able to grow in a wide variety of habitats and conditions. Both species-specific traits and general regulatory mechanisms help tune bacterial gene expression programs to specific demands and contexts. A relevant regulatory mechanism is Rho-dependent termination of transcription (RDTT), which monitors the coupling of transcription and translation during bacterial gene expression¹⁻⁵. Uncoupling of the transcription and translation machineries, either because of a ‘program bug’ (e.g. nonsense mutation) or a contextual event (e.g. sRNA- or riboswitch-induced translation impediment), provides Rho access to intragenic RDTT signals in the nascent transcript (**Figure 1A**). Rho can then use its ATPase-driven activity to dissociate RNA polymerase (RNAP), thereby ending transcription of the corresponding gene or operon^{2,3,6}. Beside surveillance of transcription-translation coupling, a major function of RDTT is the silencing of pervasive transcription, mostly in regions antisense to genes where transcription elongation complexes (TECs) are not protected from Rho by translating ribosomes⁷⁻¹⁰. Yet, a ribosome-free TEC is not the sole requirement for efficient RDTT as diffuse RNA structure/sequence determinants (e.g. C>G skewed sequence in *Escherichia coli*) or action of cofactors also play an important role. Remarkably, conditional RDTT signals controlled by riboswitches, RNA-binding proteins, or sRNAs have been found in 5'-untranslated and intergenic regions where they contribute to a variety of regulatory mechanisms^{4,11-15}. The broad regulatory function of Rho is also illustrated by its implication in the maintenance of bacterial genome integrity through the disruption of deleterious transcriptional R-loops and stalled TECs^{16,17}.

RDTT has long been regarded as essential in Gram-negative bacteria but dispensable in Gram-positive species (reviewed in ¹⁸). As a result, most studies aimed at elucidating the mechanism of RDTT have focused on the *Escherichia coli* paradigm. These studies support that *E. coli*'s Rho (_{Ec}Rho) preferentially binds RNA at C>G sequence-skewed and poorly

structured *Rut* (*Rho utilization*) sites. Once bound to a *Rut* site, the E_c Rho hexamer switches to a closed ring conformation entrapping RNA within its central channel and becomes competent for ATP-dependent RNA translocation. The classical RDTT model posits that such directional ATPase-fueled movement from a *Rut* ‘anchoring’ site towards the transcript 3’-end allows Rho to catch up with RNAP and then disrupt the TEC (see: ¹⁹⁻²² and references within). However, a series of cryoEM structures from two recent studies^{23, 24} supports an alternative ‘allosteric’ model whereby E_c Rho first binds ribosome-free RNAP²⁵. The Rho-RNAP interaction involves the N-terminal face of the E_c Rho hexamer (**Figure 1A**) and is stabilized by cofactors NusA and NusG that both bind E_c Rho and the RNAP^{23, 24}. An additional role of NusA may be to help guide the RNA chain from the RNA exit channel of RNAP towards the central channel of the E_c Rho hexamer²⁴, which remains open in the cryoEM structures. Recognition of a *Rut* site upon transcript scanning by E_c Rho’s primary binding site (PBS) would sequentially trigger allosteric inactivation of RNAP, closure of the E_c Rho ring and activation of its ATPase-fueled motor capacity, and finally TEC dissociation by the E_c Rho motor^{23, 24}. In some instances, RDTT can also initiate at suboptimal *Rut* sites with the help of the transcription factor NusG^{7, 26-28}. This could stem from the ability of *E. coli*’s NusG (E_c NusG) to stimulate closure of the E_c Rho ring around suboptimal RNA substrates^{29, 30}. The direct action of E_c NusG on RNAP, limiting termination-resistant backtracked states, may also be particularly relevant at suboptimal RDTT terminators (²⁴ and references within). Overall, these rather lax RNA recognition rules combined with high intracellular concentrations of E_c Rho and E_c NusA/G factors sustain RDTT at hundreds of sites throughout the *E. coli* genome^{7, 10, 31-33}.

Renewed interest in RDTT beyond the *E. coli* paradigm has spurred from the recent findings that *rho* inactivation also triggers genome-wide pervasive (mostly antisense) transcription in Gram-positive species such as *Bacillus subtilis*³⁴, *Staphylococcus aureus*⁸, or *Mycobacterium tuberculosis*⁹. Contrary to early expectations, depletion of Rho in these species

can have significant consequences, from changes in cell differentiation or virulence programs in Firmicutes³⁴⁻³⁶ to rapid death for *M. tuberculosis*⁹. The recent discovery of conditional regulatory mechanisms involving RDTT in the industrially important bacterium *Corynebacterium glutamicum*³⁷ also pinpoints to RDTT regulation being more widespread and decisive than previously envisioned. Yet, significant differences in genome compositions, metabolisms, or ecosystems are likely to affect how RDTT is carried out in phylogenetically divergent species. One central question is how RDTT is tuned to the specific features of individual transcriptomes, which can significantly vary in sequence skewness and degrees of secondary structure. Another key issue is whether the role of RDTT cofactors such as NusA/G is conserved throughout the bacterial kingdom.

Although the sequence of Rho is relatively well conserved among bacteria, a large amino acid (aa) insertion is often found in the N-terminal domain (NTD) of the protein (**Figure 1B,C** and **Figure S1**)³⁸. This occurrence is particularly frequent in Actinobacteria and Bacteroidetes, implying that the NTD insertion domains (NIDs) have important roles in these phyla³⁸. The NIDs are poorly conserved but are often R/K-rich in Actinobacteria and Q/N-rich in Bacteroidetes³⁸. The role of the NID has been investigated for two GC-rich Actinobacteria (*Micrococcus luteus* and *M. tuberculosis*) where it apparently helps the Rho factor to deal with structured transcripts³⁹⁻⁴². The NID also promotes promoter-proximal transcription termination *in vitro*³⁹⁻⁴¹, much like *Ec*NusG does with *Ec*Rho^{43,44}, suggesting that the NID may substitute, at least in part, for NusG function in Actinobacteria^{40,45}. By contrast, the role(s) of the NIDs in Rho factors from low G+C bacteria, in particular from Bacteroidetes, has not been determined yet.

In this work, we have prepared and characterized the Rho factor from the opportunistic pathogen *Bacteroides fragilis* (*Bf*Rho) to evaluate the role of the NID from a low G+C species (43.1% GC-richness vs. 50.8% for *E. coli* and 65.6% for *M. tuberculosis*). Growth sensitivity

to the Rho-specific inhibitor bicyclomycin (BCM)⁴⁶ and transposon inactivation⁴⁷ experiments point to an important role of Rho in *B. fragilis*. Consistent with these observations, we find that $_{Bf}Rho$ is a functional NTP-dependent motor that is able to disrupt RNA-DNA duplexes and transcription elongation complexes *in vitro*. Much like *M. tuberculosis* Rho ($_{Mtb}Rho$), $_{Bf}Rho$ needs its large NID to bind RNA and work effectively. Contrary to $_{Mtb}Rho$ however, $_{Bf}Rho$ cannot deal effectively with structured transcripts or trigger promoter-proximal transcription termination. In these respects, $_{Bf}Rho$ performance is more akin to (but weaker than) that of $_{Ec}Rho$. Notably, $_{Bf}Rho$ activity can be regulated by NusA and NusG factors and inhibited by BCM. Altogether, our data illustrate how Rho enzymatic features are tuned to the composition of the bacterium transcriptome. Furthermore, they demonstrate that NIDs can be necessary in low G+C species and, thus, can encompass functions beyond helping Rho dealing with highly structured transcripts.

RESULTS

Analysis of the $_{Bf}Rho$ sequence and preparation of protein variants to probe $_{Bf}Rho$ features

The archetypal $_{Ec}Rho$ hexamer is composed of six identical protomers, each made of two distinct domains^{1,2}. The NTD carries the PBS motifs involved in Rho recognition of YC-rich (Y being a C or U residue) transcript *Rut* sites (**Figure 1A,C** and **Figure S1**)⁴⁸. The NTD also carries the residues contacting RNAP in the recent cryoEM structures of the $_{Ec}Rho:RNAP$ complex^{23, 24}. The C-terminal domain (CTD) carries the motifs necessary for intersubunit cohesion and for ATP-dependent RNA translocation. The CTD notably includes the Walker A and B motifs forming ATPase pockets at subunit interfaces and the "catalytic Glu", 'Arg valve' and 'Arg finger' residues required for catalysis of ATP hydrolysis^{49, 50}. The CTD also contains the secondary binding site (SBS) Q-loop and R-loop motifs that allosterically move RNA

through the hexamer central channel as a function of the chemical state of the ATPase pockets (**Figure 1A** and **Figure S1**)⁴⁹.

As already mentioned, Rho factors from *Bacteroidetes* frequently contain large NIDs³⁸. This is the case of the 688 aa-long B_f Rho factor, which carries an even longer NID than does M_{tb} Rho (270 aa and ~30kDa versus 144 aa and ~15kDa) (**Figure 1B, C**). The sequence identity/similarity between both NIDs (B_f Rho-NID and M_{tb} Rho-NID) is actually very low (**Figure S1**). Their predicted isoelectric points are also significantly different (8.98 for B_f Rho-NID vs. 5.20 for M_{tb} Rho-NID) despite similar contents in basic residues (18.1 vs 17.3%). The higher pI of B_f Rho-NID suggests that the domain might be an even better RNA interactor than M_{tb} Rho-NID⁴². While both NIDs are predicted to be intrinsically disordered by XtalPred⁵¹, an intriguing additional feature of B_f Rho-NID is the presence of a 33 aa-long, asparagine/glutamine-rich sequence that displays characteristics of sequences able to form β -amyloids (**Figure S2**). Such prion-like Q/N-rich NID motifs appear to be frequent in *Bacteroidetes* Rho factors, except in the *Prevotella* genus (**Figure S3**). They are also found in other phyla, as in the Rho factor from Gram-positive *Clostridium botulinum*, which is indeed able to form prion-like aggregates compromising its RDTT activity^{52, 53}.

The remaining B_f Rho sequence is well conserved, for instance displaying 77.1% similarity and 56.7% identity with the NID-less E_c Rho sequence. All the residues and motifs critical for the RNA binding and catalytic activities of E_c Rho are highly conserved in B_f Rho (**Figure S1**). Notably and in contrast to M_{tb} Rho⁴¹, B_f Rho does not contain indels in the CSD-like RBD region that carries the PBS residues anchoring E_c Rho to *Rut* sites^{48, 54}. The Q- and R-loops that translocate RNA in the hexamer central channel^{49, 55, 56} are also highly conserved between B_f Rho and E_c Rho (**Figure S1**). Three aa substitutions are noticeable in the ATPase Walker motifs of B_f Rho (P→Q and A→T in motif A and L→F in motif B; **Figure S1**) but correspond to side-chains that do not contact ATP directly in E_c Rho^{49, 50}. Finally, all the aa targeted by the

natural inhibitor BCM near the ATPase pocket^{57, 58} are also conserved in B_f Rho (**Figure S1**). Thus, the B_f Rho factor does not display strikingly unusual sequence features, except for its very large and basic NID.

To probe the activity of the B_f Rho factor and the properties of its NID, we have prepared wild-type (WT) B_f Rho and derivatives lacking the full NID (ΔB_f Rho) or only the putative prion-like Q/N-rich motif (aa 264-296; B_f Rho $_{\beta\text{del}}$). For sake of comparison, we have also prepared WT M_{tb} Rho and its NID-less variant (ΔM_{tb} Rho)⁴¹. All proteins were purified to >90% homogeneity and yielded high-resolution mass spectrometry (HR-MS) peaks consistent with their calculated masses after proteolytic cleavage of the starting methionine (data not shown)⁵⁹. Diffusion light scattering and gel filtration experiments support that all proteins form monodisperse species, most likely hexamers (**Figure S4**)⁵⁹. The B_f Rho and B_f Rho $_{\beta\text{del}}$ proteins display anomalously low electrophoretic mobilities on SDS-PAGE gels when compared to protein standards or to ΔB_f Rho (**Figure S4**). This band ‘shift’ effect cannot be assigned easily to a specific feature of the B_f Rho-NID since many factors, including single amino acid substitutions, can strongly affect SDS-PAGE migration⁶⁰.

The B_f Rho factor is a RNA-dependent NTP hydrolase

To assess the substrate and cofactor preferences of B_f Rho, we determined its steady-state NTP hydrolysis activity in the presence of various rNTPs and polyribonucleotide homopolymers. We observed that the ATPase activity of B_f Rho is strongly stimulated by the presence of poly[rC], reaching a plateau value of ~60 ATP/hexamer/s at saturating poly[rC] concentrations (**Figure 2A**). As for E_c Rho⁶¹, the ATPase rate displays a strong sigmoidal dependence on poly[rC] concentration (Hill coefficient ~5; $K_{d,\text{app}}$ ~1.5 μM), suggesting cooperativity among RNA binding sites (**Figure 2A**). By contrast, the ATPase activity of M_{tb} Rho has an hyperbolic,

non-cooperative dependence on poly[rC] concentration up to a maximum value of ~35 ATP/hexamer/s⁴¹. Other polyribonucleotide cofactors are much less efficient than poly[rC] at stimulating B_f Rho's ATPase (**Figure 2B**), a feature also shared with E_c Rho^{39, 62}. Another similitude between B_f Rho and E_c Rho is their capacity to hydrolyze the four rNTP substrates with comparable efficiencies (**Figure 2C**)³⁹ whereas evolutionary distinct Rho factors from *M. tuberculosis*⁴¹, *Micrococcus luteus*³⁹ and *Streptomyces lividans*⁶³ are more discriminatory, especially against CTP. Deletion of the full NID (ΔB_f Rho) or that of the smaller Q/N-rich motif (B_f Rho $_{\beta\text{del}}$) do not significantly affect ATP hydrolysis under saturating poly[rC] conditions (**Figure 2B**).

Taken together, these data show that B_f Rho is a RNA-dependent NTPase that is more promiscuous and efficient than M_{tb} Rho⁴¹. The large B_f Rho-NID does not confer cofactor/substrate preference features that differ significantly from those of E_c Rho. The lower steady-state ATPase rate of B_f Rho as compared to E_c Rho (**Figure 2B**) likely stems from suboptimal, NID-less core components, possibly including the three non-conservative aa substitutions in the Walker motifs (see section above).

B_f Rho is a moderately efficient RNA-DNA helicase with 5'→3' directionality

To assess the molecular motor features of B_f Rho, we performed helicase experiments with model RNA-DNA hybrid substrates^{41, 64}. We observed that B_f Rho is able to unwind a relatively long RNA-DNA hybrid (57 base pairs [bp]) substrate bearing a 5' single-stranded RNA (ssRNA) overhang (**Figure 2D**). This substrate is also unwound by E_c Rho (at a ~5-fold higher rate) but not by M_{tb} Rho (**Figure 2D**, graph and **Table S2**), which works poorly on long RNA-DNA duplex regions⁴¹. The B_f Rho factor could not unwind a RNA-DNA substrate bearing a

3' ssRNA instead of a 5' ssRNA overhang (**Figure S5A**), supporting that the enzyme behaves as a 5'→3' RNA translocase, as do the *Ec*Rho^{64, 65} and *Mtb*Rho⁴¹ enzymes.

The NID of BfRho is important for productive RNA binding

While *Bf*Rho and *Bf*Rho_{βdel} unwind the 57 bp duplex bearing a 5' ssRNA overhang with comparable efficiencies, the Δ *Bf*Rho derivative is a significantly less efficient helicase under our standard experimental conditions (**Figure 2D** and **Table S2**). To determine the origin of this difference, we performed equilibrium-binding measurements with the *Rut*-containing RNA strand (**Figure 2D**, inset) as a substrate. The *Bf*Rho and *Bf*Rho_{βdel} factors display comparable affinities for this RNA substrate, which are only 2-4 fold lower than affinities determined for *Ec*Rho and *Mtb*Rho (**Table 1**). By contrast, the Δ *Bf*Rho derivative displays a ~14 fold lower affinity than *Bf*Rho (**Table 1**). Consistent with this binding defect, the duplex unwinding activity of Δ *Bf*Rho can be strongly stimulated upon increasing the enzyme concentration, while there is not such a dramatic effect for the full-length *Bf*Rho (**Figure S5B**). Taken together, these data demonstrate that the NID contributes significantly to *Bf*Rho binding to RNA.

