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A bacteriophage capsid protein is an inhibitor of a conserved transcription terminator of various bacterial pathogens.

Gairika Ghosh[1], Jayavardhana Reddy, Susmit Sambhare and Ranjan Sen[*]

Laboratory of Transcription, Center for DNA Fingerprinting and Diagnostics, Tuljaguda Complex, 4-1-714 Mozamjahi Road, Nampally, Hyderabad-500 001, India.

email: rsen@cdfd.org.in; Telephone: 91 40 24749428.

[1] Graduate Studies, Manipal University, Manipal, Karnataka-576104, India

[*]Corresponding author

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Abstract.

Rho is a hexameric molecular motor that functions as a conserved transcription terminator in majority of the bacterial species, which is a potential drug target. Psu is a bacteriophage P4 capsid protein that inhibits *E.coli* Rho by obstructing its ATPase and translocase activities. Here, we explored the anti-Rho activity of Psu for the Rho proteins from different pathogens. Sequence alignment and homology modelling of Rho proteins from pathogenic bacteria revealed the conserved nature of the Psu-interacting regions in all these proteins. We chose Rho proteins from various pathogens like, *Mycobacterium smegmatis, Mycobacterium bovis, Mycobacterium tuberculosis, Xanthomonas campestris, Xanthomonas oryzae, Corynebacterium glutamicum, Vibrio cholerae, Salmonella enterica and Pseudomonas syringae*. The purified recombinant Rho proteins of these organisms showed variable rates of ATP hydrolysis on the poly (rC) as substrate and were capable of releasing RNA from the *E. coli* transcription elongation complexes. Psu was capable of inhibiting these two functions of all these Rho proteins. *In vivo* pull down assays revealed direct binding of Psu with many of these Rho proteins. *In vivo* expression of *psu* induced killing of *M. smegmatis*, *M. bovis, X.campestris, and S.enterica,* indicating Psu-induced inhibition of Rho proteins of these strains under physiological conditions. We propose that the "universal" inhibitory function of the Psu protein against the Rho proteins from both the gram-negative and gram-positive bacteria could be useful for designing peptides having anti-microbial functions, and these peptides could be a part of synergistic antibiotic treatment of the pathogens through compromising the Rho functions.

Importance

Bacteriophage-derived protein factors modulating different bacterial processes could be converted into unique antimicrobial agents. Bacteriophage P4 capsid protein Psu is an inhibitor of *E. coli* transcription terminator, Rho. Here we show that apart from antagonising

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E. coli Rho, Psu is able to inhibit Rho proteins from various phylogenetically unrelated gram-negative and gram-positive pathogens. Psu upon binding to the Rho, exerted the inhibition by affecting the ATPase and RNA release functions of these Rho proteins. The expression of Psu *in vivo* kills various pathogens like, *Mycobacterium* and *Xanthomonas*. Hence, Psu could be useful to identify peptide sequences having anti-Rho activities and might be a part of synergistic antibiotic treatment against the pathogens.

Key words: Rho, Psu, transcription termination, RNA polymerase, pathogen

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Introduction

The Rho-dependent transcription termination in bacteria is an important step in regulation of gene expression (1-5). The homo-hexameric transcription terminator, Rho is a RNA-dependent helicase motor that functions as a transcription terminator. Upon its association with the *rut* site on nascent RNA emerging from the elongation complex (EC) (6, 7), it becomes ATPase competent and translocates along the RNA towards the EC, and eventually dislodges the latter (8). Recent studies have revealed that Rho-dependent termination is involved and instrumental in regulating many physiological processes (9). This multifaceted functional outcome of the Rho-dependent termination process makes Rho an ideal target of different bactericidal agents. With the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains, it has become necessary to look for alternative avenues and additional drug targets. It is likely that due to its involvement in many physiological pathways, Rho-inhibition could be part of a synergistic antibacterial treatment strategy.

The Psu (polarity suppression) is an unconventional capsid organizing protein of the bacteriophage P4 that moonlights as a specific inhibitor of Rho (10). It binds and antagonizes Rho *in trans* by creating mechanical hindrance to Rho translocation (11) via a physical blockade of the RNA exit point of the Rho central channel upon formation of a V-shaped cap-like knotted homodimer structure (12, 13). Psu is an α-helical protein having a solvent-exposed flexible C-terminal domain (helices 6 and 7; Ref. 12) that is responsible for its association with Rho, and a N-terminal domain that sustains the stability of the protein (14). Overexpression of Psu in *E. coli* induces lethality due to robust antitermination at the Rho-dependent terminators throughout the genome. Rho contains an unstructured loop-forming stretch of residues in its C-terminal domain that binds with Psu (figure 1B), which is assisted by another helical region that surrounds the Rho central channel (13).

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We hypothesized that the bacteriophage derived Psu might be useful for functioning as a platform to design new antagonist(s) of the Rho protein, especially those made from the C-terminal helices 6 and 7. So far, the antagonism of Psu was demonstrated for the Rho protein from the model organism *E. coli*. In order to establish it as a platform for drug-design, it is imperative to prove universal antagonism of Psu against various Rho proteins both from the gram-positive and gram-negative bacteria. In this report, we have chosen and characterized the biochemical activities of the Rho proteins of selected bacterial pathogens like, *Mycobacterium tuberculosis*, *Salmonella enterica*, *Vibrio cholerae*, *Xanthomonas oryzae*, *Xanthomonas campestris*, *Pseudomonas syringae* and *Corynebacterium glutamicum*. *In vitro* biochemical assays showed that Psu is capable of inhibiting the ATPase functions of these Rho proteins. These Rho proteins were unable to terminate at a Rho-dependent terminator in the presence of Psu. Psu also inhibited RNA release by these Rho from a stalled EC and showed direct interaction with these proteins. *In vivo* expression of *psu* in the *M. smegmatis*, *M. bovis* and *X. campestris* induced severe growth defects to the hosts, and it inhibited the *S. enterica* Rho functioning in *E. coli*. These results strongly indicated that Psu is capable of functioning as a bona fide antagonist of Rho proteins from a wide spectrum of bacterial species. We further speculate that derivatives of Psu could be a part of synergistic antibiotic treatment by offering bacterial pathogens with compromised Rho functions.

Results

Selection of different pathogenic bacteria.

To establish the universal antagonism of the bacteriophage P4 coat protein, Psu, against the Rho proteins from a diverse set of bacteria, we have chosen the bacterial species, *Salmonella*, *Vibrio*, *Xanthomonas*, *Pseudomonas* that are the Gram-negatives, and *Mycobacteria* and *Corynebacteria* are from the Gram-positive category. They are pathogens for a wide variety of organisms; *Xanthomonaus oryzae* (Rice), *Xanthomonas campestris*

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(Cabbage, Mustard) and *Pseudomonas syringae* (wide variety of hosts) are the phyto-pathogens, while *Salmonella typhi*, *Vibrio cholerae*, *Mycobacterium tuberculosis*, and *Cornebacterium diptherae* are human pathogens. For each of the human pathogens, we have carried out experiments also with their non-pathogenic porotypes such as *M. smegmatis* and *M. bovis* (BCG) and *C. glutamicum*. The phylogenetic tree, constructed based on the 16S rRNA sequence of these bacterial species is shown in the figure 1A, and it confirms the diversity of these pathogens. Majority of these organisms are slow-growing species having contrasting features compared to *E. coli*, and it is expected that the Rho proteins in each of these species function under varied physiological conditions. Therefore, it is important to establish the inhibitory power of Psu against each of them individually.

Comparison of Rho proteins from different pathogens.