BfRho can trigger termination of transcription with E. coli RNAP

To determine if *Bf*Rho is capable to terminate transcription, we performed *in vitro* RDTT experiments with the RNAP of *E. coli* and a DNA template encoding the Rho-dependent terminator λ tR1 (**Figure 3A**)^{56, 66}. First, we compared the transcription patterns obtained under standard conditions (see methods) with the *Ec*Rho, *Mtb*Rho, Δ *Mtb*Rho, and *Bf*Rho factors. As described previously⁶⁷, transcription termination with *Ec*Rho occurs mostly at three cluster sites (sites I, II and III) in the downstream part of the λ tR1 region (**Figure 3B**, lane 2). By contrast,

the termination window of $M_{tb}Rho$ (i.e., the region encompassing all RNA release sites) is much larger and starts upstream from the $\lambda tR1$ region (**Figure 3B**, lane 4) while the NID-less $\Delta M_{tb}Rho$ factor displays an intermediate RDTT pattern that includes the three cluster sites of E_cRho (lane 6), as expected⁴¹. The B_fRho factor is also able to induce RDTT with our heterologous transcription system (**Figure 3B**, lane 8) but its RDTT pattern is more akin to the pattern of E_cRho (lane 2) than to that of $M_{tb}Rho$ (lane 4) despite the presence of the large and basic B_fRho -NID. RDTT is less efficient for B_fRho than for E_cRho but is similarly inhibited by BCM, with IC_{50} values of ~ 13 and $\sim 42 \mu M$ for B_fRho and E_cRho , respectively (**Figure S6A**).

The RDTT patterns of B_fRho and E_cRho are also similar in the presence of the NusG factor from *E. coli* (E_cNusG). In both cases, E_cNusG stimulates RDTT and slightly expands the termination window towards the upstream $\lambda tR1$ boundary (**Figure 3B**, lanes 3 and 9). The effect of E_cNusG is more dramatic with $\Delta M_{tb}Rho$. In this case, the RDTT window is almost completely shifted upstream from the $\lambda tR1$ region (**Figure 3B**, lane 7), with an upstream RDTT boundary even closer to the promoter than for the full-length $M_{tb}Rho$ (lane 4). This observation is consistent with the idea that $M_{tb}Rho$ -NID has a role similar to E_cNusG ⁴⁵. However, we note that E_cNusG also slightly changes the termination pattern of $M_{tb}Rho$, which starts at an ‘earlier’ (closer to promoter) termination stop point (**Figure 3B**, lane 5). This may be due to indirect effects on the TEC since E_cNusG is unable to bind $M_{tb}Rho$ directly⁴⁵. Similar results were obtained with bead-immobilized TECs (**Figure S7**), confirming that the Rho-dependent changes are due to RNA release events and, thus, to RDTT (see also discussion in ³³).

Next, we assessed the transcription termination capabilities of the $B_fRho_{\beta del}$ and ΔB_fRho mutants. While $B_fRho_{\beta del}$ triggered RDTT in patterns highly similar to those observed for WT B_fRho (**Figure 3C**, compare lanes 1, 2 with lanes 5, 6), ΔB_fRho was unable to induce termination efficiently, even in the presence of E_cNusG (lanes 3, 4). This RDTT deficiency could not be

alleviated upon increasing $\Delta_{Bf}Rho$ concentration (**Figure S6B**), suggesting that $BfRho$ -NID is also important for interactions with the TEC.

We also assayed RDTT with the various Rho variants in the presence of the NusA factor from *E. coli* ($EcNusA$). The factor delays the RDTT process with $EcRho$ ^{24, 68}, resulting in a downstream shift of the λ R1 termination window (**Figure 3D**, lane 3). A NusA-dependent delay is also observed with $MtbRho$ but RDTT still starts closer to the promoter than with $EcRho$ (**Figure 3D**, lane 5). The delaying effect of $EcNusA$ is more dramatic with $\Delta_{Mtb}Rho$ and $BfRho$, resulting in nearly complete losses of RDTT signals (**Figure 3D**, lanes 7 and 9). A similar result was obtained with $BfRho_{\beta del}$ while $\Delta_{Bf}Rho$ remained completely inactive even in the presence of both $EcNusG$ and $EcNusA$ (**Figure S6C** and data not shown). The NIDs thus do not shield the $MtbRho$ and $BfRho$ factors from the action of $EcNusA$. Their roles are distinct and not interchangeable with that of $EcNusA$.

Overall, these data demonstrate that $BfRho$ is a *bona fide* transcription termination factor. They also show that $BfRho$ -NID is strictly required for productive interaction with the TEC, which contrasts with the less critical role of $MtbRho$ -NID in the conditions of our RDTT assay. The two NIDs also differ in their capacities to direct their cognate Rho factor towards early (promoter-proximal) RDTT sites while neither NID appears to work as a NusA surrogate.

BfRho cannot trigger RDTT at a structurally constrained terminator

To better comprehend the role of $BfRho$ -NID, we also performed transcription experiments with a DNA template encoding the structurally constrained *pgaA* terminator from the 5' leader region of the *pgaABCD* operon of *E. coli* (**Figure 4A**)⁶⁹. Part of the terminator *Rut* site can be sequestered in a hairpin-like RNA structure that prevents interaction with $EcRho$ and inhibits

RDTT (**Figure 4B** and **4C**, lane 3)⁶⁹. Binding of the regulatory protein CsrA to the upstream arm of the hairpin prevents formation of the RNA structure, thereby allowing *Ec*Rho to access to the full *Rut* site and to trigger RDTT (**Figure 4B** and **4C**, lane 5)⁶⁹. *Ec*NusG can also switch on the *pgaA* terminator with *Ec*Rho, albeit with a lower efficiency than CsrA (**Figure 4C**, lane 4)⁶⁹.

The *Bf*Rho factor is unable to induce efficient RDTT at the *pgaA* terminator, even in the presence of CsrA or *Ec*NusG (**Figure 4C**, lanes 7 and 8). Similar results were obtained with the *Bf*Rho_{βdel} and Δ*Bf*Rho mutants (data not shown). By contrast, *Mtb*Rho can trigger highly efficient RDTT, even in absence of CsrA or *Ec*NusG (**Figure 4D**, lanes 7-9). As with the λ*tR1* terminator (see above), the termination window is larger and starts closer to the promoter than for *Ec*Rho. RDTT with Δ*Mtb*Rho displays intermediate features, requiring CsrA or NusG for activation but starting at earlier positions than with *Ec*Rho (**Figure 4D**, lanes 10-12). These observations provide the first direct proof for a prominent role of *Mtb*Rho-NID in the handling of structured transcripts by *Mtb*Rho^{41, 42}. They also prove that the *Bf*Rho-NID lacks this capacity and may in fact prevent RDTT at the structurally constrained *pgaA* terminator. The last conjuncture is inferred from the lack of RDTT with *Bf*Rho in the presence of CsrA (**Figure 4C**, lane 7). Binding of CsrA to the *pgaA* leader prevents formation of the hairpin-like structure but also occludes part of the mRNA (**Figure 4B**). This steric constraint next to the *Rut* site could be particularly unfavorable to the *Bf*Rho factor, which bears six bulky NIDs around its ring structure (**Figure 1C**, inset). Alternatively, other features of the *pgaA* terminator may not match *Bf*Rho requirements as well as does the λ*tR1* terminator.

The predicted prion-like NID motif does not significantly affect *Bf*Rho behavior

Although the PLAAC server⁷⁰ predicted the presence of a prion-like sequence in B_f Rho-NID (**Figure S2**), we did not detect significant differences in RNA binding or enzymatic activities between B_f Rho and the B_f Rho $_{\beta\text{del}}$ mutant, even after several months of storage as 50% glycerol solutions at -20°C. This was surprising given that the amyloidogenic motif in *C. botulinum* Rho (C_b Rho) readily promotes the formation of inactive C_b Rho aggregates^{52, 53}. However, the prion-like sequence is longer in C_b Rho (63 vs 33 aa) and yields higher PLAAC prion-likelihood scores (43 vs 23). We thus analyzed the C_b Rho and B_f Rho sequences with the conceptually distinct PrionW algorithm⁷¹. PrionW identified a prion-like motif in C_b Rho but did not replicate the PLAAC findings for B_f Rho (data not shown). Consistent with the PrionW rather than PLAAC prediction, we did not detect formation of prion-like B_f Rho aggregates with the amyloid dye Thioflavin-T. Slight decreases of basal Thioflavin-T fluorescence were observed after incubation of B_f Rho or B_f Rho $_{\beta\text{del}}$ for several days at 25°C (**Figure 5A**) rather than the characteristic enhancement of Thioflavin-T fluorescence upon amyloid formation⁵³. Using circular dichroism (CD), we also did not observe significant changes in the β -sheet content of B_f Rho or B_f Rho $_{\beta\text{del}}$ after several days of incubation at 25°C (**Figure 5B**). These data argue against a self-sufficient ability of the Q/N-rich NID motif to drive dramatic changes in B_f Rho structure, in particular formation of cross- β amyloids. To assess if more subtle changes could affect B_f Rho, we performed helicase activity measurements after 2h of pre-incubation of the B_f Rho and B_f Rho $_{\beta\text{del}}$ proteins at 37°C. We assumed that this delay would be sufficient to detect physiologically relevant inactivation effects since it matches the generation time of *B. fragilis* in minimal media⁷². However, the pre-incubation period did not affect the helicase activities of B_f Rho and B_f Rho $_{\beta\text{del}}$ (**Figure S5C**), which are thus comparably stable enzymes. Taken together, these data support that the Q/N-rich NID motif of B_f Rho does not readily promote formation of inactive, prion-like aggregates.

DISCUSSION

Although RDTT appears to be widespread in bacteria³⁸, most studies have focused on E_c Rho or on RDTT in *E. coli* (or in close species such as *Salmonella*). However, even in Gram-negative bacteria, RDTT may not always be as critical as in *E. coli* or may take significantly different forms. For instance, a number of Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Proteus vulgaris*, or *Neisseria meningitides* are highly resistant to Rho's inhibitor BCM⁷³ or contain BCM biosynthesis gene clusters⁷⁴ without carrying Rho mutations known to confer BCM resistance³⁸. Rho sequences also often contain large deletions or insertions³⁸ that could highlight phylogenetically distinct RDTT mechanisms or that could be detrimental and indicative of a lesser importance of RDTT in the corresponding species. Of particular notice is the prevalence of large N-terminal domain insertions (NIDs) in Rho factors from Bacteroidetes³⁸, a phylum of Gram-negative bacteria containing many low G+C species such as *B. fragilis*. In addition to their unusual sizes, the Bacteroidetes Rho-NIDs also frequently contain putative prion-like sequences (**Figures S2 and S3**) that may promote functional inactivation, as observed for the phylogenetically distinct C_b Rho⁵². Despite these unusual features, the representative B_f Rho factor from *B. fragilis* displays a rather orthodox enzymatic behavior when benchmarked against E_c Rho. Indeed, B_f Rho hydrolyzes NTPs in an RNA-dependent manner, with poly[rC] as preferred cofactor (**Figure 2A-C**); it unwinds RNA-DNA duplexes in an ATP-dependent and 5'→3' directional manner (**Figure 2D** and **Figure S5A**); it can dissociate *E. coli*'s TECs at the same sites than E_c Rho and, in doing so, is similarly stimulated by NusG and delayed by NusA (**Figure 3**). In all these biochemical activities, however, B_f Rho is less efficient than E_c Rho. This may reflect experimental conditions that are more adequate for E_c Rho than for B_f Rho. For instance, B_f Rho may prefer RNA substrates with distinct (e.g. AT-richer) sequence contents or may form termination-prone contacts with *B. fragilis* RNAP that are not fully recreated with *E. coli* RNAP. We note that the side-chain

contacts between E_c Rho and E_c RNAP that are best resolved in the recent cryoEM structures^{23, 24} are poorly conserved in *B. fragilis* and *M. tuberculosis* (**Table S3** and **Figure S8**). Alternatively, the lower biochemical activity of B_f Rho may stem from intrinsic features such as, possibly, the non-conservative mutations in its Walker motifs (**Figure S1**) or the steric bulk imposed by the NIDs around the hexamer (~40% of protein mass; **Figure 1C**). A lower Rho activity may be an adaptive trait in a bacterium having a slower metabolism than *E. coli*⁷². In any case, our data support that B_f Rho is a *bona fide* transcription termination factor that is also capable to disrupt RNA-DNA duplexes efficiently and may thus contribute to protect the *B. fragilis* genome against transcriptional R-loops, as is the case of E_c Rho in *E. coli*^{75, 76}. The presence of a large NID is thus not a marker of Rho inactivation in *B. fragilis*. On the contrary, B_f Rho-NID stimulates Rho activity, at least in the conditions of our helicase (**Figure 2D**) and transcription termination (**Figure 3**) assays. In line with this hereby-demonstrated enzymatic proficiency, the B_f Rho factor seems to be essential in *B. fragilis*. This is inferred from transposon inactivation experiments that failed to detect viable transpositions in the *rho* gene⁴⁷ and from the BCM sensitivity of tens of *B. fragilis* strains⁴⁶. Our data support that this BCM sensitivity stems from direct inhibition of the B_f Rho factor (**Figure S6**) and, thus, that RDTT is a valid pharmacological target in the opportunistic *B. fragilis* pathogen.

Until now, only NID-bearing Rho factors from the high G+C *Mic. luteus* (M_{ic} Rho) and *M. tuberculosis* species had been characterized^{39-42, 45}. In both cases, the NID increases Rho affinity for RNA, favors promoter-proximal RDTT in a manner similar to NusG, and stimulates RDTT with structurally constrained transcripts (**Figure 4D**)^{40, 41}. In the case of B_f Rho, the NID also significantly increases Rho affinity for RNA (**Table 1**) but does not promote promoter-proximal RDTT (**Figure 3B**) or RDTT with a structurally constrained transcript (**Figure 4C**). Moreover, B_f Rho-NID appears to be strictly required for termination activity (**Figure 3C**) whereas both M_{ic} Rho and M_{tb} Rho remain able to trigger promoter-distal RDTT when deprived

of their NID (**Figure 3B**)^{40, 41}. This may be due to the comparably lower affinity of $\Delta_{Bf}Rho$ for RNA (**Table 1**)⁴⁰, a feature that is by itself surprising given that the primary RNA binding site (PBS) domain contains disruptive indels in $_{Mic}Rho$ and $_{Mtb}Rho$ but not in $_{Bf}Rho$ (**Figure S1**)³⁹. One possible explanation is that the poorly conserved, N-terminal NHB domain (**Figure 1C** and **Figure S1**) is able to mitigate the lack of NID in $_{Mic}Rho$ and $_{Mtb}Rho$ but not in $_{Bf}Rho$. The NHB domain has been proposed to electrostatically help Rho's PBS to catch the RNA chain⁷⁷, a role that could be strictly devoted to the basic NID in $_{Bf}Rho$. Alternatively, the NID may form contacts with RNAP that are especially critical for $_{Bf}Rho$. In support of this proposal is the observation that $\Delta_{Bf}Rho$ retains ATPase and helicase activities (**Figure 2**) but has no significant transcription termination activity even at high concentration (**Figure 3C** and **Figure S6B**).

It is worth considering the hypothetical roles of $_{Bf}Rho$ -NID in light of the recent cryoEM structures of the $_{Ec}Rho:_{Ec}RNAP$ complex^{23, 24}. The structures support a model for RDTT whereby Rho hitchhikes on ribosome-free RNAP while scanning the emerging transcript for a *Rut* site (**Figure 1A**). The orientation of the $_{Ec}Rho$ hexamer in the structures suggests a rather convoluted and sterically constrained path of the transcript at the Rho:RNAP interface and around Rho (**Figure 1A**)^{23, 24}. It is envisioned that *Rut* recognition by Rho's PBS at the Rho:RNAP interface triggers allosteric destabilization of the TEC by the bound Rho hexamer^{23, 24}. Importantly, the positions of NID insertions (corresponding to residues 43-45 in $_{Ec}Rho$) are located on the outer edge of the Rho ring facing RNAP (**Figure 6A**, residues in purple). Assuming that this Rho:RNAP configuration is conserved in *B. fragilis*, the NIDs may be in ideal positions to make peripheral contacts with RNAP, thereby extending the Rho:RNAP interface (**Figure 6A**, cartoon). This could be critical to compensate for otherwise poorly conserved Rho:RNAP interactions (**Table S3** and **Figure S8**)²³. The NIDs may also be adequately located on the ring periphery to help guide the transcript from the RNA exit channel of RNAP to the inner Rho channel and, possibly, to extrude the RNA 5'-end from the

Rho:RNAP complex (**Figure 6A**). A similar ‘guiding’ role has been ascribed to $_{Ec}NusA$ ²⁴ but our RDTT data (**Figure 3D** and **Figure S6C**) argue against interchangeability between NIDs and $_{Ec}NusA$. The cooperative capture of *Rut* sequences by both the NIDs and $_{Bf}Rho$ ’s PBS is also possible; however, the physical separation between ‘external’ NIDs and a ‘buried’ PBS (**Figure 6A**) makes it less easily envisioned than for the classical RDTT model of ‘tethered tracking’ (**Figure 6B**)¹⁹. Further structural characterization of the Rho:RNAP complex, including from species that are phylogenetically distinct from *E. coli*, will be needed to clarify these important aspects.

The *B. fragilis rho* gene is significantly GC-richer than the whole genome (48.2% vs 43.1%), a feature that usually stresses the importance of the corresponding gene and its belonging to the core genome⁷⁸. Intriguingly, the upstream section of the gene (until the end of the NID-encoding region) displays a strong sequence bias when compared to the downstream section (**Figure 6C**). Similar sequence biases were found in other Bacteroidetes *rho* genes but not in the *rho* gene of *M. tuberculosis* (**Figure 6D** and data not shown). These observations suggest that the unusual NTD of $_{Bf}Rho$ is the product of relatively recent insertion(s) of foreign DNA into the *rho* gene. This may have helped tune $_{Bf}Rho$ activity to the AT-rich transcriptome of *B. fragilis* (e.g. by increasing binding to less C-rich *Rut* sites) or to specifics of its transcription machinery (**Table S3** and **Figure S8**). Alternatively, the acquisition of an unusually long NTD-encoding sequence (**Figure 1B**) could have been a source of new, evolutionary advantageous properties. Notably, the presence of a putative prion-like Q/N-rich sequence in $_{Bf}Rho$ -NID (**Figure S2**) suggested that $_{Bf}Rho$ could form amyloid structures, as was observed previously for the $_{Cb}Rho$ factor of *C. botulinum*⁵². Functional amyloids support a diversity of advantageous functions in microorganisms, from the structural scaffolding of biofilms to the regulation of the life cycle or response to stresses⁷⁹. However, we were unable to detect formation of $_{Bf}Rho$ amyloids or significant structural/activity differences between

B_f Rho and the B_f Rho $_{\beta\text{del}}$ variant (**Figures 5 and S5C**). In retrospect, this is consistent with prion prediction scores that are lower for B_f Rho than for c_b Rho (see results). Notwithstanding, we cannot exclude that B_f Rho promptly self-assembles only under specific conditions. Many ‘facultative’ functional amyloids have been described, which adopt the amyloid-forming conformation only upon a specific trigger such as a pH change, post-translational modification, cleavage of the peptide chain, or presence of a metal ion, nucleic acid, or protein ‘nucleator’⁷⁹. A minimalistic amyloidogenic core sequence such as the Q/N-rich sequence found in B_f Rho-NID (**Figure S2**) might help keep such structural transition ‘conditional’. Further work will be needed to explore this intriguing possibility.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and enzymes were purchased from Sigma-Aldrich and New England Biolabs, respectively. BCM was purchased from Santa Cruz Biotechnology. Nucleoside triphosphates were purchased from GE-Healthcare while radionucleotides were from PerkinElmer. Synthetic oligonucleotides were obtained from Eurogentec. Polynucleotide fragment (> 300 nt) stocks were prepared as described previously⁵⁹ from polynucleotide batches obtained from Santa Cruz Biotechnology and Midland Certified Reagent Company (Midland, Texas). The DNA templates containing the λ tR1 and *pgaA* terminators used in transcription termination experiments with *E. coli* RNAP were prepared as described previously^{33,69}. RNA substrates were obtained by *in vitro* transcription of PCR amplicons with T7 RNAP and purified by denaturing polyacrylamide gel electrophoresis (PAGE), as described⁵⁹. Genomic DNA from *B. fragilis* type strain NCTC 9343 was obtained from the DSMZ repository (Germany). Plasmid for over-expression of WT B_f Rho (pET28b- B_f Rho) was prepared by subcloning the B_f Rho coding sequence within plasmid pET28b (Novagen). To this

end, the B_f Rho coding sequence was PCR amplified from genomic DNA using primers BfRho-F and BfRho-R (**Table S1**), digested with the KpnI and XhoI enzymes, and then subcloned within the KpnI and XhoI restriction sites of the pET28b plasmid. Plasmids for overexpression of the ΔB_f Rho and B_f Rho $_{\beta del}$ variants were engineered from the pET28b- B_f Rho plasmid using the NEBuilder HiFi DNA Assembly kit (NEB) and, respectively, primers ΔB_f Rho-F and ΔB_f Rho-R or βdel BfRho-F and βdel BfRho-R (**Table S1**), following manufacturer's instructions. The sequences of DNA templates and plasmids were verified by capillary DNA sequencing (Genoscreen, France). Plasmid pET28b-NusA for overexpression of the *E.coli* NusA protein with a C-terminal His6 tag was custom synthesized by Genscript.

Preparation of proteins. The E_c NusG, E_c Rho, M_{tb} Rho and ΔM_{tb} Rho proteins were prepared and purified as described previously^{41, 59}. The B_f Rho, ΔB_f Rho, and B_f Rho $_{\beta del}$ proteins were prepared following similar procedures. Briefly, they were over-expressed as N-terminal His6 tag fusions in Rosetta 2(DE3) cells (Merck-Millipore) harboring the appropriate pET28b plasmid derivative. They were purified by affinity chromatography on a HisTrap HP column (GE-Healthcare) with a 10-500 mM imidazole gradient and by ion exchange chromatography on a SP- (B_f Rho and B_f Rho $_{\beta del}$) or Q- (ΔB_f Rho) sepharose FF column (GE-Healthcare) with a 0.15-1M NaCl gradient. The NusA protein was overexpressed in standard BL21(DE3) cells harboring the pET28b-NusA plasmid and purified by HisTrap chromatography, as described⁸⁰. Protein identities and purities were checked by liquid chromatography coupled to HR-MS at the CBM mass spectrometry facility, Orléans, France. All proteins were stored at -20°C as micromolar solutions in 100 mM KCl, 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, and 50% (v/v) glycerol. Concentrations of Rho factors are expressed in hexamers throughout the manuscript.

Preparation of duplex substrates. Duplexes were assembled by mixing 10 pmoles of ³²P-end labeled RNA transcript with 12 pmoles of complementary oligonucleotide in annealing buffer (150 mM potassium acetate, 20 mM HEPES, pH 7.5, 1 mM EDTA). Mixtures were heated at 95°C and slowly cooled to 20°C. Duplexes were then purified by native 7 % PAGE and stored at -20°C in helicase buffer (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.5 mM DTT, and 150 mM potassium acetate), as described⁵⁹.

NTP hydrolysis assay. The NTP hydrolysis activities of the Rho enzymes were determined as described previously with a thin layer chromatography-based ³²P radiometric assay⁶⁴ or with the EnzCheck Phosphate Assay kit (Molecular Probes)^{56, 81}. Control experiments confirmed that both assays yield comparable results. Reaction mixtures contained 20 nM Rho, 1 mM NTP and the indicated concentration of RNA cofactor (e.g. poly[rC] at a standard concentration of 10 μM in rC residues) in NTPase buffer (1 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, 0.1 mM sodium azide). Experiments were performed at 37°C.

Equilibrium binding assays. Equilibrium Rho-RNA dissociation constants were determined using a filter-binding assay, as described previously^{64, 82}. Briefly, ~10 fmoles of ³²P-labeled RNA substrate were mixed with various amounts of Rho in 100 μl of binding buffer (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.5 mM DTT, 150 mM potassium acetate, and 20 μg/ml BSA). After incubation for 10 min at 30°C, the samples were filtered through stacked [top] nitrocellulose (Amersham Protran) and [bottom] cationic nylon (Pall Biodyne B) membranes using a Bio-dot SF apparatus (Biorad). The fractions of free and Rho-bound RNA (retained on the nylon and nitrocellulose membranes, respectively) as a function of Rho concentration were then determined by phosphorimaging of the membranes using a Typhoon FLA9500 imager and dedicated ImageQuant TL v8.1 software (GE healthcare).

Duplex unwinding assay. Helicase reactions were performed as described previously⁸³ with ³²P-labeled duplex substrates. Briefly, 5 nM substrate was mixed with 20 nM Rho in helicase buffer and incubated for 3 min at 30°C. Then, 1 mM MgCl₂, 1 mM ATP, and 400 nM oligo trap (unlabeled oligonucleotide having the same sequence than the released ‘reporter’ strand) were added to the helicase mixture before further incubation at 30°C. Reaction aliquots were taken at various times and mixed with two volumes of quench buffer (30 mM EDTA, 0.75% SDS, 150 mM sodium acetate, 6 % Ficoll-400) before being loaded on 8% polyacrylamide gels that contained 1X TBE and 0.5% SDS. Detection and quantification of gel bands were performed by phosphorimaging with a Typhoon FLA9500 imager as described⁸¹.

Transcription termination experiments. Standard transcription termination experiments were performed as described previously³³. Briefly, DNA template (0.1 pmol), *E. coli* RNAP (0.45 pmol), Rho (0 or 1.4 pmol), and Superase-In (0.5U/μL; Ambion) were mixed in 18 μL of transcription buffer (40 mM Tris-HCl, pH 8, 0, 5 mM MgCl₂, 1,5 mM DTT, and 100 or 150 mM KCl for experiments with the λtR1 or *pgaA* terminator, respectively) and incubated for 10 min at 37°C. Whenever necessary (see text and figure legends), NusG (2.8 pmol), NusA (2.8 pmol), CsrA (2.8 pmol), and/or BCM (0-3 nmol) were also added to the premixes. Then, 2 μL of initiation solution (2 mM ATP, GTP, and CTP, 0.2 mM UTP, 2.5 μCi/μL of ³²P-αUTP, and 250 μg/mL of rifampicin in transcription buffer) were added to the reaction mixtures before further incubation for 20 min at 37°C. Transcription reactions were stopped with 4 μL of EDTA (0.5 M), 6 μL of tRNA (0.25 mg/mL), and 80μL of sodium acetate (0.42 M) before precipitation at -20°C with 330 μL of ethanol. Reaction pellets were dissolved in denaturing loading buffer (95% formamide, 5 mM EDTA), and analyzed by denaturing 7% polyacrylamide gel electrophoresis (PAGE) and Typhoon FLA9500 phosphorimaging (GE-Healthcare). Gamma

settings of gel images have been optimized to facilitate the comparison of termination signals⁴¹. Transcription termination efficiencies with the λ tR1 terminator were estimated from gel band intensities after normalization for distinct ³²P- α U incorporation levels in the various transcript species, as described previously⁵⁶.

Circular Dichroism (CD). Experiments were performed with 10 μ M (in monomers) solutions of the BfRho and BfRho β del proteins in CD buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.5). The solutions were incubated in a dry bath at 25°C in the dark. At various times, CD spectra of the protein solutions were recorded at 20°C on a Jasco J-810 spectropolarimeter from 260 to 200 nm using a 1-mm quartz cell, a scan rate of 50 nm/min, a response time of 4 s, a bandwidth of 2 nm, and a resolution of 1 nm. Each CD spectrum represents the average of five scans, with the buffer subtracted.

Amyloid detection assay. The assay was performed with the Thioflavin-T dye as described previously⁵³, with minor modifications. Briefly, the BfRho and BfRho β del proteins at a concentration of 20 μ M (monomers) in CD buffer were incubated at 25°C in the dark. Aliquots were withdrawn at various times and mixed gently with an equal volume of a 50 μ M aqueous solution of Thioflavin-T dye. Fluorescence emission spectra were recorded on a CLARIOstar Plus microplate reader (BMG labtech) set for excitation at 440 nm. Background fluorescence of the Thioflavin-T dye in CD buffer was subtracted from the spectra.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Rho-dependent termination of transcription (RDTT). **(A)** Schematic depiction of transcription-translation coupling in *E. coli* (top) and how the lack of ribosome allows Rho binding to RNAP²³ and, eventually, to a *Rut* site within the nascent transcript (bottom). In 5'untranslated regions (UTRs), Rho may first bind RNAP and trigger RDTT. If the 5'UTR is too short or does not encode a *Rut* site promoting RDTT, a ribosome may displace Rho from RNAP once the ribosome-binding site (RBS) has been transcribed. For sake of clarity, cofactors NusA and NusG are not shown and only three subunits of the Rho hexamer are depicted with the PBS sites facing RNAP in orange (diagram not to scale). Once bound to the *Rut* site (blue segment), the Rho hexamer presumably triggers allosteric destabilization of the TEC²³⁻²⁵. Full disruption of the TEC likely requires closure of the Rho ring around the transcript and ATP-dependent RNA translocation as depicted (small back arrows)²³. **(B)** Distribution of NID lengths from 1259 phylogenetically diverse Rho factors³⁸. NIDs < 20 aa are not shown. **(C)** Diagram showing the arrangement of key motifs within the primary sequence of Rho subunits (numbering is for _{Bf}Rho; role of specific residues is based on information obtained with _{Ec}Rho). *Rut* recruitment is ensured primarily by PBS residues (orange arrows)⁸⁴ within the RNA binding domain (RBD) of Rho; the N-terminal helix bundle (NHB) domain may help⁷⁷. The large NID of _{Bf}Rho (sequence in **Figure S1**) is depicted by a purple triangle. SBS residues translocating RNA through the _{Ec}Rho hexamer channel⁴⁹ are depicted by green arrows. A model of the _{Bf}Rho Rho hexamer based on the _{Ec}Rho structure (PDB #3ICE) with purple spheres representing the NIDs (one per subunit, not to scale) is shown inset. Note that the NIDs are probably unstructured in absence of cofactors (see main text).

Figure 2: Standard ‘molecular motor’ properties of the B_{f} Rho factor. **(A)** The steady-state ATP hydrolysis of B_{f} Rho is stimulated by poly[rC]. **(B)** ATPase activity as a function of RNA ligand (with B_{f} Rho; left graph) or Rho factor (with poly[rC]; right graph). **(C)** The identity of the NTP hardly affects the NTP hydrolysis activity of B_{f} Rho (with poly[rC]). Unless specified otherwise, the concentrations of NTP and RNA polymer in the NTPase assays were 1 mM and 10 μ M (in nucleotide residues), respectively. Graphs show mean \pm error values from at least two independent experiments. **(D)** Duplex unwinding activity. The B_{f} Rho helicase is able to unwind a 57 bp-long RNA-DNA duplex bearing a 5’-single stranded RNA tail containing a synthetic *Rut* site optimized for E_{c} Rho⁶⁴, as depicted. Unwinding reactions were performed under standard conditions with 20 nM Rho and 5 nM of the RNA-DNA substrate. The graph shows unwinding time courses (fraction means from three independent experiments) for the various factors identified on the right. A representative 9% PAGE gel illustrates the helicase activity of B_{f} Rho.

Figure 3: The B_{f} Rho factor triggers RDTT with *E. coli* RNAP and the prototypical λ tR1 terminator. **(A)** Schematic of the DNA template used. **(B)** The termination windows of B_{f} Rho and E_{c} Rho are similar. **(C)** The NID of B_{f} Rho is critical for RDDT activity. **(D)** RDTT with either the M_{tb} Rho or B_{f} Rho factor is sensitive to the delaying effect of E_{c} NusA. Representative denaturing PAGE gels show the products of transcription termination experiments performed under standard conditions with *E. coli* RNAP and the λ tR1 template in the presence of various Rho factors and with or without E_{c} NusA or E_{c} NusG (indicated above gel lanes).