Earlier studies from our laboratory, revealed the existence of a conserved disorganized loop region connecting the N-terminal to the C-terminal domains of the *E. coli* Rho, to be the main interacting site of Psu (figure 1B-D) (13). A multiple sequence alignment analysis revealed that this particular loop region is well conserved in different Rho proteins (figure 1B). To establish the structural conservation of this loop region, we built homology models of Rho proteins from different bacterial pathogens that we have chosen for our studies using the published structure of *E.coli* Rho as the template (figures 1C and D; PDB 3ICE) (15). The modelling showed that the Psu-binding loops on different Rho proteins are surface exposed and accessible to the solvent. However, the size and the finer structural details of the loops varied in the different Rho proteins. The structural conservation of the Psu-binding regions of all these Rho proteins is suggestive of the Psu-Rho interaction in these species.

Characterization of the Rho proteins from different pathogens.

Rho proteins of the bacterial species that we have chosen for our study were not characterized except those from the *S. enterica* (16) and *M. tuberculosis* (17). Therefore,

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120 characterization of all these Rho proteins are important before attempting to establish the
121 inhibitory property of Psu against them. We cloned and purified the recombinant versions of
122 all these Rho proteins from *E.coli* (figure 2A). We were unable to express the *M. smegmatis*
123 Rho in different varieties of the BL21 (DE3) cells under different conditions, thus we
124 proceeded *in vitro* experiments only with the *M. tuberculosis* Rho.

125 We used poly (rC), the best co-factor that induces the ATPase function of the *E.coli*
126 Rho, to measure the rates of ATPase activities of all these different Rho proteins (figures 2 B-
127 D). The rates are tabulated in figure 2D. The rate of ATPase activity of the *Salmonella* Rho
128 was observed to be comparable to that of the *E. coli*, whereas that exhibited by the Rho
129 proteins of *M. tb*, *C. glutamicum* and *P. syringae* were the slowest in the presence of poly
130 (rC) (> 10-fold slower; note the scale differences of the X-axes in the different plots). The
131 same rates obtained for the Rho proteins of *V.cholerae*, *X. campestris* and *X.oryzae* were 2.5-
132 4-fold slower than their *E.coli* counterpart.

133 *V. cholerae*, a Gram-negative bacterium belongs to a different family from that of the
134 *E. coli* (see figure 1A). Rho from this species has several residual differences in the primary
135 RNA binding site (PBS; figure S1) compared to that of *E. coli*. The *Xanthomonas* species are
136 Gram-negative plant pathogens having high GC content in their genome. The *X. oryzae* and
137 *X. campestris* Rho sequences are almost similar to each other with only three amino acids
138 differences in the PBS, while having considerable differences with their *E. coli* counterpart.
139 Both the *Xanthomonas* species have a 7 amino acids insertion at the beginning of the N-
140 terminal domain (NTD) and several residue changes in the PBS, ATP-binding sites and
141 secondary RNA binding sites (SBS) when compared to the *E. coli* Rho. *Pseudomonas*
142 *syringae* is also a Gram-negative plant pathogen and its Rho protein has residue differences
143 from the *E.coli* Rho in both the PBS and SBS regions. The *Mycobacteria* and
144 *Corynebacteria* are gram-positive organisms having highly GC-rich genome and their Rho

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proteins have an extended NTD insertion, a smaller insertion (six amino acids in *M. tuberculosis* and eleven amino acids in *C. glutamicum*) as well as amino acid sequence differences from the *E.coli* Rho in the PBS. Some of them also have alterations in the residues in both the ATP-binding sites and the SBS (see figure S1).

Even though the homology model building (figure 1C and D) suggests an overall similarity in the 3D structure of all these Rho proteins, these diversities at the amino acid sequence level might have affected the sequence specific interactions between the poly(rC) and the Rho-PBS. It is possible that the poly(rC) is not the optimum substrate for many of these Rho proteins. Weak interactions at the PBS is likely to be the major reason for the slower ATPase activities. In addition to the sequence variations, the Rho proteins from the gram-positive bacteria have an extended NTD insertion, which could have caused some effects. Moreover, all the organisms, other than *Salmonella* and *Vibrio*, have distinctively longer generation times or slower growth rates, compared to that of *E. coli.* This specific trait is also consistent with the slow rate of ATPase activity of their respective Rho proteins.

In spite of this wide range of ATPase activities of all these Rho proteins, we have used this basic property of Rho because it is a hallmark for any ATPases to study the effect of Psu using either poly(rC) (both long and oligomers) or natural RNA with *E.coli* Rho-dependent terminator sequences as the co-factor.

Inhibition of ATPase activity by the Psu protein.

Psu inhibits the ATPase activity of the *E. coli* Rho quite efficiently (11). We performed the ATPase activity assays of different Rho proteins both in the presence and absence of the Psu in a similar way as described before. In addition to the WT Psu, we have also used a non-functional Psu mutant, ΔCTD10 (having a 10 amino acids deletions in its C-terminal; 11) as a negative control. Using poly(rC) as an inducer, we observed that the ATPase activities of the Rho proteins from the *M.tb* and the *C.glutamicum* were efficiently

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inhibited by Psu (figure 3A), whereas the inhibition was moderate for the Rho proteins from both the *Xanthomonas* species. In the presence of poly(rC), we did not observe significant inhibition of the ATPase activities of the Rho proteins from the *Salmonella*, *Pseudomonas* and *Vibrio* species. We repeated the experiments with a shorter poly(rC) oligo, rC_{25}, to slow down the rate of ATPase activity (18). Now Psu was able to inhibit the ATPase activity of the Rho from the *Salmonella sp.* However, the ATPase function of the Rho proteins from the *Vibrio* and *Pseudomonas* still was mildly affected by Psu. It should be noted that in case of the *E. coli* Rho, we used a natural RNA having *λtR1* terminator sequence because its ATPase activity on the poly(rC) is too fast and could not be inhibited by the Psu (11). The mutant Psu failed to illicit inhibition to any of the Rho proteins used in this study. These results indicate that in general, Psu-mediated inhibition occurred more readily when the rates of ATPase activity were slow. The ineffectiveness of Psu on the ATPase functions of the *Pseudomonas* Rho protein suggests that it may not be an efficient inhibitor for this protein.

Inhibition of in vitro transcription termination by Psu protein.

Psu is capable of inhibiting *E.coli* Rho-dependent termination in an *in vitro* purified system (11). Next, we assayed the Psu mediated inhibition of the transcription termination functions of the different Rho proteins chosen for this study using the *E.coli* transcription system. We have used a linear DNA template carrying the λt_{R1} terminator sequence cloned downstream of a strong T7A1 promoter (figure 3B). On this template, a 22-mer EC (EC_{23}) was made first and then was chased with 20 μM NTPs through the terminator region in the presence of only Rho or Rho with WT Psu (figure 3B). We used lower concentrations of NTPs, to check the activity of Psu. Under this stringent condition the *E.coli* Rho is very efficient for termination, but under the same condition, among all the Rho proteins only those from *M. tb.*, *S. enterica* (figure 3B) and *C. glutamicum* (figure S2B) were able to terminate an elongating *E.coli* RNAP (see figure S2A for the inability of the other Rho

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proteins). In the presence of WT Psu, significant fractions of transcripts were found to read-through the terminator regions (figure 3B, lanes 3, 5, 7 and the bar diagrams) when the *M. tb* and the *S. enterica* Rho proteins were used, whereas the effects of Psu was moderate on the *C. glutamicum* Rho (figure S2B). These results indicate that Psu is capable of inhibiting the termination function of the Rho proteins from these pathogens.