Figure 4: RDTT with the structurally constrained *pgaA* terminator of *E. coli*. **(A)** Schematic of the DNA template used. The template encodes the full *pgaA* 5’ leader, which includes the CsrA-

regulated Rho-dependent *pgaA* terminator⁶⁹. **(B)** A hairpin-like structure shields part of the *pgaA* *Rut* site. Binding of the CsrA protein prevents formation of the hairpin structure and allows *Ec*Rho to interact productively with the *pgaA* transcript⁶⁹. **(C)** The *Bf*Rho factor cannot terminate transcription at the *pgaA* terminator. **(D)** *Mtb*Rho triggers early, promoter proximal RDTT with the *pgaA* terminator even in absence of CsrA or NusG.

Figure 5: The *Bf*Rho factor does not spontaneously form β -amyloids. Thioflavin-T fluorescence emission **(A)** and circular dichroism **(B)** spectra of the *Bf*Rho and *Bf*Rho β del proteins after 0 to 7 days of incubation at 25°C in 100 mM NaCl, 10 mM Phosphate, pH 7.5 buffer. The β -sheet content of the proteins was predicted from the CD spectra by using the BETSEL server⁸⁵.

Figure 6: Role of the *Bf*Rho-NID. **(A)** Model based on the recent cryoEM structures of the *Ec*Rho:RNAP complex^{23, 24}. Residues 43-45 of *Ec*Rho are shown in magenta on the filled-sphere structure of the complex (PDB #6XAS) to appreciate where NIDs would be protruding from the hexameric *Bf*Rho ring. In the model, the NIDs would contribute to stabilize the Rho:RNAP complex and, possibly, help keep the RNA chain into the path required for transcript scanning by the Rho factor until a *Rut* site is detected. **(B)** In the classical RDTT model, The NIDs would cooperate with the PBS to anchor Rho to the *Rut* site and to stabilize the *Rut*-Rho interaction during ATP-dependent ‘tethered tracking’ of the hexamer motor along RNA towards the RNAP. At present, it cannot be totally excluded that the CryoEM-like and ‘tethered tracking’ models represent distinct, physiologically relevant pathways^{23, 24}. **(C & D)** The graphs show the distributions of A and C residues versus G and T residues along the sequences of the genes encoding *Bf*Rho **(C)** and *Mtb*Rho **(D)**. Percentages were calculated with a 20 nt sliding window.

Note that the AC>GT sequence bias in the upstream portion of the *Bjrho* gene is not detected with a standard %GC vs. %AT plot (**Figure S9**).