As majority of the Rho proteins were not able to terminate the elongating *E.coli* RNAP, we hypothesized that these Rho proteins might be able to release RNA from a stalled elongation complex (EC) as kinetic coupling between Rho and the RNAP is not required if the latter is stalled on the DNA template. So, we designed a set-up where the EC is stalled on a template bound to magnetic beads, at a particular position inside the *trpt'* terminator (an *E.coli* Rho-dependent terminator) region using lac repressor as a roadblock (figure 3C). In this set-up, the terminated RNA would be released in the supernatant. After stalling the EC, all the NTPs were removed by washing the beads, following which Rho plus ATP either in the absence or presence of Psu were added, and the RNA release was observed in the supernatant (figure 3D). In the absence of WT Psu, Rho proteins from all the organisms, except *Pseudomonas* (figure S2C) could release RNA very efficiently. It should be noted that for most of the Rho proteins from pathogens took longer time to release RNA from a divergent EC made of the *E. coli* RNAP (figures 3D and E). In the presence of WT Psu, the RNA release by all the Rho proteins used in this study was significantly reduced from the stalled EC (figures 3D and E). RNA polymerase release assays (equivalent to RNA release assays), with the mutant Psu, ΔCTD10, showed that it was unable to inhibit the dislodging of the polymerases as compared to its WT counterpart from the stalled ECs by the various Rho proteins (figure S3A). These observations strongly indicate that WT Psu is capable of specifically inhibiting the Rho proteins from several pathogenic species that is comparable to that observed for the *E. coli* Rho inhibition by this bacteriophage protein. It should also be

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noted that the Psu-induced inhibition of the RNA release efficiencies by different Rho proteins are qualitatively correlated with that observed for their respective ATPase assays. As the Rho-dependent termination function is a fundamental biological process of the prokaryotic world, we postulate that Psu would be able to inhibit these Rho proteins in the presence of their respective transcription system. As *Pseudomonas* Rho was not able to release RNA from this stalled EC, we refrain to conclude that Psu would be an efficient inhibitor of Rho of this bacterium.

Specific interaction of Psu with Rho proteins from different pathogens.

Earlier a specific Psu- *E. coli* Rho complex formation was only demonstrated *in vivo* by co-overexpressing both the proteins in the same strain (11, 13, 14). We failed to get a stable *in vitro* complex with the purified components. This is most likely due to the mis-folding propensity of the unusually knotted dimer conformation of Psu (12). Following the same procedure as before (11), we co-overexpressed a His-tagged Psu and a non-His tagged WT Rho from two different pET vectors and pulled down the complex using the Ni-NTA beads. Typically, after induction, the cell lysate was directly loaded onto Ni-NTA columns. The eluted fractions of WT Psu contained significant amounts of Rho proteins of *E. coli*, *S. enterica* and *V. cholerae* (figure 4B). For the *X. campestris* and *X. oryzae* Rho, both Rho and Psu were overexpressed in separate strains, and then the two lysates were mixed and loaded onto the Ni-NTA column. In all these cases, ~30% of Rho proteins were found to be associated with Psu that is comparable with what we observed for the *E.coli* Rho. *P.syringae* Rho was unable to bind to Psu even under *in vivo* conditions, which again indicated that Psu might not be an efficient antagonist of this Rho protein (figure S3B). These interactions were specific as they were 4 to 7-fold weaker when a Psu mutant, ΔCTD10 Psu, defective in inhibiting the Rho protein (11), was used in the various experiments (figure 4B). The expression levels of *M. tuberculosis* and *C. glutamicum* Rho proteins were very poor to

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measure the complex formation by this procedure. These results suggest *in vivo* complex formation of Psu with some of the Rho proteins, at least in the cytoplasm of the *E.coli*, and we concluded that this interaction plays important role in inhibiting the Rho function.

Psu-induced cell mortality upon expression in the pathogens.

So far, we have established that the bacteriophage P4 coat protein, Psu, is capable of inhibiting *in vitro* functions of Rho proteins from a diverse set of bacteria most likely via a direct interaction with the latter. In order to design a potent inhibitor of Rho from Psu, it is imperative that the latter's expression in different bacterial strains should cause lethality or toxicity. We chose two of the *Mycobacterium* (gram positive) and one of the *Xanthomonas* (gram-negative) strain to study the *in vivo* effects of Psu, based on their availability and ease of handling. As the *Salmonella* Rho complements the deficiency of the *E.coli* Rho *in vivo*, we used an *E.coli* strain expressing *Salmonella* Rho to monitor the *in vivo* effects of the Psu. As the Rho proteins of *M. tb* and *M. bovis* have the same sequence and also due to the relative ease of handling of the *M. bovis* strains compared to that of the *M. tb.* ones, we monitored the *in vivo* effect of Psu in a *M. bovis* strain.

The Rho proteins of *Salmonella enterica* and *E. coli* are 99% homologous and thus the former can complement *E. coli* Rho *in vivo*. We deleted the chromosomal copy of *rho* of an *E. coli* MG1655 strain and supplied the *S.enterica* Rho from a plasmid pHYD3011 (pRS1801). Subsequently, the strain was transformed with an IPTG- inducible plasmid having WT *psu* cloned under a P_{tac} promoter (figure 5A). When this strain was streaked on plates containing the increasing concentrations of IPTG, causing the expression of Psu, severe growth inhibition was observed (figure 5A) that is similar to what was observed for *E.coli* Rho earlier (11, 14). This indicated that Psu inhibits *S. enterica* Rho efficiently *in vivo*.

Xanthomonas campestris is a Gram-negative plant pathogen (infecting cabbage, mustard etc.) that produces a diffusible signal factor (DSF) responsible for the cell-cell

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communication and different pathogenic aspects of the bacteria. We cloned the WT and the ΔCTD20 *psu* (having a 20 amino acids deletion in its C-terminal; 11) under the control of a DSF-inducible promoter (P_{eng}, pRS1574 and pRS1845, respectively) so that the naturally produced DSF will induce the expression of the Psu inside the *X. campestris* (figure 5B). We have used the strains, Xcc8004 (DSF producing) and Xcc8523 (*rpfF* mutant; DSF deficient) and transformed them with either pRS1574 (WT *psu*) or with pRS1845 (ΔCTD20 *psu*) or with pRS1508 (empty vector). WT Xcc8004 did not produce any transformants when transformed with pRS1574, whereas Xcc8523 produced healthy colonies under the same conditions. Healthy colonies were also formed by both the strains when they were transformed with the empty vector pRS1508 or with the Psu mutant expressing pRS1845 (figure 5B). These observations indicated that *rho* is essential in *X. campestris* and its specific inhibition by Psu leads to cell mortality.

We used *M. smegmatis* strain mc^2155 and *M. bovis BCG* to monitor the effect of *in vivo* expression of Psu . We electroporated *M. smegmatis* with the pSTKT plasmids having WT *psu* (pRS1724), ΔCTD20 *psu* (pRS1837; 20 amino acid deletion at the C-terminal) and an empty vector (pRS1511). In this vector, the *psu* genes are cloned under anhydrous tetracyclin (ATc) inducible P_{tet} promoter. The WT psu transformants were very small and few in number, and upon re-streaking they exhibited very poor growth (figure 5C), whereas the transformants expressing ΔCTD20 *psu* or having only the vector, upon re-streaking, grew well (figure 5C). We also performed similar experiments using the *M. bovis BCG* strain. There were very few transformants with heterogeneous sizes when the strain was transformed with pRS1724, whereas high number of transformants were observed when it was transformed with either pRS1837 or pRS1511 (figure 5D). Upon re-streaking these transformants, the WT *psu* expressing colonies showed very poor growth compared to those expressing the mutant *psu.* Interestingly, in all the cases described (figures 5C and D), the

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295 WT Psu inhibited growth even in the absence of the inducer, ATc. Basal level expression of
296 Psu was sufficient to cause severe growth defects in both of these *Mycobacterium* strains.
297 Therefore, we concluded that Psu functions as a strong inhibitor of the *Mycobacterium* Rho
298 *in vivo*.

299

300 **Discussion**

301 The bacteriophage P4 capsid coat protein moonlights as a strong inhibitor of the
302 *E.coli* transcription terminator, Rho (11). Rho is highly conserved protein among the
303 prokaryotes (figure 1), and hence we hypothesized that Psu may function as a "universal"
304 inhibitor of this transcription terminator from different bacteria. Here we tested the inhibitory
305 properties of the Psu protein against a diverse set of the Rho proteins (figure 1) and showed
306 unequivocally that Psu efficiently antagonizes the two most important functions of Rho,
307 namely the ATPase activity and the transcription termination function (figure 3), which
308 indicates a direct complex formation between the Psu and Rho proteins from different
309 species. We demonstrated this complex formation *in vivo* with the Rho proteins from four
310 representative bacteria (figure 4). Most importantly, we showed that *in vivo* expression of the
311 Psu protein induces mortality of four pathogenic bacteria by antagonizing their transcription
312 termination function (figure 5). We concluded from the aforementioned results that this
313 *E,coli* bacteriophage protein is capable of functioning as an universal antagonist of
314 transcription termination of a wide range of bacteria (both gram positive and gram negative).
315 We hypothesize that this phage protein could be useful for designing peptide-inhibitor(s) of
316 the Rho.

317 It is interesting to note that Psu functions across genus boundaries when P4 is such a
318 narrow host range phage. . Most of the bacteriophage derived protein factors target conserved

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host machineries, like RNA polymerases etc. The recently solved structures of the *E.coli* transcription modulators from the lambdoid phages, N (19) and Nun (20) in complex with the transcription elongation complex revealed that these factors target conserved regions around the active center of the RNAP. Hence, it could be predicted that these transcription regulators would also function with RNA polymerases from other bacteria. Similarly, even though Psu is a unique protein of *E.coli* phage P4, it targets the universal ATPase domain present in this conserved transcription termination factor Rho of different bacteria. Therefore, the host range of the phages and the nature of the cellular targets may not necessary be correlated.

The Rho protein could be a potent drug target for following several reasons. I) It is structurally and functionally highly conserved in most of the bacteria (1), which makes drug designing possible for the Rho proteins from different pathogens based on the structure of the *E.coli* Rho (15). II) The Rho-dependent termination is involved in many important physiological processes [9], affecting any of them could cause lethality of the bacteria. III) It has been recently shown that the Rho-dependent termination is involved in the pathogenicity of the *Mycobacterium tuberculosis* (21). Bicyclomycin is the only known antibiotic that binds to Rho (22-24), but its application is limited due to its inability to cross the cell membrane of the gram positive pathogens and due to its high cytotoxicity (25, 26). In this backdrop, the present study establishing the potential of Psu to inhibit Rho proteins from wide varieties of pathogens could enable us to design new Rho inhibitor(s). Compromising the Rho function might also be used as a component of co-synergistic treatment regime together with other antibiotics, which would have great implications in the treatment of Multi-drug resistant (MDR) and Extensively-drug resistant (XDR) bacterial strains.

We have shown earlier that the helical C-terminal 20 amino acids region of Psu makes direct contacts with Rho (12, 13). It is revealed from biochemical probing and its crystal structure (13) that the helices 6 and 7 forming the C-terminal Rho binding region are

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surface exposed and forms the tip of the arms of the V-shaped knotted dimer of Psu. It appears from the structure that the rest of the Psu dimer functions as a scaffold to hold these helices nearer to the Psu-binding loop region (see figure 1) of the Rho so that the local concentrations of the helices increases due to the reduction of the entropy of the C-terminal domain of the former. We speculate that the sequences of these helices could function as a template to design peptide inhibitors of the Rho protein. Ideally, these peptide sequences must have higher affinities for the Rho protein so that they can bind in the absence of rest of the Psu-scaffold. It would be interesting to know whether these two helices or their sequence variations in isolation could interact with Rho and elicit the antagonizing function.

The bacteriophages are the large reservoirs of unique proteins that are capable of modulating many bacterial machineries for their own advantage. Like the Psu, most of these protein modulators do not have any homology with the known proteins from all the three kingdoms. The modulation of the host machineries by them are usually executed through direct interactions with the key host proteins and regulatory DNA and RNA sequences, leading to complete shutdown of the host metabolism and the eventual killing. In addition to the effectiveness of these phage modulators, they are small in size, which makes them more amicable to function as a platform for designing new peptide inhibitors. A crude estimate measures the number of bacteriophage particles to be 10^{31} (27). Therefore, theoretically, this reservoir is capable of producing "infinite" numbers of these modulators specific for each of the pathogenic bacteria, and the number surpasses the capacity of the chemical libraries that are presently in use. We envision that the bacteriophage-modulator libraries, products of natural origin, could become a cheaper alternative to the chemical libraries.

Materials and Methods.

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Materials. NTPs were purchased from GE Healthcare. [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]CTP (3000 Ci/mmol) were obtained from Jonaki, BRIT (Hyderabad, India). Antibiotics, IPTG, lysozyme, DTT and BSA were from U.S. Biochemical Corp. Restriction endonucleases, polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. WT *E. coli* RNA polymerase holoenzyme was purchased from Epicenter Biotechnologies and New England Biolabs. Streptavidin coated magnetic beads were from Promega. Taq DNA polymerase was obtained from Roche Applied Science. Ni-NTA-agarose beads were from Qiagen.

Details of the bacterial strains and plasmids are described in Table 1.

Cloning, expression and purification of recombinant Rho proteins of different bacterial species: Genomic DNA was isolated from the pathogens *Salmonella enterica*, *Corynebacterium glutamicum*, *Xanthomonas oryzae*, *Xanthomonas campestris* and *Pseudomonas syringae* using the genomic DNA isolation kit (Invitrogen). The *rho* genes were PCR-amplified from the genomic DNA using Deep Vent DNA polymerase (NEB) and were cloned in *NdeI-XhoI* sites of the pET28b vector. All the clones were sequenced. *E. coli* and *M. tuberculosis* Rho were available from the lab-stock (11, 17). *Vibrio cholerae* Rho cloned in pET28a was a gift from Prof U. Sen (SINP, Kolkata). The Rho proteins were overexpressed in BL21 (DE3) cells and purified using Ni-NTA beads (Qiagen) as per the manufacturer's protocol. The proteins eluted from the Ni-NTA columns were further purified by passing through a HiTrap Heparin HP sepharose column (GE healthcare).

ATPase activities of Rho: The rates of RNA-dependent ATP hydrolysis of Rho proteins of different bacterial species were measured using poly (rC) as template. ATP hydrolysis was assayed by monitoring the release of Pi from ATP that was observed on the polyethylenimine TLC plates using 0.75 M KH_2PO_4 (pH 3.5) as a mobile phase buffer. The hydrolysis reactions were performed in T buffer (25mM Tris-HCl (pH 8.0), 50mM KCl, 5mM $MgCl_2$, 1

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393 mM DTT, and 0.1 mg/ml BSA) at 37°C. The rate of ATP hydrolysis of 1mM ATP mixed
394 with [γ-^{32}P] ATP (3500 Ci/mmol; BRIT, India) were measured using 50 nM of each Rho
395 from different bacterial species. The reactions were initiated by the addition of 20μM RNA.
396 Aliquots were removed, and the reactions were stopped with 1.5 M formic acid at various
397 time points. Release of Pi was analysed by exposing the TLC sheets to a Phosphor-Imager
398 screen and subsequently by scanning using FLA 9000 (Typhoon).