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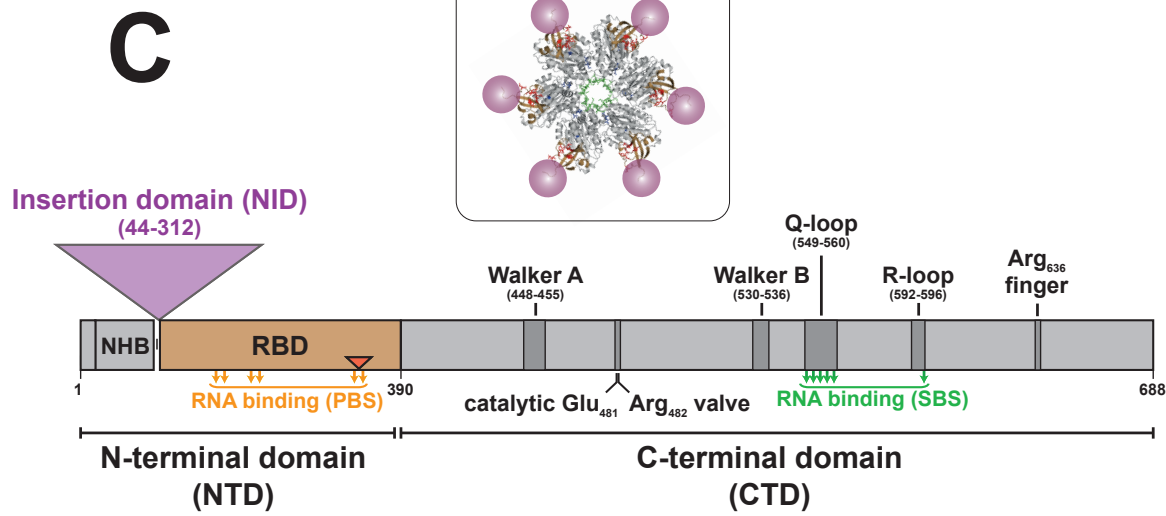
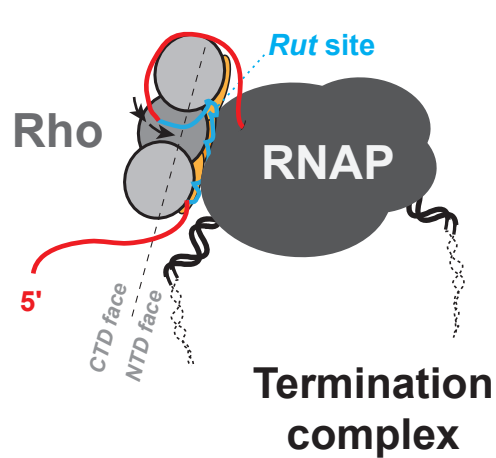
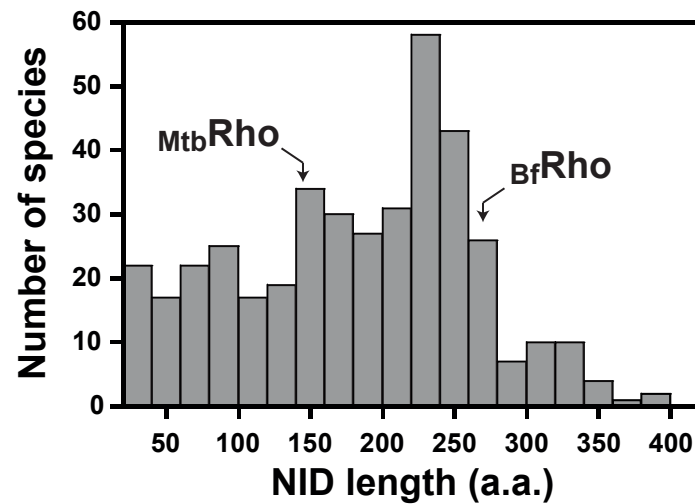
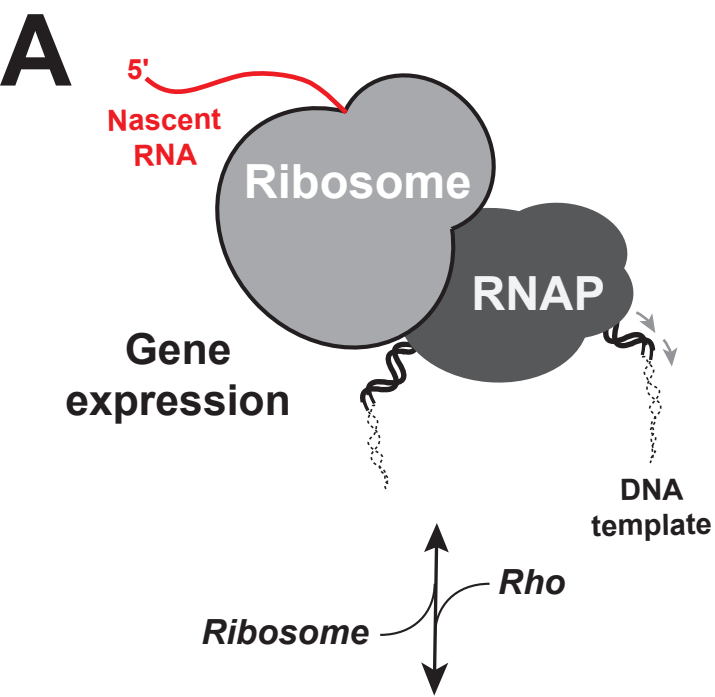
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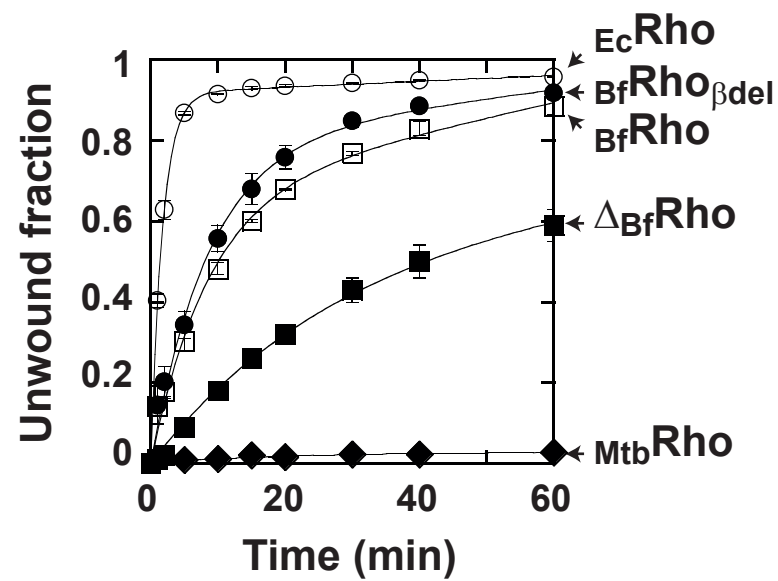
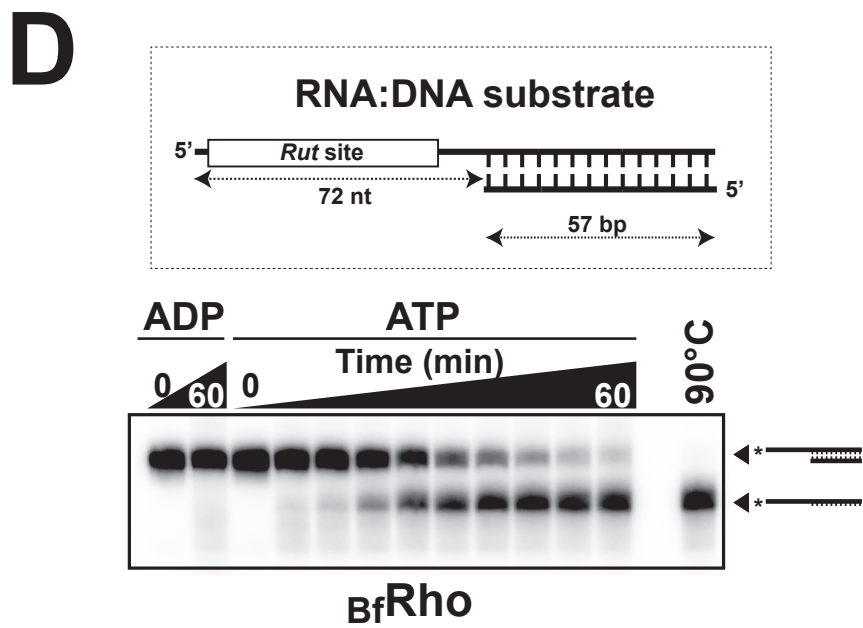
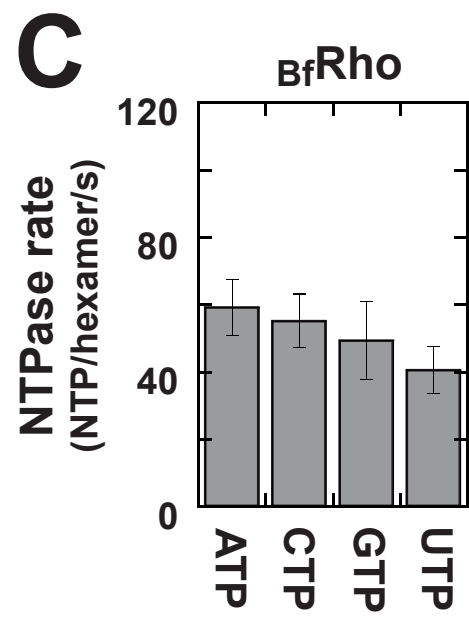
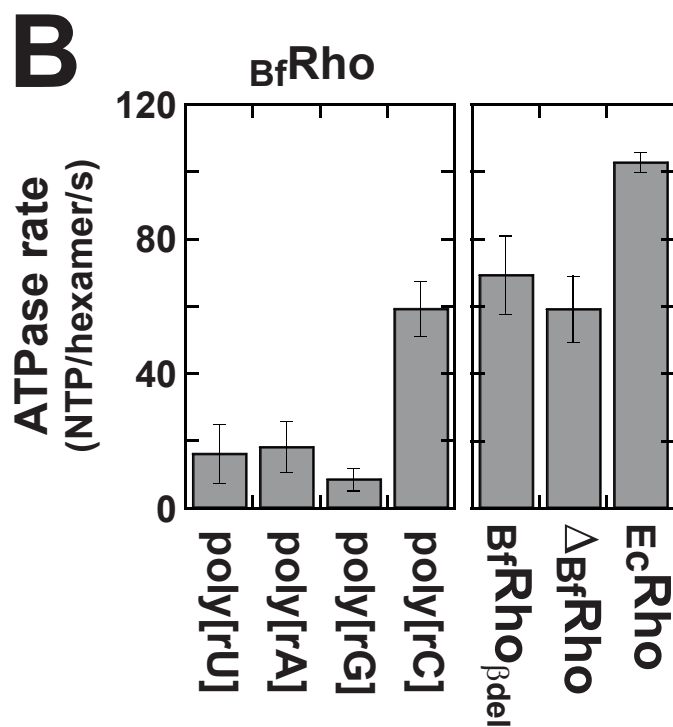
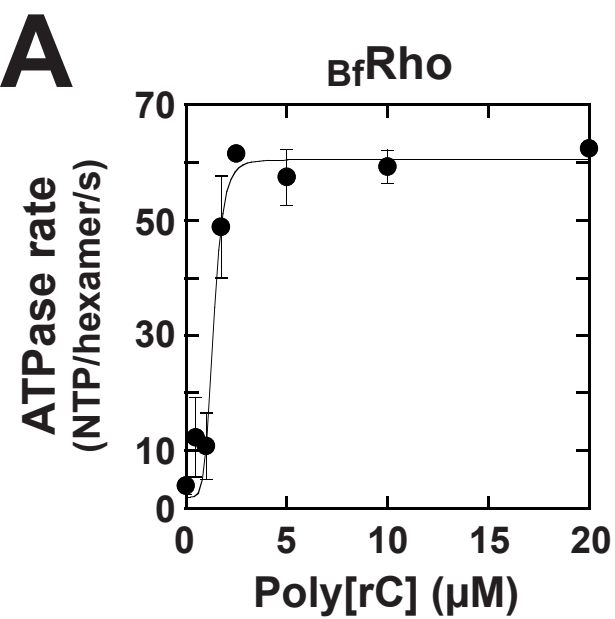
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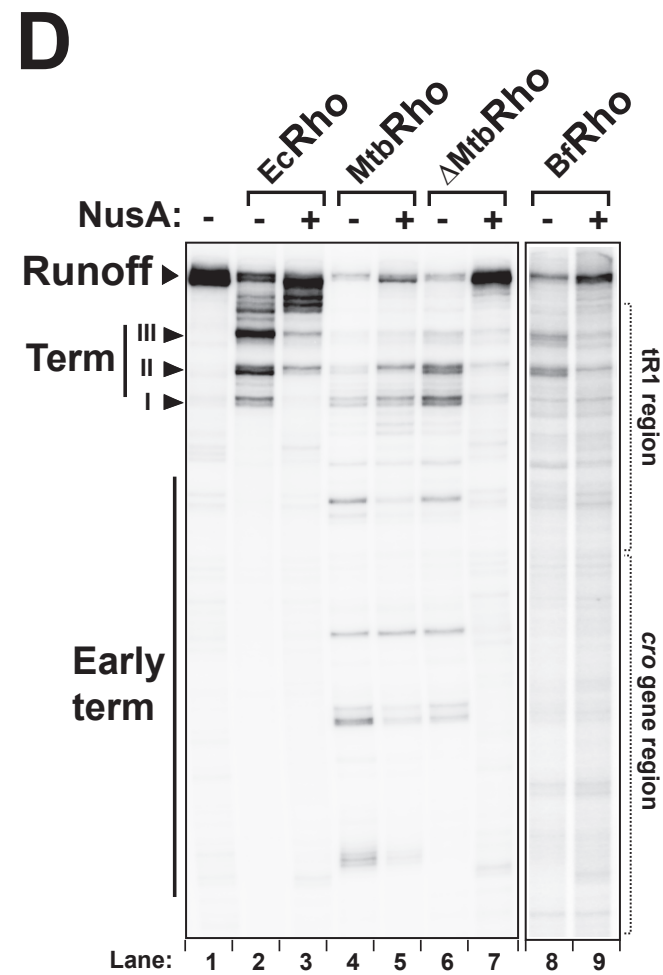
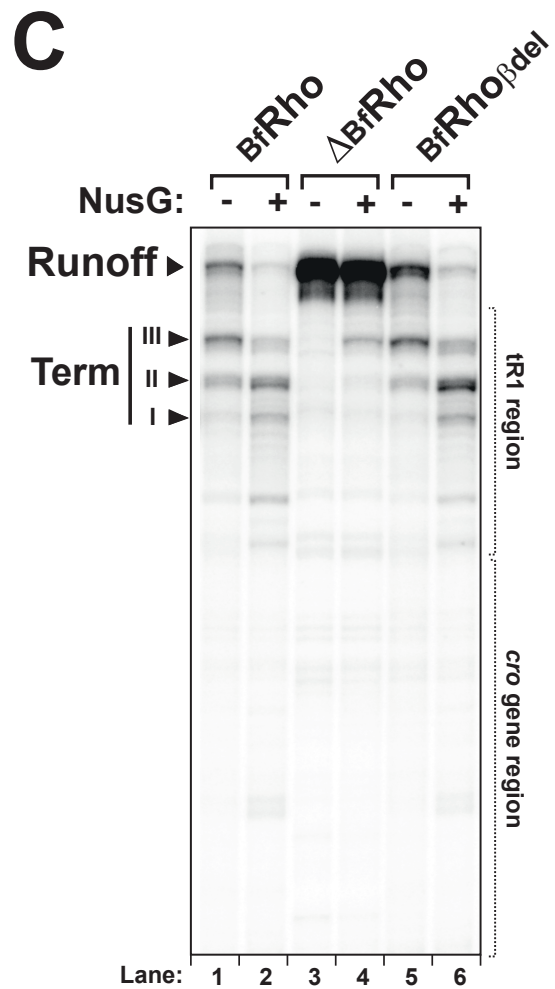
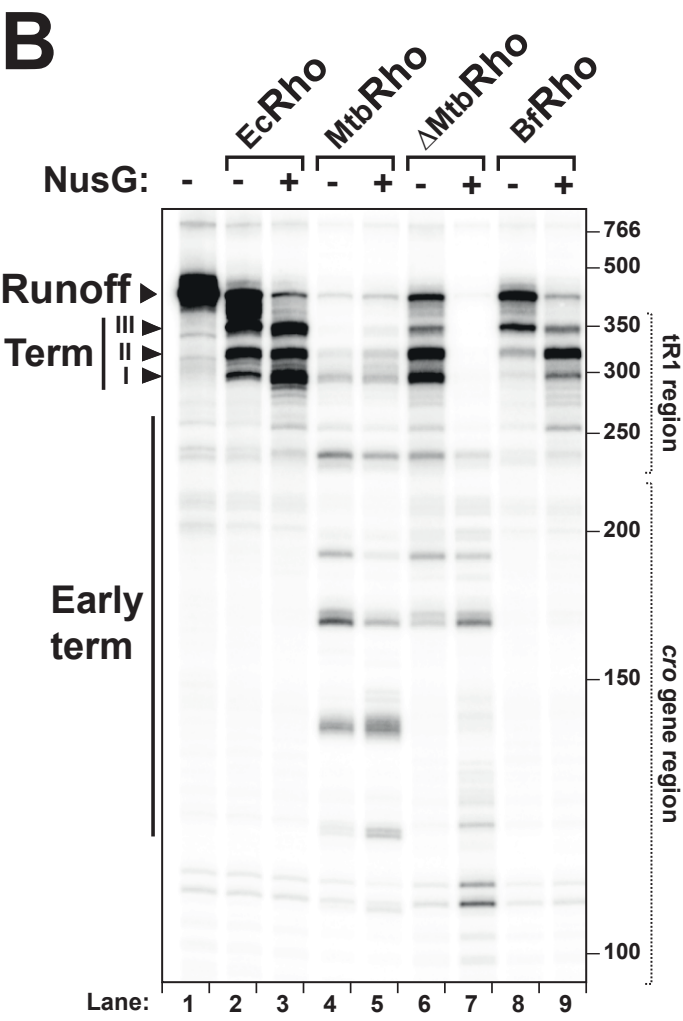
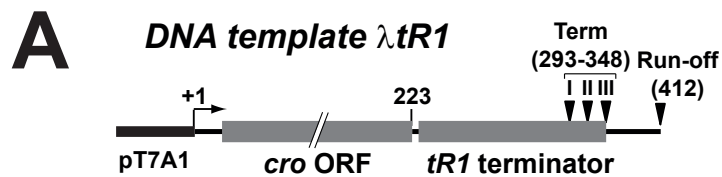
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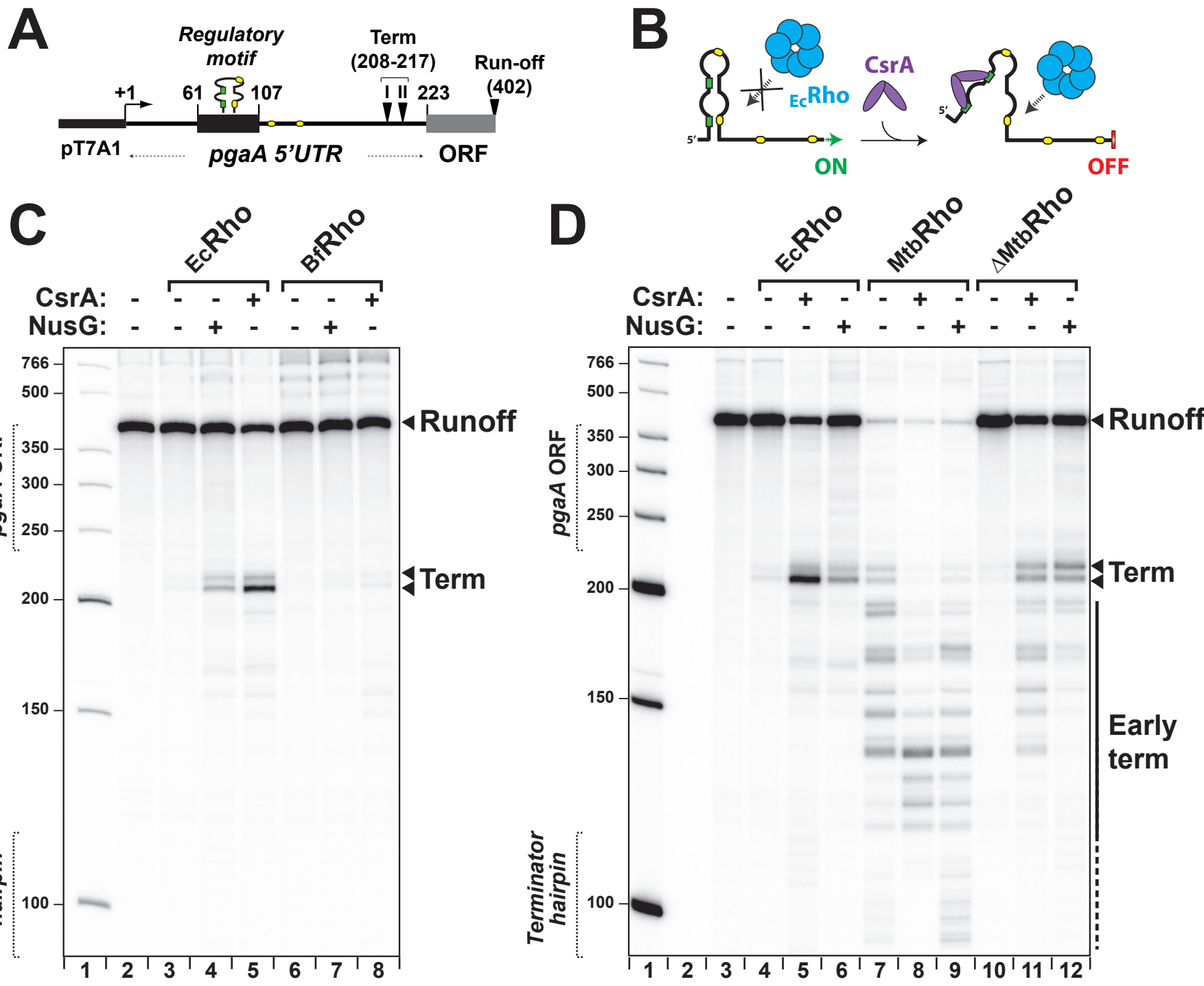
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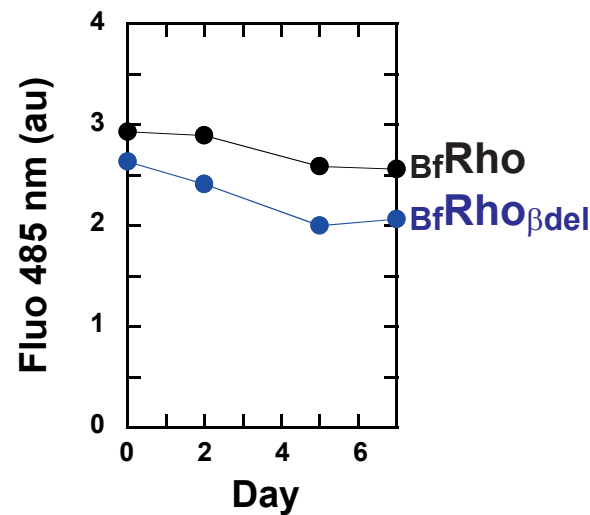
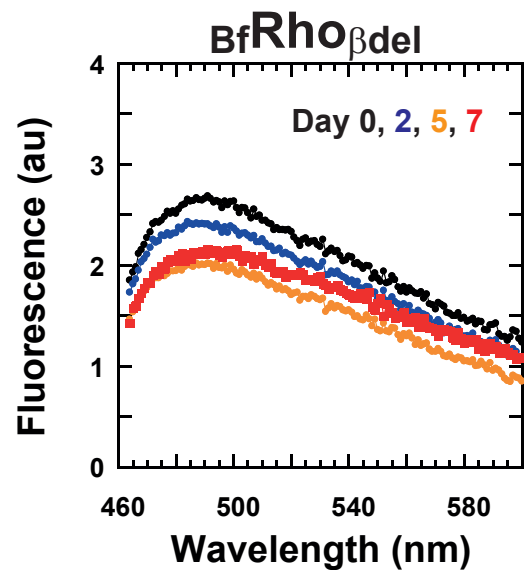
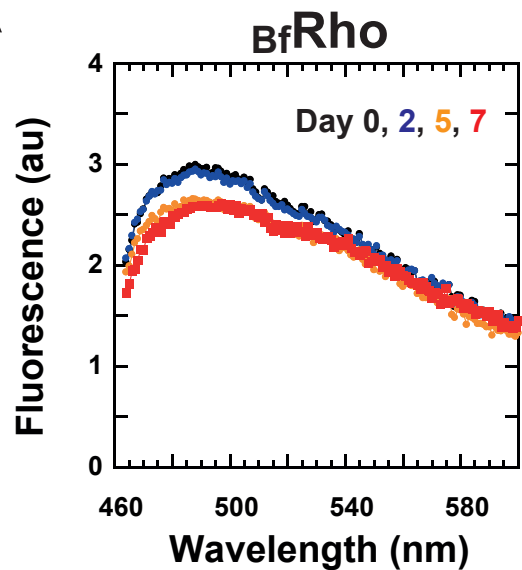
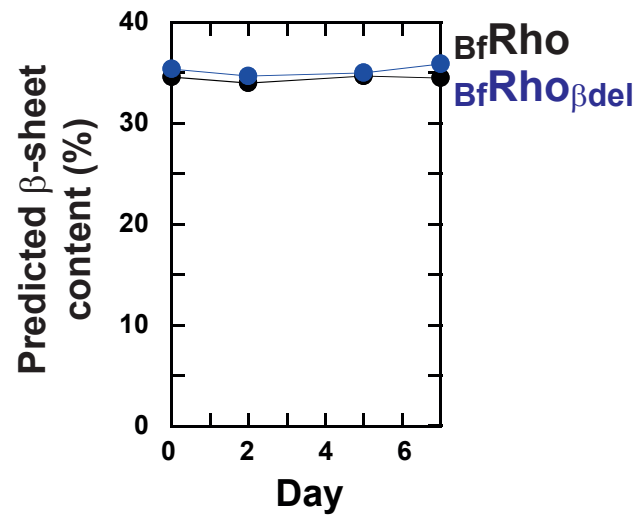
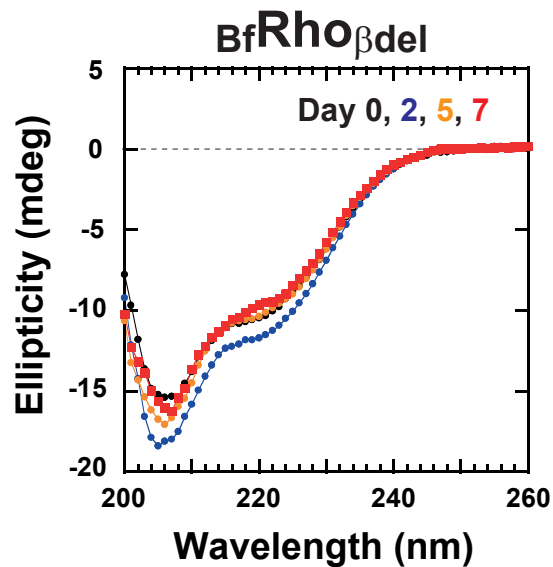
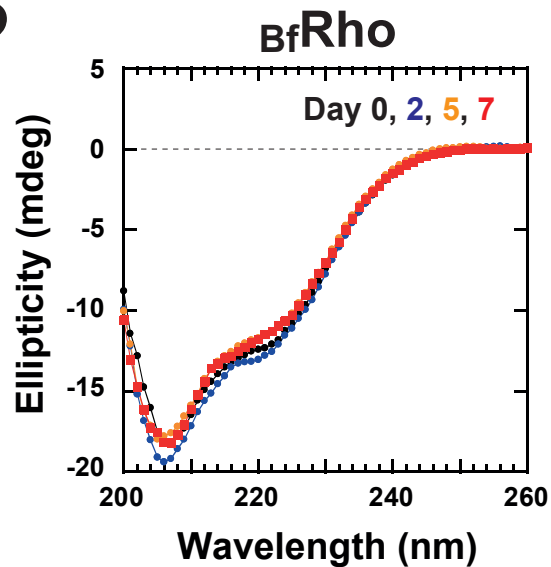
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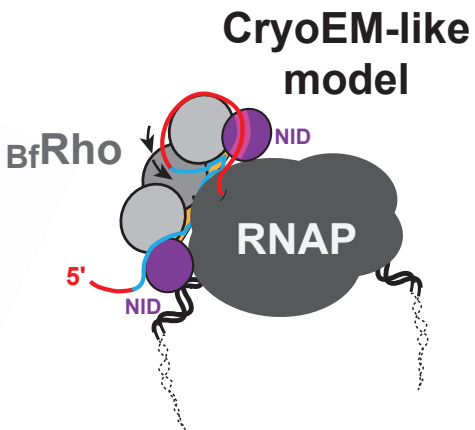
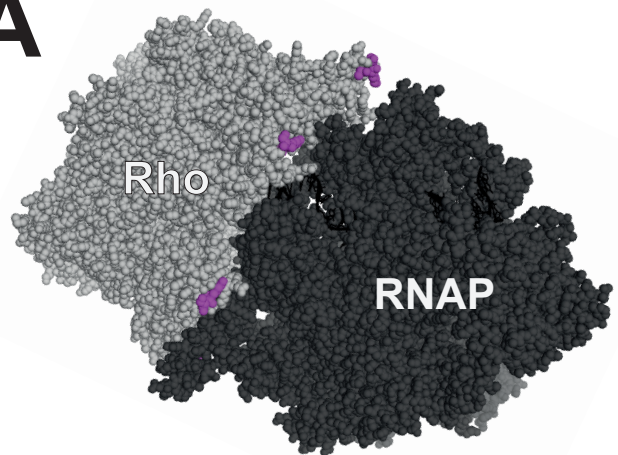
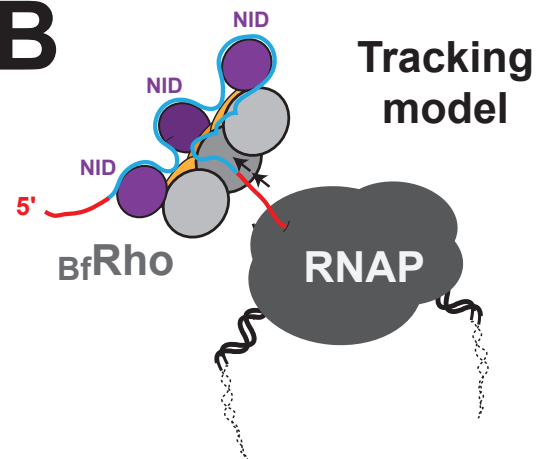
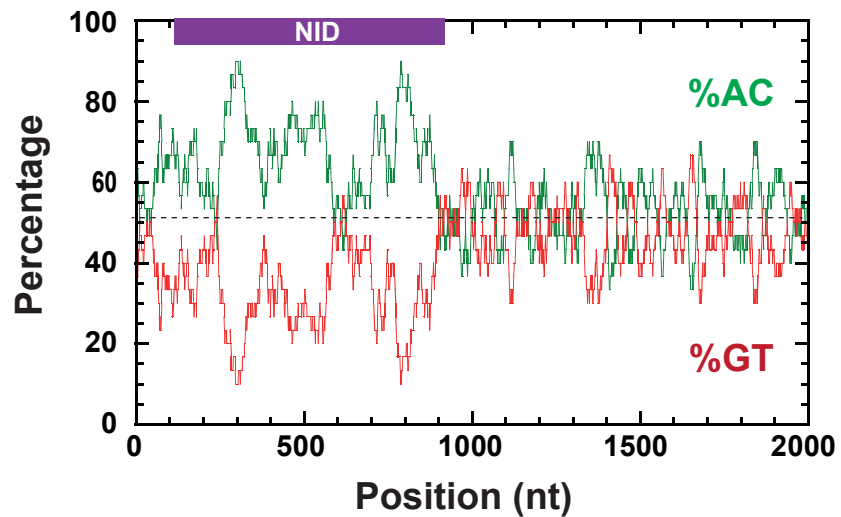
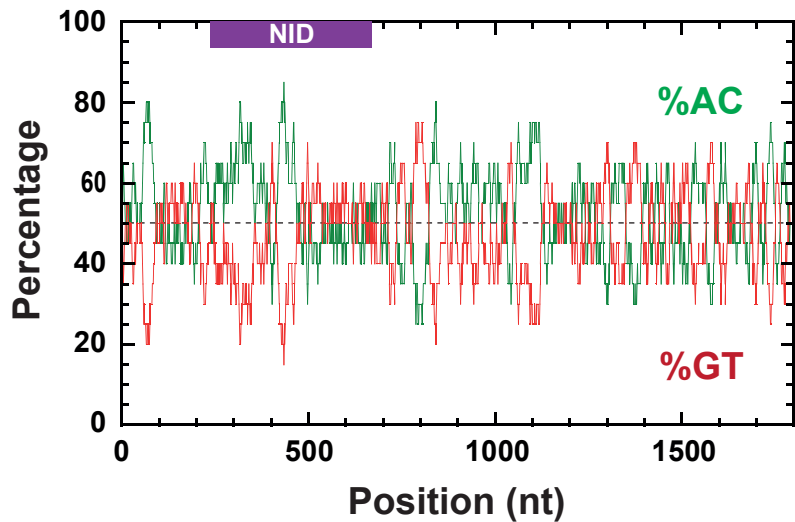