399 Psu-mediated inhibition of the Rho ATPase activities were measured using λt_{R1} RNA
400 as template for *E. coli* Rho. This RNA has one of the strongest *rut* sites for *E. coli* Rho
401 protein. Poly (rC) was used for all other Rho proteins except *Salmonella* Rho. Oligo rC_{25} was
402 used in case of *S.enterica* Rho. Reactions were initiated with the Rho proteins in the presence
403 or absence of the WT and ΔCTD10 Psu proteins and were stopped at a final time point where
404 maximum hydrolysis occurred. Other reaction conditions were same as described above.
405 *Templates for in vitro transcription assays:* Linear DNA templates for *in vitro* transcription
406 and RNA release assays were made by PCR amplification from the plasmids, pRS604 (P_{T7A1}-
407 λt_{R1}) and pRS106 (P_{T7A1}-*trpt'*) using the oligo pairs RS58/RS147 and RS83/RS177,
408 respectively. To form a road-block (RB) on the template, a 22-bp lac operator sequence was
409 inserted after the *trpt'* terminator sequence using a downstream primer (RS177) having the
410 lac operator sequence (28). A transcription elongation road-block is formed at this sequence
411 in the presence of lac repressor. To immobilize the DNA templates to the streptavidin-coated
412 magnetic beads (Promega), a biotin group at the 5'-end of the templates was incorporated by
413 using a biotinylated primer (RS83).
414 *In vitro transcription assays: In vitro* transcription assays were performed on P_{T7A1}-λt_{R1}
415 template, where transcription was initiated from the strong T7A1 promoter and elongation
416 occurs through the Rho-dependent terminator, λt_{R1}. Transcription reactions were performed
417 in the presence of rifampicin in the transcription buffer (T-buffer; 25mM Tris-HCl (pH 8.0),

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418 5mM $MgCl_2$, 50mM KCl, 1mM DTT, and 0.1mg/ml BSA). At first, a 23-mer elongation
419 complex, EC_{23}, was formed by mixing 5nM DNA template, 25nM RNA polymerase, 175µM
420 adenylyl (3'-5')-uridine, 5µM each of the ATP and GTP and 2.5µM CTP. [α-^{32}P]CTP
421 (3000Ci/mmol, BRIT, Hyderabad) was used for labeling the transcripts. This EC was then
422 chased with 20µM NTPs for 15 min. Samples were run onto 8% sequencing gel and analyzed
423 by FLA-9000 PhosphorImager (Typhoon).

424 *RNA release assays from RB complex.* For the RNA release assays from the stalled ECs (RB),
425 the P_{T7A1}-*trpt'*-lacO template immobilized on streptavidin-coated magnetic beads was used.
426 100 nM lac repressor was added to the DNA templates to form a RB. On this template, at
427 first the EC_{23} was formed by initiating the reactions with 175µM adenylyl (3'-5')-uridine,
428 5mM GTP, 5mM ATP, 2.5mM CTP, and [α-^{32}P]CTP (3000 Ci/mmol). This complex was
429 then chased with 250 µM each of the NTPs. Following the chase, excess NTPs were removed
430 by washing the beads thoroughly. 50 nM Rho and 5 µM Psu (when required) were added to
431 the RB and 10µl of samples were removed at the indicated time points for each Rho (that
432 varied for different Rho proteins), separated into S (half of the supernatant) and P (other half
433 of supernatant + pellet) on the magnetic stand. The rest of the procedures were same as
434 described above. The fractions of released RNA [2S/{S + (S+P)}] were measured for each of
435 the Rho proteins.

436 *RNA polymerase release assays from the RB complex.* RNA polymerase release from the
437 stalled ECs (RB) on P_{T7A1}-*trpt'*-lacO template immobilized on the streptavidin-coated
438 magnetic beads was carried out following the similar procedures as above. The radioactive
439 CTP was not included in these experiments. 50 nM of each types of the Rho, 5 µM WT or
440 ΔCTD10 Psu proteins were added to the RB and 10µl of samples were removed at the
441 indicated time points. Supernatant (S) and the pellet (P) fractions were separated by keeping
442 the samples against a magnetic stand; S fraction was removed directly, whereas the P

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443 fractions were re-suspended in equal volume of the T buffer. These two fractions were then
444 loaded onto a SDS PAGE, and analysed by western blotting. The RNA polymerase release
445 was detected using an anti-RpoC monoclonal antibody (Neoclone).
446 *In vivo pull down assays:* BL21 (DE3) strain was co-transformed with the plasmids pET28
447 (*kan*^{r}) expressing WT or ΔCTD Psu proteins, and pET21 (*amp*^{r}), expressing Rho proteins
448 from *E. coli, S. enterica* and *V.cholerae*. Psu proteins were His-tagged at the N-terminus.
449 The Transformants were inoculated in 5 ml of LB and were grown at 37°C for ~3 h. The 5ml
450 culture was then added to 100 ml of LB and grown until OD_{600} ~0.3, following which 0.1mM
451 IPTG was added to induce the protein expressions and the induction was continued for 3 h.
452 The cells were then lysed in the lysis buffer (100mM NaH_2PO_4, 100mM NaCl, 10mM
453 imidazole, 1 mg/ml of lysozyme and 10 mg/ml of PMSF). The lysate was passed through Ni-
454 NTA (Qiagen) affinity columns, washed with wash buffer (100mM NaH_2PO_4, 100mM NaCl
455 and 20mM imidazole), and the proteins were eluted with the elution buffer (100mM
456 NaH_2PO_4, 100mM NaCl and 500mM imidazole). The volumes of lysate, wash buffer and
457 elution buffers were kept same for loading of the same amount of proteins in each lane. Rho
458 proteins of *X. oryzae, X. campetsris, M. tuberculosis* and *C. glutamicum* could not be co-
459 overexpressed with Psu. Thus, the Rho proteins of *Xanthomonas* species and both types of
460 the Psu proteins were overexpressed separately and then the lysates of the two were mixed
461 and pull-down was carried out in the same way as described above. The mixing was done for
462 at least 2hrs. Expression levels of Rho proteins of *M. tb* and *C. glutamicum* were very low, so
463 the mixing experiments were not attempted.
464 *In vivo growth inhibition assays. 1) S. enterica Rho inhibition in E. coli: S. enterica rho* was
465 cloned in pHYD3011 under the control of P_{BAD} promoter. pHYD3011carrying *S. enterica rho*
466 was transformed into the *E. coli* MG1655 WT strain. The chromosomal *rho* was subsequently
467 deleted from the MG1655 strain. *S. enterica rho* complemented *E. coli rho* after the deletion

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of the chromosomal *rho*. These strains were transformed then with pNL150 expressing *psu* cloned under an IPTG-inducible promoter, P_{tac}, and the transformants were streaked on the plates containing different IPTG concentrations.

II) M. smegmatis mc^{2}155 and M. bovis: WT *psu*, and $\Delta 20_{CTD}$ *psu* (20 amino acid deletions at the C-terminal) were PCR-amplified from the pNL150-*psu* plasmid by Deep Vent DNA polymerase (NEB), and were cloned in the EcoRI / HindIII sites of pSTKT plasmid, a Mycobacterial / *E.coli* shuttle vector having a P_{tetO} (ATc, anhydrous tetracycline inducible). Next we electroporated WT and$\Delta 20_{CTD}$ *psu* and the empty vector into the *M. smegmatis mc^{2}155* strain following the published protocol (29). Transformants were grown at 37°C for 4 days in the Diffco Middlebrook 7H10 agar plates supplemented with 10% OADC (oleic albumin dextrose catalase) and 0.2% glycerol in the presence of 10µg/ml Kanamycin and were further purified in higher concentration (25µg/ml) of Kanamycin containing media. Subsequently, colonies were patched on the plates to study the effect of *psu* expression on the cell viability of *M. smegmatis*. In the same way, the *M. bovis* strain was transformed with plasmids having different *psu* derivatives following the procedures described above. The transformants were grown for 3 weeks under same conditions as used for *M. smegmatis*. The transformants expressing WT or mutant Psu proteins were re-streaked subsequently.

III) X. campestris and X. oryzae: WT and ΔCTD20 *psu* were cloned in SalI/HindIII sites of pKLN55, a *Xanthomonas* shuttle vector having a *Peng* promoter (DSF, diffusible signal factor-inducible endoglucanase gene promoter). *X. campestris strains* Xcc 8004 WT (DSF producing) and Xcc 8523 (*rpf*F mutant; DSF non-producing) were electroporated with the pKLN55 plasmids with and without the WT or mutant *psu*. The transformants were grown in Peptone Sucrose agar media at 28°C.

Multiple sequence alignment and homology modelling of Rho protein of different species: All sequences of Rho proteins from selected bacterial species were submitted to Clustal Omega

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493 online server (30) to obtain the multiple sequence alignment. The phylogenetic tree was built
494 from the multiple sequence alignment of 16S RNA by using SeaView software (31) and by
495 the distance BioNJ (32). Homology modelling of all the Rho proteins were performed by
496 MODELLER version 9v17 (33) software. The *E. coli* Rho crystal structure (PDB code:
497 3ICE) was used as template for the modelling. The best model was selected on the basis of
498 DOPE (Discrete optimized protein energy), a statistical method optimized for model
499 assessment. All Rho monomers from different species were superimposed in the UCSF
500 Chimera Version1.11 software (34) and the Psu-binding regions were highlighted in each of
501 the Rho monomers.
502
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512
513 **References**.
514 1. Banerjee S, Chalissery J, Bandey I, Sen R. 2006. Rho-dependent transcription
515 termination: more questions than answers. J Microbiol 44:11-22.
516 2. Boudvillain M, Nollmann M, Margeat E. 2010. Keeping up to speed with the
517 transcription termination factor Rho motor. Transcription 1:70-75.

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518 3. Ciampi MS. 2006. Rho-dependent terminators and transcription termination.
519 Microbiology 152:2515-2528.
520 4. Peters JM, Vangeloff AD, Landick R. 2011. Bacterial transcription terminators: The
521 RNA 3'-end chronicles. J Mol Biol 412:793-813.
522 5. Ray-Soni A, Bellecourt MJ, Landick R. 2016. Mechanisms of Bacterial Transcription
523 Termination: All Good Things Must End. Annu Rev Biochem 85:319-347.
524 6. Dombroski AJ, Platt T. 1988. Structure of rho factor: an RNA-binding domain and a
525 separate region with strong similarity to proven ATP-binding domains. Proc Natl Acad Sci
526 85:2538-2542.
527 7. Kalyani BS, Muteeb G, Qayyum MZ, Sen R. 2011. Interaction with the nascent RNA
528 Is a prerequisite for the recruitment of Rho to the transcription elongation complex in vitro. J
529 Mol Biol 413:548-560.
530 8. Steinmetz EJ, Platt T. 1994. Evidence supporting a tethered tracking model for
531 helicase activity of Escherichia coli Rho factor. Proc Natl Acad Sci 91:1401-1405.
532 9. Grylak-Mielnicka A, Bidnenko V, Bardowski J, Bidnenko E. 2016. Transcription
533 termination factor Rho: a hub linking diverse physiological processes in bacteria.
534 Microbiology 162:433-447.
535 10. Ranjan A, Banerjee R, Pani B, Sen U, Sen R. 2013. The moonlighting function of
536 bacteriophage P4 capsid protein, Psu, as a transcription antiterminator. Bacteriophage
537 3:e25657.
538 11. Pani B, Banerjee S, Chalissery J, Muralimohan A, Loganathan RM, Suganthan RB,
539 Sen R. 2006. Mechanism of inhibition of Rho-dependent transcription termination by
540 bacteriophage P4 protein Psu. J Biol Chem 281:26491-26500.

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12. Banerjee R, Nath S, Ranjan A, Khamrui S, Pani B, Sen R, Sen U. 2012. The first structure of polarity suppression protein, Psu from enterobacteria phage P4, reveals a novel fold and a knotted dimer. J Biol Chem 287:44667-44675.

13. Ranjan A, Sharma S, Banerjee R, Sen U, Sen R. 2013. Structural and mechanistic basis of anti-termination of Rho-dependent transcription termination by bacteriophage P4 capsid protein Psu. Nucleic Acids Res 41:6839-6856.

14. Pani B, Ranjan A, Sen R. 2009. Interaction Surface of Bacteriophage P4 Protein Psu Required for Complex Formation with the Transcription Terminator Rho. J Mol Biol 389:647-660.

15. Thomsen ND, Berger JM. 2009. Running in reverse: the structural basis for translocation polarity in hexameric helicases. Cell 139:523-534.

16. Housley PR, Leavitt AD, Whitfield HJ. 1981. Genetic analysis of a temperature-sensitive Salmonella typhimurium rho mutant with an altered rho-associated polycytidylate-dependent adenosine triphosphatase activity. J Bacteriol 147:13-24.

17. Kalarickal NC, Ranjan A, Kalyani BS, Wal M, Sen R. 2010. A Bacterial Transcription Terminator with Inefficient Molecular Motor Action but with a Robust Transcription Termination Function. J Mol Biol 395:966-982.

18. Chalissery J, Banerjee S, Bandey I, Sen R. 2007. Transcription Termination Defective Mutants of Rho: Role of Different Functions of Rho in Releasing RNA from the Elongation Complex. J Mol Biol 371:855-872.

19. Said N, Krupp F, Anedchenko E, Santos KF, Dybkov O, Huang YH, Lee CT, Loll B, Behrmann E, Bürger J, Mielke T, Loerke J, Urlaub H, Spahn CMT, Weber G, Wahl MC. 2017. Structural basis for λN-dependent processive transcription antitermination. Nat Microbiol 2:17062.

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Journal of Bacteriology JB Journal of Bacteriology

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565 20. Kang JY, Olinares PD, Chen J, Campbell EA, Mustaev A, Chait BT, Gottesman ME,
566 Darst SA. 2017. Structural basis of transcription arrest by coliphage HK022 Nun in an
567 Escherichia coli RNA polymerase elongation complex. Elife e25478.
568 21. Botella L, Vaubourgeix J, Livny J, Schnappinger D. 2017. Depleting Mycobacterium
569 tuberculosis of the transcription termination factor Rho causes pervasive transcription and
570 rapid death. Nat Commun 8:14731.
571 22 Zwiefka A, Kohn H, Widger WR. 1993. Transcription Termination Factor rho: The
572 Site of Bicyclomycin Inhibition in Escherichia coli. Biochemistry 32:3564-3570.
573 23. Kohn H, Widger W. 2005. The molecular basis for the mode of action of
574 bicyclomycin. Curr. Drug Targets Infect Disord 5:273-95.
575 24. Lawson MR, Dyer K, Berger JM. 2016. Ligand-induced and small-molecule control
576 of substrate loading in a hexameric helicase. Proc Natl Acad Sci 113:13714-13719.
577 25. Nishida M, Mine Y, Matsubara T, Goto S, Kuwahara S. 1972. Bicyclomycin, a new
578 antibiotic. 3. In vitro and in vivo antimicrobial activity. J Antibiot 25:582-93.
579 26. Bambeke VF, Glupczynski Y, Plésiat P, Pechère JC, Tulkens PM. 2003. Antibiotic
580 efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the
581 future of antimicrobial therapy. J Antimicrob Chemother 51:1055-65.
582 27. Hendrix RW. 2002. Bacteriophages: evolution of the majority. Theor Popul Biol
583 61:471-80.
584 28. Dutta D, Chalissery J, Sen R. 2008. Transcription termination factor Rho prefers
585 catalytically active elongation complexes for releasing RNA. J Bio Chem 283:20243-20251.
586 29. Goude R, Parish T. 2009. Electroporation of mycobacteria. Methods Mol Biol
587 465:203-215.