A**B**

A**B****C***Bfrho* gene**D***Mtbrho* gene

A large insertion domain in the Rho factor from a low-G+C Gram-negative bacterium is critical for RNA binding and transcription termination activity

Isabelle Simon, Mildred Delaleau, Annie Schwartz, and Marc Boudvillain

(Supplementary information)

SUPPLEMENTARY METHODS

Single round transcriptions. Single-round ‘on-beads’ transcription termination experiments were performed as described previously (1) with minor modifications. The biotinylated λ -tR1 DNA templates (30 nM) were mixed with *E. coli* RNAP holoenzyme (36 nM) in transcription initiation buffer (40 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1mM DTT, 0.05 mg/mL BSA, 0.4U/ μ L Superase-In [Ambion]) and incubated at 37°C for 5 min. The mixture was supplemented with 12 μ M ApU, 6 μ M ATP, 6 μ M GTP, 0.95 μ M UTP, and 1 μ Ci/ μ L [α^{32} P]-UTP, incubated for 10 min at 37°C, and then chilled on ice. The concentration of KCl was raised to 0.1 M before immobilization of the halted transcription complexes on streptavidin-coated magnetic beads (Ademtech, Pessac, France). Then, rNTPs (75 μ M each, final concentrations) and rifampicin (25 μ g/mL) were added to initiate chase reactions. After incubation for 10 min at 37°C, supernatants and bead pellets were separated on a MagRack (Cytiva) and their transcript compositions analyzed by 8% denaturing PAGE and Typhoon-Trio phosphorimaging.

Supplementary Table 1: plasmids			
Plasmid	Method	Source DNA	Primers
pET28b-BfRho	subcloning of PCR fragment	Genomic DNA from <i>B. fragilis</i> type strain NCTC 9343	5'GGCAGCCATATGATGTATAACATCATTCAATTG AACG (BfRho-F) 5'GGTGGTGGTGGTGGTGGTCTCGAGTTAGCTGTTC ATGCTCATCAGG (BfRho-R)
pET28b-ΔBfRho	HiFi DNA Assembly	pET28b-BfRho	5'GGCGGCAGCGGCTATGAATTTGATGATATCCT CACCGG (ΔBfRho-F) 5'GCCGCTGCCGCCTTGTTTCGTCGAGGATTTTG (ΔBfRho-R)
pET28b-BfRhoβdel	HiFi DNA Assembly	pET28b-BfRho	5'GCCACGAGATGCCGCCGAAGCCGCACC (βdelBfRho-F) 5'TTCGGCGGCATCTCGTGGGCGAATCACACGCG (βdelBfRho-R)

Supplementary table 2 : helicase reaction parameters^a				
		Burst phase		Linear phase
Protein	Condition^b	Amplitude (A)	k_{exp} (min⁻¹)	k_{lin} (X100 min⁻¹)
<i>E_c</i>Rho	Standard	0.92 ± 0.03	0.58 ± 0.01	0.08 ± 0.01
<i>B_f</i>Rho	Standard	0.67 ± 0.07	0.12 ± 0.02	0.4 ± 0.2
	10X	1.00 ± 0.03	0.07 ± 0.02	-
	Pre-incubation	0.76 ± 0.03	0.13 ± 0.01	0.2 ± 0.1
Δ<i>B_f</i>Rho	Standard	0.54 ± 0.28	0.04 ± 0.02	0.2 ± 0.3
	10X	0.93 ± 0.02	0.14 ± 0.01	-
<i>B_f</i>Rho_{βdel}	Standard	0,90 ± 0.02	0.10 ± 0.01	0.02 ± 0.04
	Pre-incubation	0,86 ± 0.06	0.09 ± 0.01	0.2 ± 0.1
<i>M_{tb}</i>Rho	Standard	0.02 ± 0.05	0.06 ± 0.08	0.01 ± 0.05

^a Reaction data points were fitted to an equation describing the kinetic regimen determined previously for *E_c*Rho: $F_p = A \times (1 - e^{-k_{exp}t}) + k_{lin} \times t$, where F_p is the fraction of product formed, A is the amplitude of the exponential (burst) phase of the reaction, and k_{exp} and k_{lin} are the rate constants of the exponential and linear phases of the reaction, respectively (2-4).

^b In “standard” conditions, the RNA-DNA substrate was preincubated with 20 nM Rho for 5 min at 30°C before initiating the helicase reaction with ATP. In “10X” conditions, Rho concentration was raised to 200 nM while in “pre-incubation” conditions, Rho proteins were first incubated for 2h at 37°C (to probe their stability) before being used in the helicase reactions. Note that linear steady-state phases were no longer detectable under “10X” conditions.

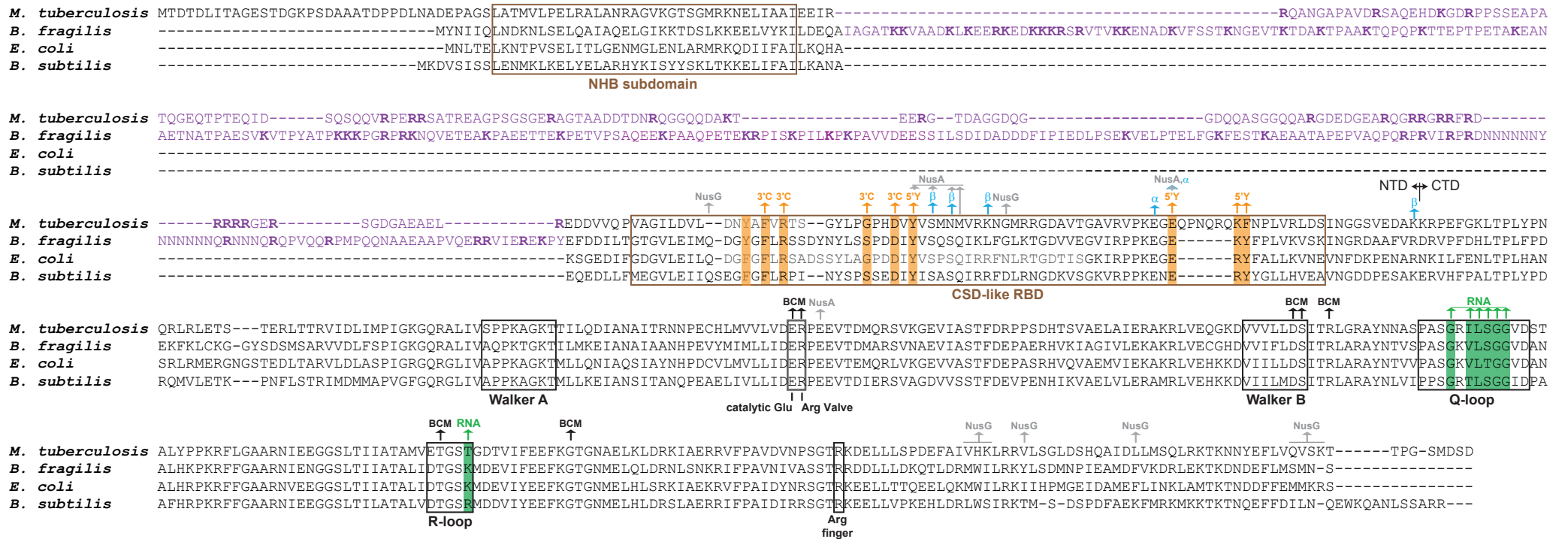
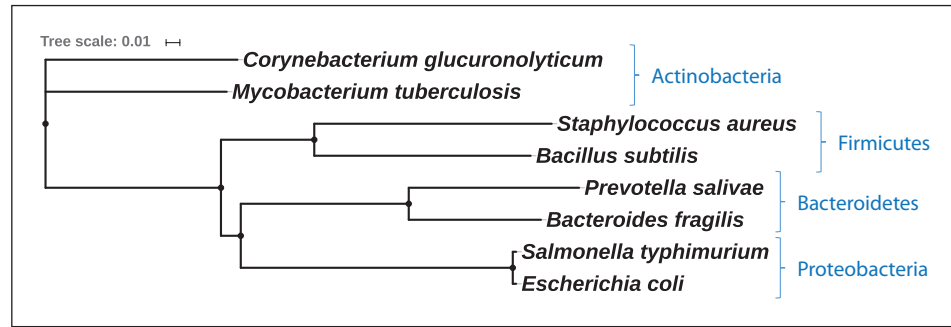
Supplementary Table 3 : conservation of Rho:TEC contacts ^a							
Rho			Interaction partner				Ref.
Ec	Bf	Mtb	identity	Ec	Bf	Mtb	
S82	S353	S254	RNAP β	K1022	Y1006	del ^b	(5)
R87	K358	R259	RNAP β	D491	D452	T410	(5)
R88	L359	K260	RNAP β	D485	E446	E404	(5)
			RNAP β	Y1018	F1002	del ^b	(5)
			NusA	E136	T143	E113	(6)
R102	R373	R274	RNAP β	D1019	I1003	del ^b	(5)
K105	K376	K277	RNAP β	H1023	K1007	del ^b	(5)
E106	E377	E278	RNAP α	K298	K302	K222	(6)
			RNAP β'	K39	L35	K29	(6)
			RNAP β'	R60	R56	R50	(6)
E108	E379	Q286	RNAP α	K297	K301	Q221	(6)
			RNAP β	K1027	A1011	del ^b	(5)
K115	K386	R293	RNAP β	E1016	L1000	del ^b	(5)
			NusA	E219	G226	G203	(6)
T276	T546	N451	RNAP β	P897	P864	P810	(6)

^a Conservation of residues in proteins from *E. coli* (Ec), *B. fragilis* (Bf), and *M. tuberculosis* (Mtb). Residues not conserved are in red. The loosely defined interactions (e.g. between regions rather than side-chains) reported in refs (5,6) are not listed here, although some involve lineage-specific RNAP domains (5,6)

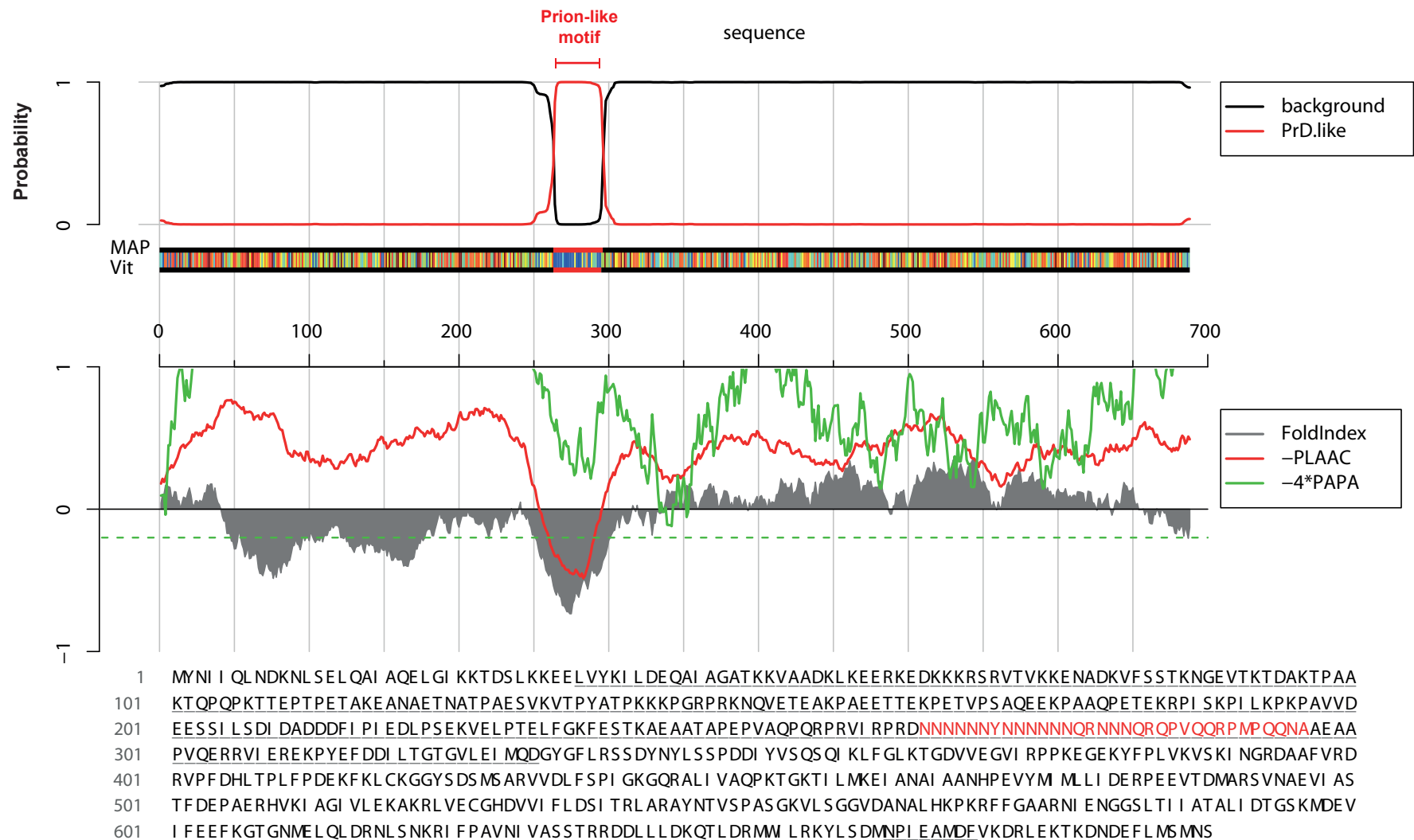
^b Deletion. See also Figure S8.

References

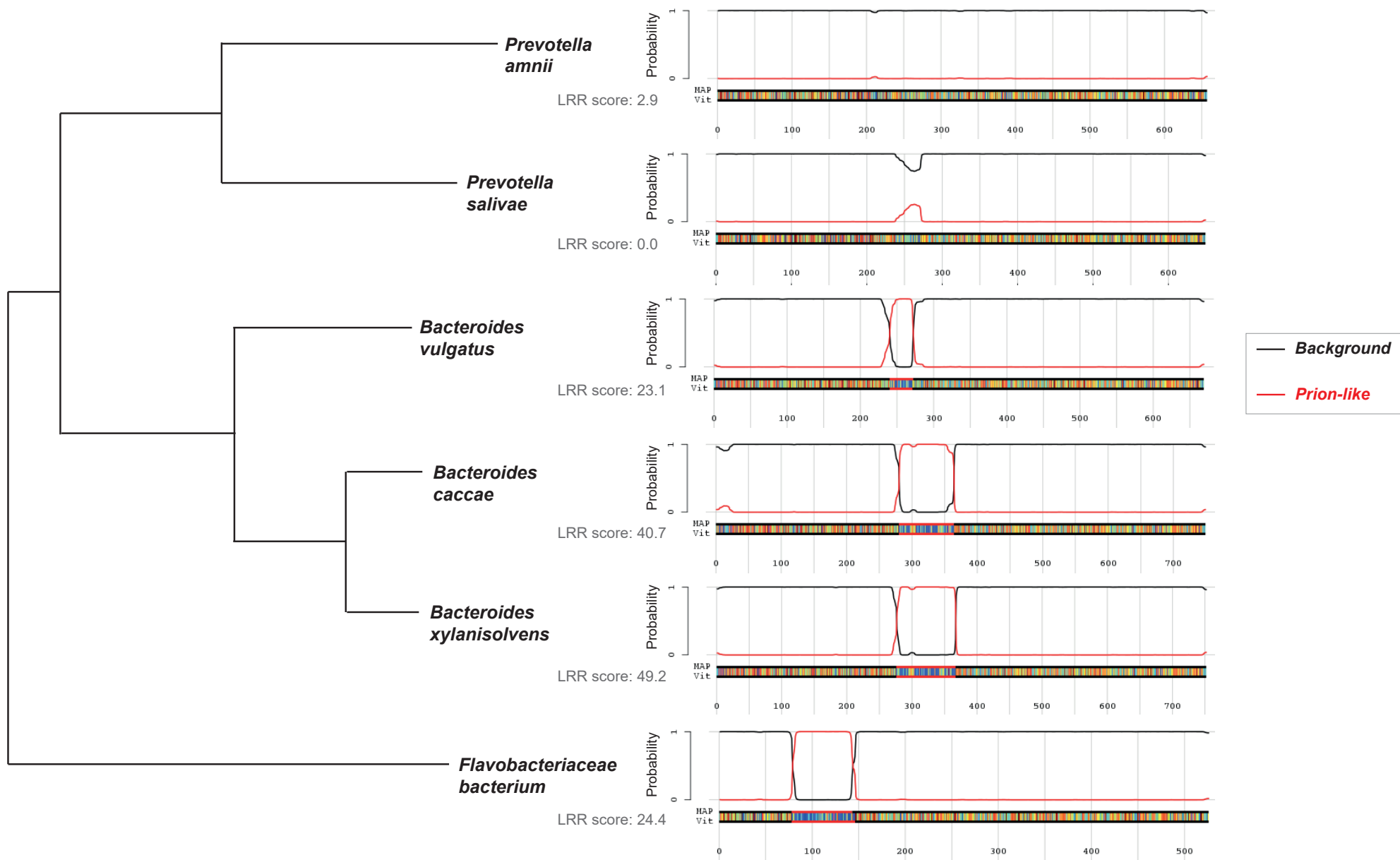
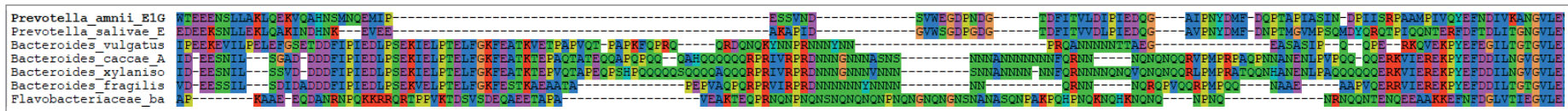
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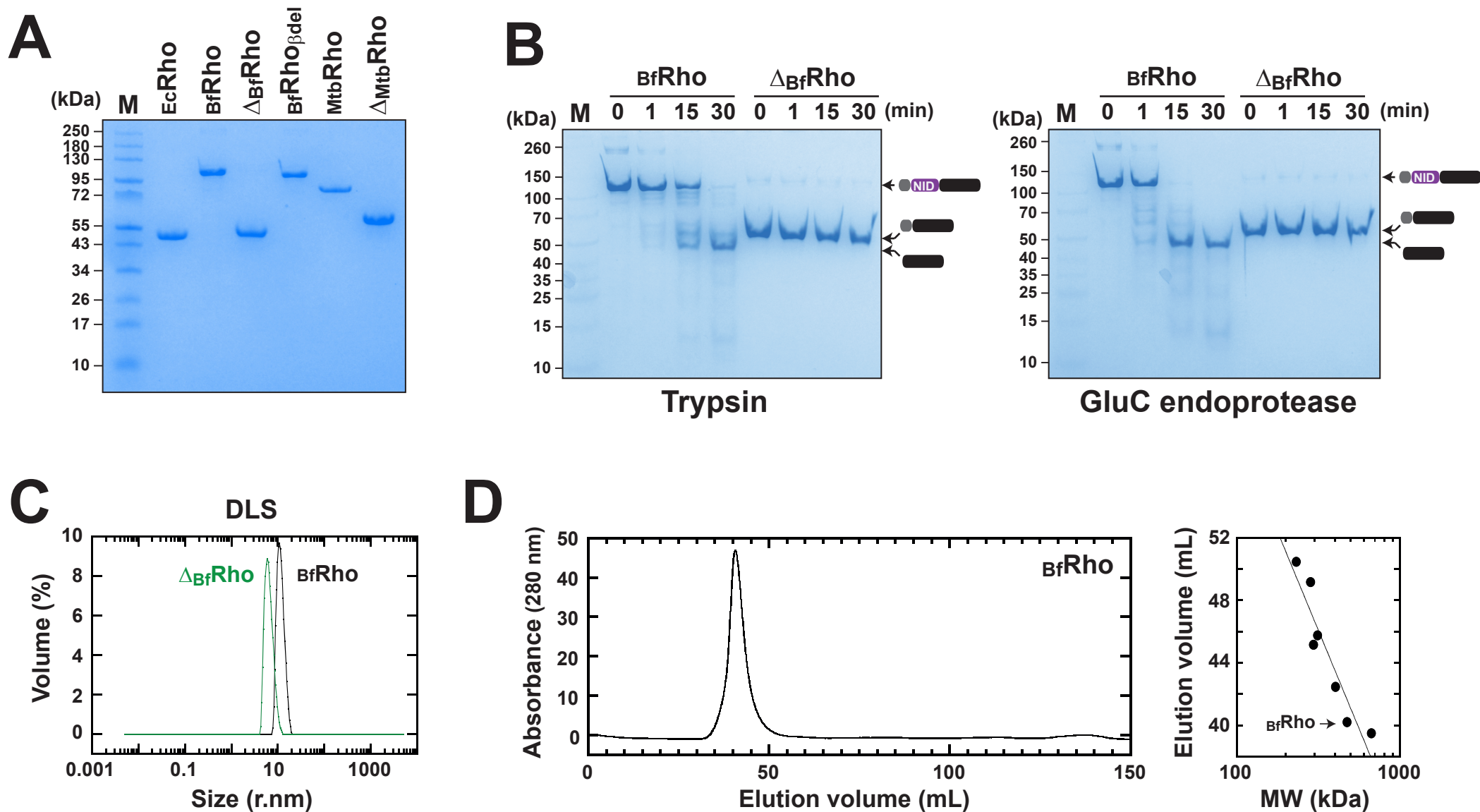
Supplementary Figure 1: Conservation of the Rho sequence across bacterial phyla. The phylogenetic tree and sequence alignment were generated with the Seaview software as described in D'Heygere et al., *Microbiology* (2013) 159, 1423-36. The NID sequences of β_{Rho} and $\text{M}_{\text{tb}}\text{Rho}$ are in purple with basic residues in bold. Features important for the function of $\text{E}_{\text{C}}\text{Rho}$ are highlighted on the sequence alignment. PBS components are highlighted in orange while SBS residues are in green. The $\text{E}_{\text{C}}\text{Rho}$ residues making the most probable contacts with $\text{E}_{\text{C}}\text{RNAP}$ and $\text{E}_{\text{C}}\text{NusA/G}$ factors in recent cryoEM and crystal structures (see main text) are indicated by blue/grey arrows.



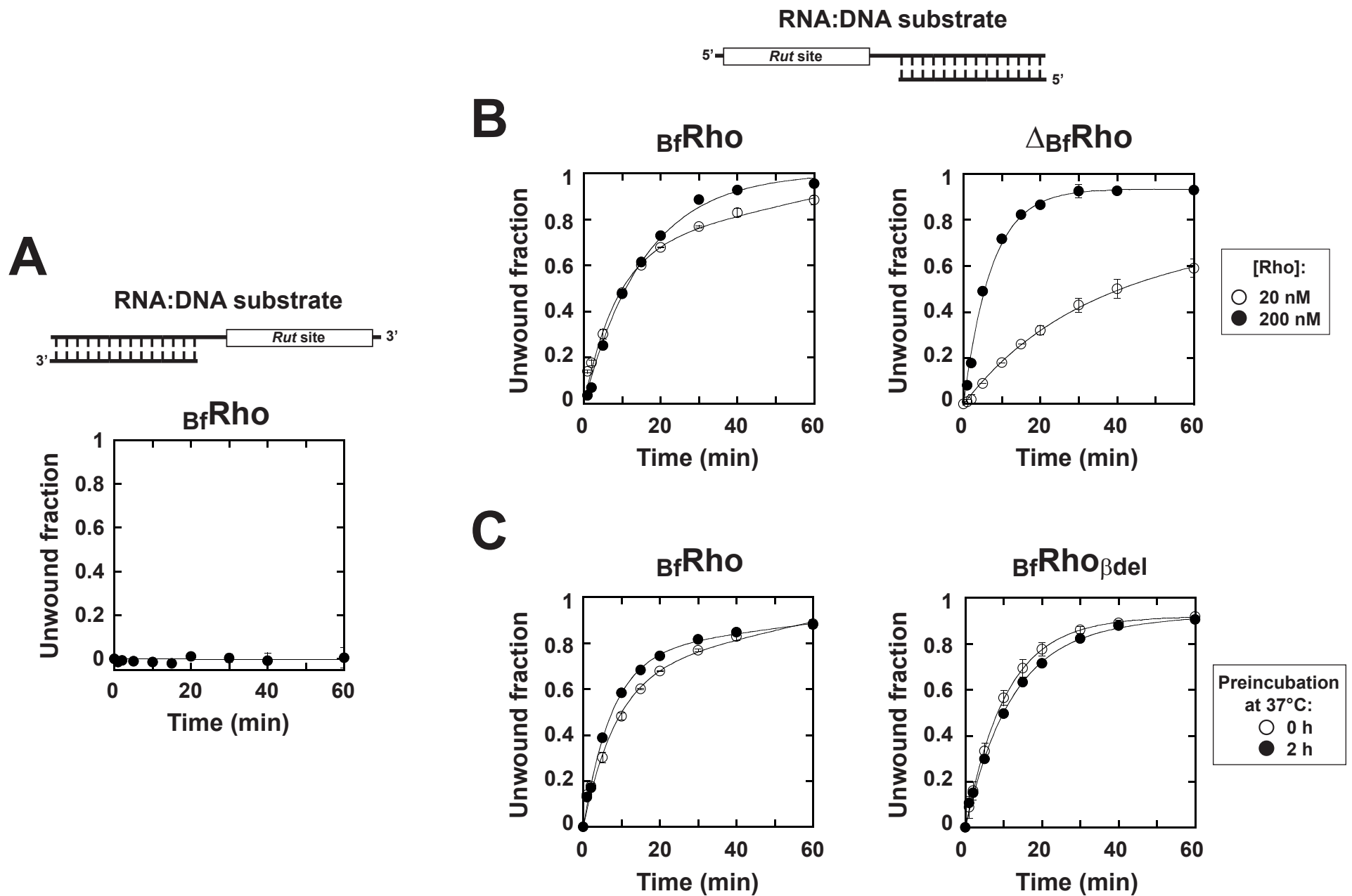
Supplementary Figure 2: Analysis of the prion-like propensity contained in the β_f Rho sequence using the PLAAC server (Lancaster et al., *Bioinformatics* (2014) doi:10.1093/bioinformatics/btu310). A screenshot of the server output is shown with the predicted prion-like sequence in the NID in red (<http://plaac.wi.mit.edu>; default parameters). The prion-likelihood LLR score is 22.6.