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588 30. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H,
589 Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-
590 quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539.
591 31. Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: A multiplatform graphical
592 user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221-
593 224.
594 32. Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple
595 model of sequence data. Mol Biol Evol 14:685-695.
596 33. Sali A, Blundell TL. 1993. Comparative protein modelling by satisfaction of spatial
597 restraints. J Mol Biol 234:779-815.
598 34. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin
599 TE. 2004. UCSF Chimera--a visualization system for exploratory research and analysis. J
600 Comput Chem 25:1605-1612.
601 35. Linderoth NA, Tang G, Calendar R. 1997. In vivo and in vitro evidence for an anti-
602 Rho activity induced by the phage P4 polarity suppressor protein Psu. Virology 227:131-141.
603 36. Newman KL, Almeida RP, Purcell AH, Lindow SE. 2004. Cell-cell signalling
604 controls Xylella fastidiosa interactions with both insects and plants. Proc Natl Acad Sci
605 101:1737-42.
606 37. Parikh A, Kumar D, Chawla Y, Kurthkoti K, Khan S, Varshney U, Nandicoori VK.
607 2013. Development of a new generation of vectors for gene expression, gene replacement,
608 and protein-protein interaction studies in mycobacteria. Appl Environ Microbiol 79:1718-
609 1729.

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611 **Legends to the Figures**

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612 **Figure 1**. *Sequential and structural homology among the Rho proteins from different*
613 *pathogenic bacteria*. (A) A phylogenetic tree, constructed based on the 16S rRNA sequences,
614 demonstrating the diversity of the different pathogenic bacteria from which the Rho proteins
615 were selected in this study. (B) The sequence alignment of the region enclosing the Psu-
616 binding sites from different Rho proteins produced by Clustal Omega software. The locations
617 of the residues responsible for the Psu binding are indicated. (C) Homology models of Rho
618 hexamers obtained by Modeller, using the crystal structure of the *E. coli* Rho as a template
619 (PDB 3ICE) [15]. Red loops on each structure indicates the unstructured Psu-binding region.
620 (D) Superimposed monomer structures of all the Rho proteins highlighting the Psu-binding
621 loops. A zoomed in view of the loops have been shown.

622 **Figure 2.** *Purification and characterization of the Rho protein activities from different*
623 *pathogenic bacteria*. (A) SDS PAGE showing the migration patterns of the recombinant Rho
624 proteins of different species. (B-D) The ATPase activities of these Rho proteins in the
625 presence of poly(rC) as substrate. The Rho proteins having similar rates of ATPase activities
626 are shown together in each of the panels as indicated. The activity of *E.coli* Rho has been
627 included all the panels for comparison. D) Table showing the values of the ATPase activities
628 of all the Rho proteins expressed in nmol/min/μg.

629 **Figure 3**. *ATPase and in vitro transcription assays showing Psu mediated inhibition of the*
630 *activities of Rho from different pathogens*. **(A)** Bar diagrams showing the fractions of ATP
631 hydrolyses by each of the Rho protein in the absence and presence of 1 μM and 5 μM Psu. In
632 control experiments, 5 μM of a Psu mutant, ΔCTD10, was used. Similar to figure 2, poly (rC)
633 was used as substrate for all the Rho proteins except for those from *E.coli* and *Salmonella*.
634 For *E.coli*, a RNA with λt_{R1} terminator was used, whereas rC_{25} oligo RNA was used for
635 *Salmonella* Rho protein. Errors were calculated from 3 independent measurements. **(B)** Left
636 panel, autoradiograms showing the *in vitro* Rho-dependent transcription termination assays

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637 using Rho proteins of *E.coli*, *S. enterica* and *M. tuberculosis* performed on a DNA template
638 having the λt_{R1} terminator (see the adjacent cartoon), both in the absence and presence of 5
639 μM Psu in the reaction mixture. Termination zone is indicated. Right panel, bar diagrams
640 showing the fractions of Run-off (RO) product under different conditions. This is calculated
641 using the formulae: (Intensity of the Run-off product)/ (Intensities of all the terminated
642 products + Intensity of the Run-off product). Errors were calculated from 3 independent
643 measurements. **(C)** Cartoon showing the stalled elongation complex (EC) at a lac repressor
644 road-block located downstream of the *trpt'* terminator. On this template, transcription is
645 initiated from the T7A1 promoter and the RNA release was induced from the stalled EC by
646 different Rho proteins as indicated in D. Template is immobilized via an attachment to a
647 magnetic bead to monitor the released RNA. **D**) Autoradiogram showing the RNA release in
648 the supernatant fractions (S: half of the Supernatant). Rest of the fractions were in the pellet
649 fraction (P: other half of the supernatant and whole of the pellet). RNA products in different
650 fractions are shown in the presence and absence of Psu. Time of incubation of the stalled
651 ECs with different Rho proteins are indicated. RB denotes the RNA product of the stalled
652 EC. **(E)** Amount of RNA released under different conditions in figure (D) are expressed as
653 bar diagrams. Fractions of released RNA was calculated as: (2S)/[(S) + (S+P)]. Errors were
654 measured from 3 independent experiments.

655 **Figure 4**. *Complex formation of Psu with different Rho proteins*. *In vivo* pull-down assays
656 showing direct complex formation between different Rho and **A)** WT Psu and **B)** ΔCTD10
657 Psu proteins. Flow through (FT) and wash (W) fractions contain the unbound Rho, whereas
658 the amounts of protein in elute (E) fraction gave the measure of the Psu-bound Rho. Both the
659 proteins bands are indicated. Fractions of Rho associated was calculated as: [E] /
660 {[FT]+[W]+[E]}. Values are shown below the gel pictures. Errors were calculated from two
661 to three independent measurements.

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Figure 5. *Psu induced lethality of different bacteria.* **(A)** MG1655 *Δrho::kan* strains expressing *Salmonella enterica* Rho from the pHYD3011 plasmid were transformed together with the second plasmid expressing WT Psu. Strains were streaked on LB plates in the presence and absence of different concentrations of the inducer IPTG as indicated**. (B)** *Xanthomonas campestris* strain Xcc8004 WT producing the inducer, DSF and Xcc8523, a DSF deficient strain (*rpf*F mutant), were transformed with pRS1574 (a Xanthmonas shuttle vector, pKLN55) (expressing. WT Psu) and pRS1508 (empty pKLN55 vector). The Xcc8004 strain was also transformed with pRS1845 expressing ΔCTD20 Psu.. Plates with the transformants are shown. Cartoon above the plates showing the location of *psu* under the control of P_{eng}. The transformants obtained upon transforming the strain Xcc8004 with pRS1508 and pRS1845 were streaked separately to show the inability of the Psu mutant to cause mortality. **(C)** *Mycobacterium smegmatis* strain mc^2155 was transformed with either pSTKT empty vector (pRS1511) or expressing either WT Psu (pRS1724) or the mutant Psu, ΔCTD20 (pRS1837). Transformants were subsequently patched on the 7H10 plates. Location of the WT or mutant *psu* under the ATc (anhydrous tetracycline) inducible promoter P_{tet} is shown as a cartoon. **(D)** The *Mycobacterium bovis* BCG strain, was transformed with the same plasmids described in (C). Transformants obtained after 3 weeks are shown, and in two of the cases, colonies were further streaked to show the growth differences of the *M. bovis* strains upon expression of either WT or mutant Psu proteins.