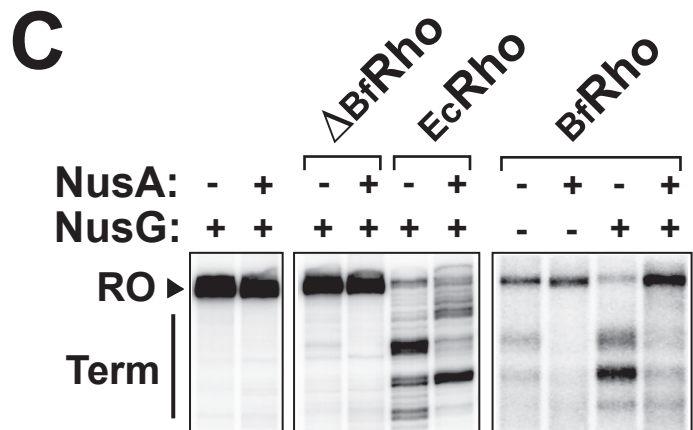
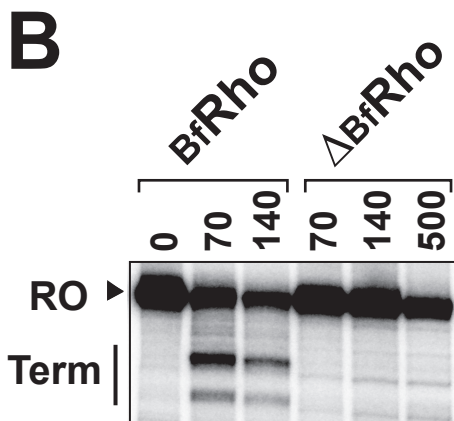
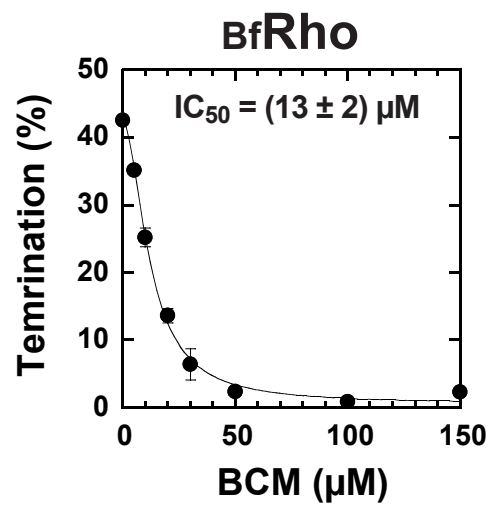
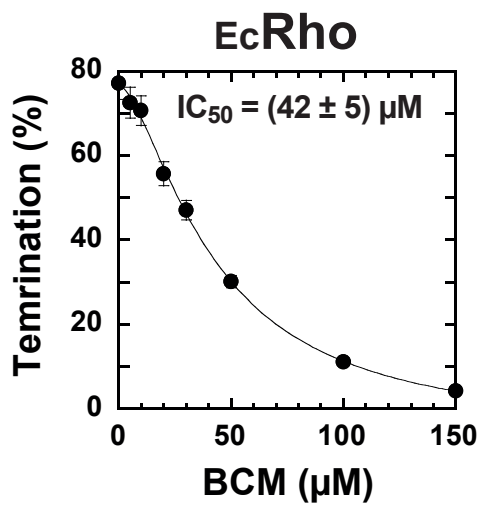
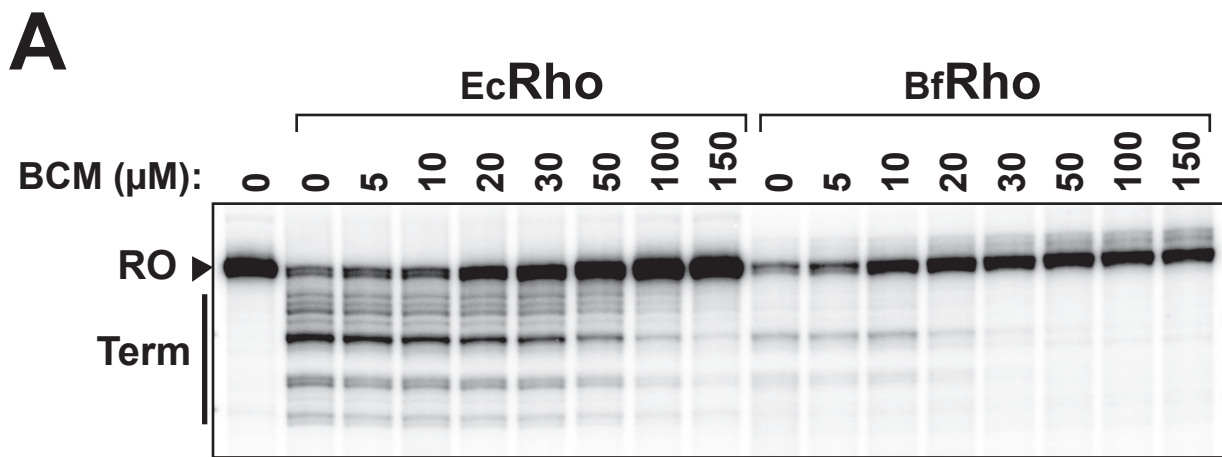
Supplementary Figure 3: Prion-like propensity of representative Bacteroidetes Rho sequences as predicted with the PLAAC server. A screenshot of the Sea-view sequence alignment zoomed on the NID region containing N/Q-rich motifs is shown at the top of the figure.



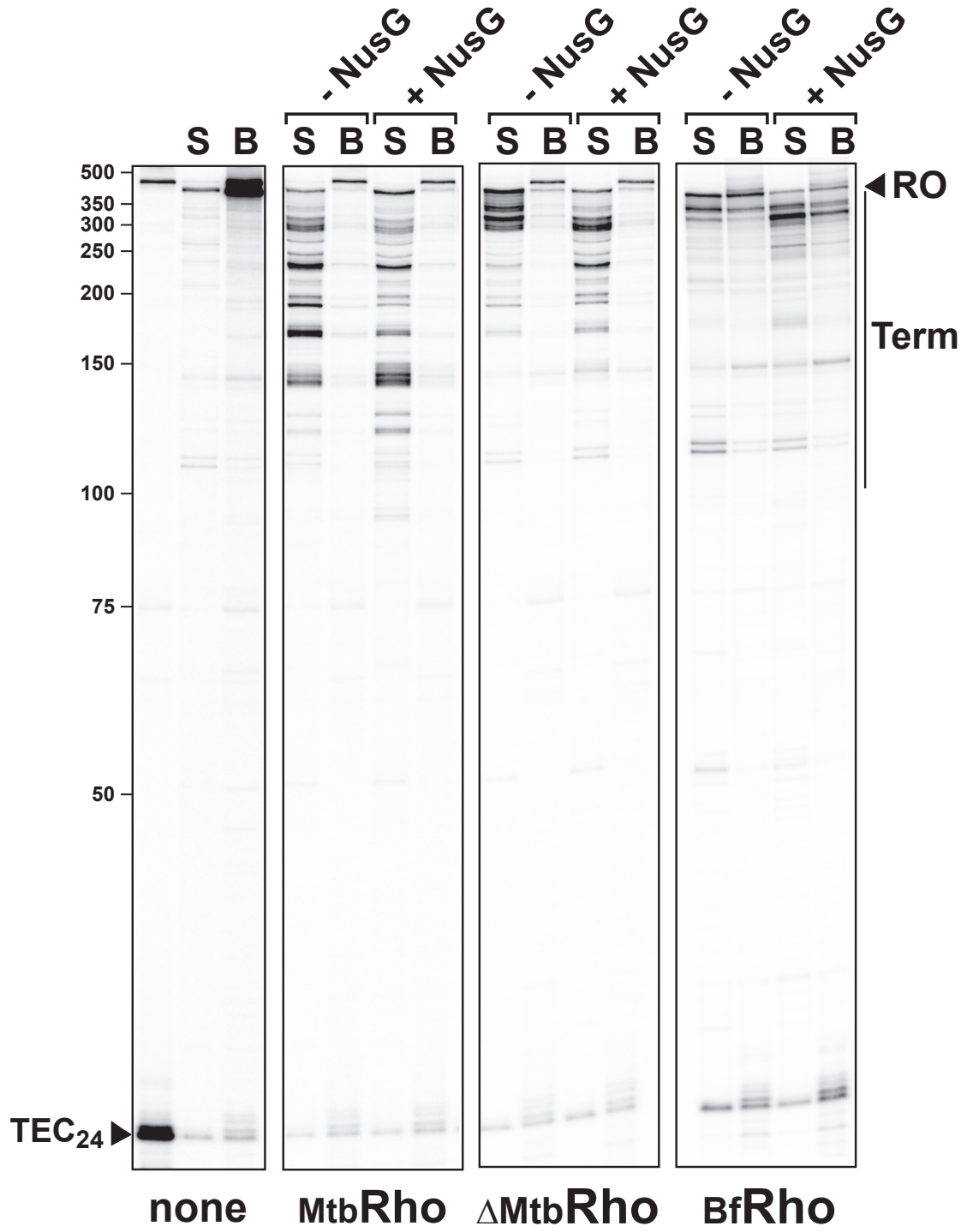
Supplementary Figure 4: (A) SDS-PAGE gel of the purified proteins used in the study. The E_c Rho (47.1 kDa) and Δ_{Bf} Rho (49.3 kDa) proteins migrate in the expected size range, the M_{tb} Rho (67.3 kDa), and $\Delta_{M_{tb}}$ Rho (52.2 kDa) display slight gel shifts as expected (Kalarickal et al., J. Mol. Biol., 2010, 395, 966-82), whereas B_f Rho (79 kDa) and B_f Rho $_{\beta del}$ (75.1 kDa) migrate much more slowly than expected (>20kDa difference in relative mobilities). Lane M corresponds to the broad range 10-250 kDa protein standard (NEB #P7712). **(B)** Protease digests of the B_f Rho and Δ_{Bf} Rho proteins support that the NID is intrinsically disordered. Reactions were performed at 37°C with 1:50 (w:w) protease in 100 mM KCl, 10 mM Tris-HCl, pH 8, 50% glycerol, 0.1 mM EDTA, 0.1 mM DTT. **(C)** Diffusion light scattering analysis indicates that B_f Rho and Δ_{Bf} Rho form monodisperse species (Zetasizer Nano S instrument). **(D)** The B_f Rho factor elutes from a Sephacryl S300 HR column (10-1500 kDa separation range) in a single peak consistent with hexamer formation. MW controls were catalase (232 kDa), E_c Rho hexamer (284 kDa), Δ_{Bf} Rho hexamer (296 kDa), $\Delta_{M_{tb}}$ Rho hexamer (314 kDa), M_{tb} Rho hexamer (404 kDa), and thyroglobulin (669 kDa).



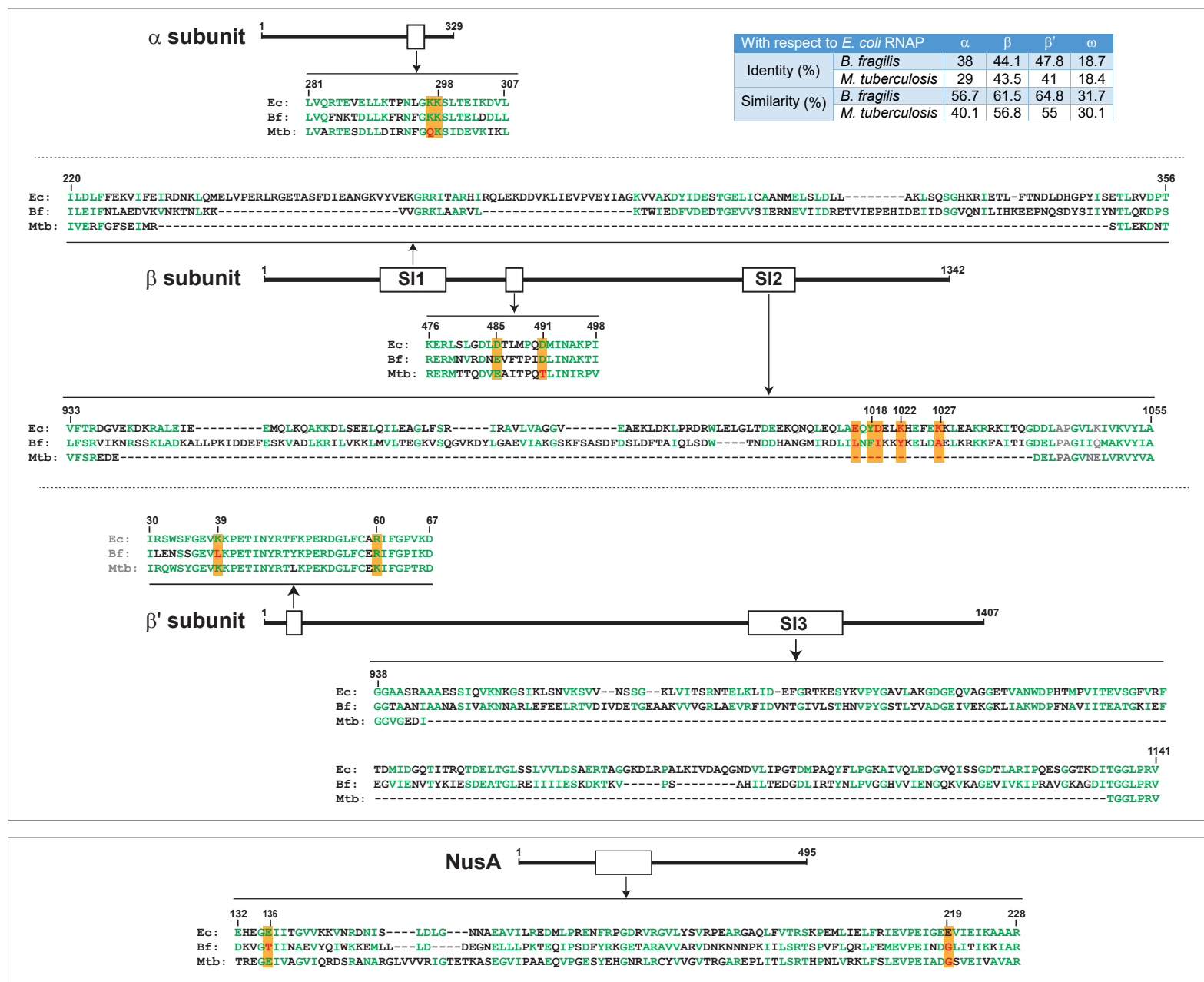
Supplementary Figure 5: RNA-DNA duplex unwinding by the B_f Rho factor. (A) B_f Rho cannot unwind a RNA-DNA duplex located upstream from the single-stranded *Rut* region; **(B)** Increasing the concentration of ΔB_f Rho stimulates duplex unwinding; **(C)** Incubating B_f Rho for long periods of time without ATP or RNA does not compromise its helicase activity. Protein stock aliquots ($\sim 1 \mu\text{M}$) were diluted to 200 nM in 1X helicase buffer before preincubation for 0 or 2h at 37°C.



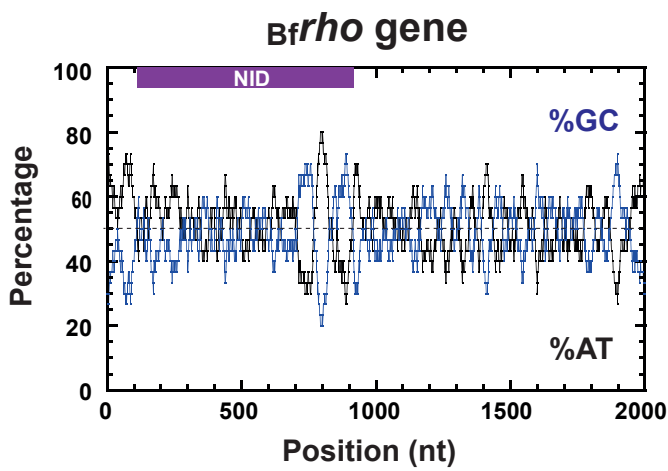
Supplementary Figure 6: Standard transcription termination experiments with the λ tR1 template. **(A)** B_f Rho is highly sensitive to the bicyclomycin (BCM) inhibitor. **(B)** Increasing the concentration of ΔB_f Rho does not alleviate its RDTT defect. **(C)** RDTT with B_f Rho is strongly delayed by E_c NusA even in the presence of E_c NusG.



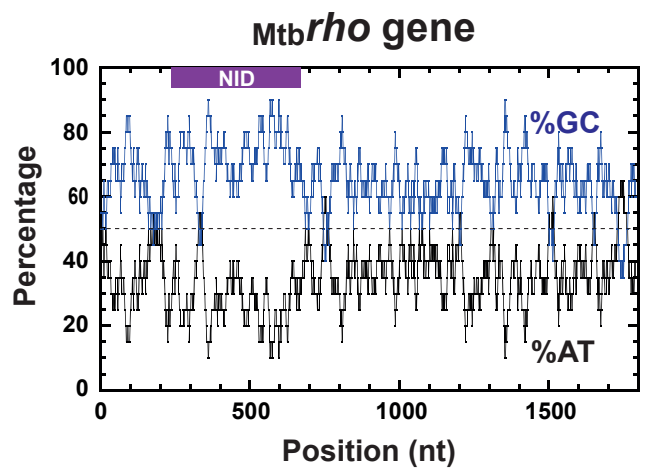
Supplementary Figure 7: Single-round transcription experiments performed with bead-affixed TECs containing biotinylated λ tR1 templates. Reactions were performed in presence of 100 mM KCl, as in figure 3. In each case, transcripts released in the supernatant (S lanes) or still bound to the beads (B lanes) are compared.



Supplementary Figure 8: Conservation of the core RNAP and NusA factor among *E. coli*, *B. fragilis*, and *M. tuberculosis*. Alignments correspond to regions involved in contacts between E_c RNAP and E_c Rho (contact residues boxed in orange with non-conserved side-chains in red) and to the lineage-specific sequence insertions present in E_c RNAP (S11, S12, S13; see Artsimovitch et al., J. Biol. Chem, 2003, 278,12344-55). Note that similar sequence insertions are found in B_f RNAP but not in M_t RNAP. Numbering is for the *E. coli* sequences.



48.2% GC (gene) vs. 43.1% GC (genome)



65.5% GC (gene) vs. 65.6% GC (genome)

Supplementary Figure 9: Distributions of G and C residues versus A and T residues along the sequences of the genes encoding *Bfrho* and *Mtbrho*. Percentages of individual bases were calculated with EMBOSS freak (Rice et al., Trends Genet., 2000, 16, 276-7) using a 20 nt sliding window.