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681 **Table 1:** Strains and plasmids used in the study

Strains	Genotype	Reference
RS 1263	*E. coli* MG1655 K12 WT	Lab stock
RS 1487	*M. smegmatis* mc^2155 WT	From Dr Sangita Mukhopadhyay
RS 1833	*X. campestris* Xcc8004 WT	From Dr Subhadeep Chatterjee
RS 1835	*X. campestris* Xcc8523 DSF-deficient *rpf*F mutant	From Dr Subhadeep Chatterjee
RS 1838	*M. bovis* BCG WT	From Dr Sanjeev Khosla
Plasmids		
pRS 96	pET21b with *E. coli rho* cloned at NdeI/XhoI site, His tag at C-terminal, Amp^R	[18]
pRS 100	pET21b with *E. coli rho* cloned at NdeI/XhoI site, Non-His tagged, Amp^R	[11]
pRS 106	pT7A1 clone at EcoRI/HindIII sites upstream of trpt' cloned at HindIII/BamHI sites of pK8641, Amp^R	[11]
pRS 258	pNL150 with P*tac*-WT *psu*, Cam^R	[33]
pRS 458	pET 28b with WT Psu cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	[11]
pRS 460	pET 28b with $\Delta 20_{CTD}$ *psu* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	[11]
pRS 553	pET28a with *M. tb rho* cloned at NdeI/XhoI site, His tag at N-terminal, Amp^R	[17]
pRS 604	pTL61T with pT7A1-Lambda *nutR-tR1-T1T2-lacZYA*; Amp^R	[14]
pRS 1508	pKLN55 *Xanthomonas* shuttle vector with P_{eng}, $Spec^R$	[36]
pRS 1511	pSTKT mycobacterial shuttle vector with P_{tetO}, Kan^R	[37]
pRS 1574	pKLN55 with WT *psu* cloned at SalI/HindIII site, $Spec^R$	This study
pRS 1670	pET28b with *P. syringae rho* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	This study
pRS 1671	pET28b with *X. oryzae rho* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	This study
pRS 1672	pET28b with *X. campestris rho* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	This study
pRS 1680	pET28b with *S. enterica rho* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	This study
pRS 1681	pET28b with *C. glutamicum rho* cloned at NdeI/XhoI site, His tag at N-terminal, Kan^R	This study
pRS 1724	pSTKT with WT *psu* cloned at EcoRI/HindIII site, Kan^R	This study
pRS 1770	pET28a with *V. cholerae rho* cloned at NdeI/BamHI site, His tag at N-terminal, Kan^R	From Dr U. Sen
pRS 1779	pET21b with *X. oryzae rho* cloned at NdeI/XhoI site, Non-His tagged, Amp^R	This study
pRS 1780	pET21b with *S. enterica rho* cloned at NdeI/XhoI site, Non-His tagged, Amp^R	This study
pRS 1781	pET21b with *V. cholerae rho* cloned at NdeI/XhoI site, Non-His tagged, Amp^R	This study
pRS 1785	pET21b with *X. campestris rho* cloned at NdeI/XhoI site, Non-His tagged, Amp^R	This study
pRS 1801	pHYD3011 with *S. enterica rho* cloned at NdeI/SalI site, Non-His	This study

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	tagged, Amp^R	
pRS 1837	pSTKT with ΔCTD20 *psu* cloned at EcoRI/HindIII site, Kan^R	This study
pRS1845	pKLN55 with ΔCTD20 *psu* cloned at SalI/HindIII site, $Spec^R$	This study
Oligos		
RS58	ATAAACTGCCAGGAATTGGGGATC; located upstream of T7A1 promoter of pRS106	[26]
RS83	ATAAACTGCCAGGAATTGGGGATC;5'-biotinylated RS58	[26]
RS147	GCGCGCGGATCCCCCCATTCAAGAACAGCAAGCAGC, reverse oligo to generate T7A1-λtR1 terminator template	[26]
RS177	TTGTGAGCGCTCACAATTCGGATATATATTAACAATTACCTG; reverse oligo with lac operator sequence, used to generate roadblock downstream of rut sites of T7A1-*trp t'* template	[26]

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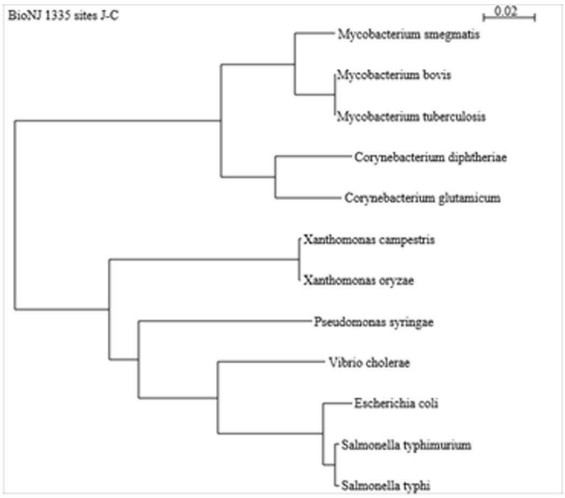
A)



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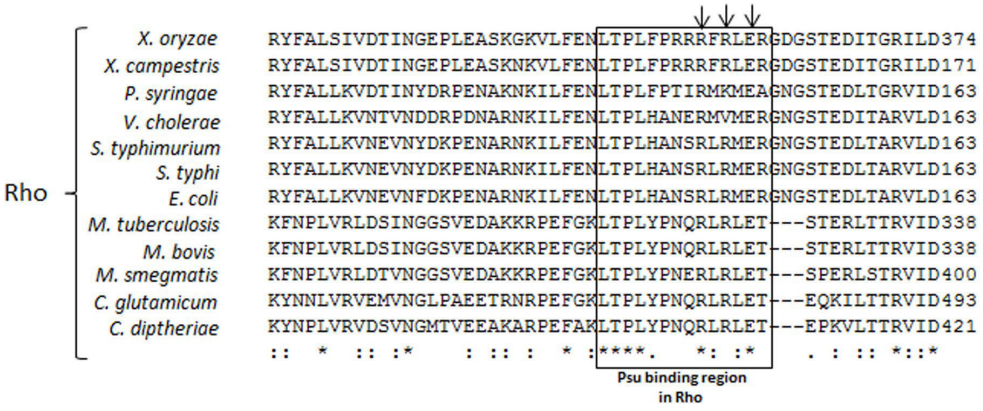

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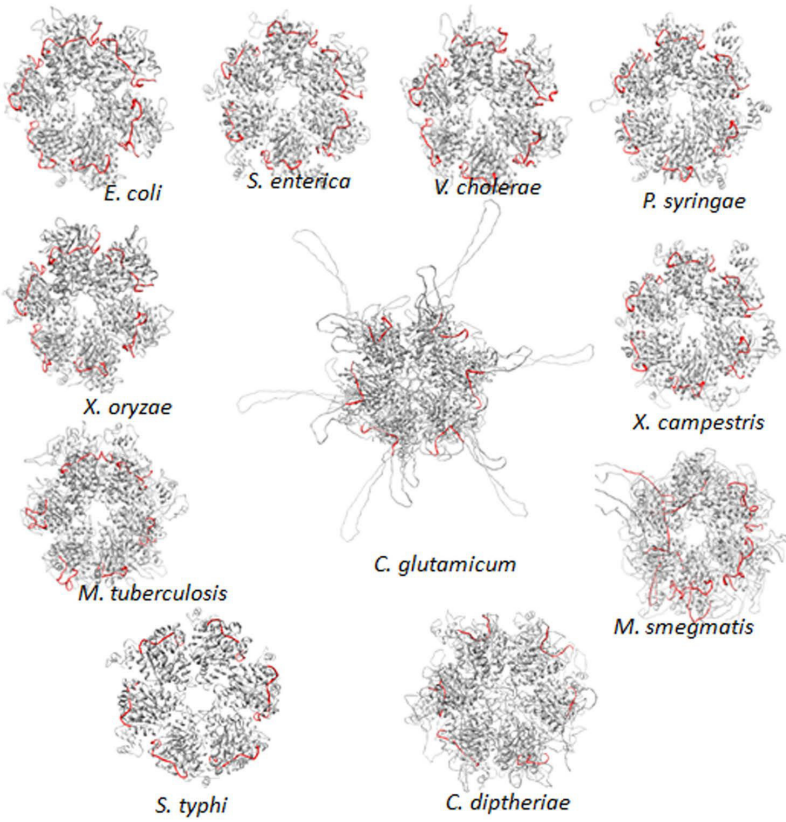

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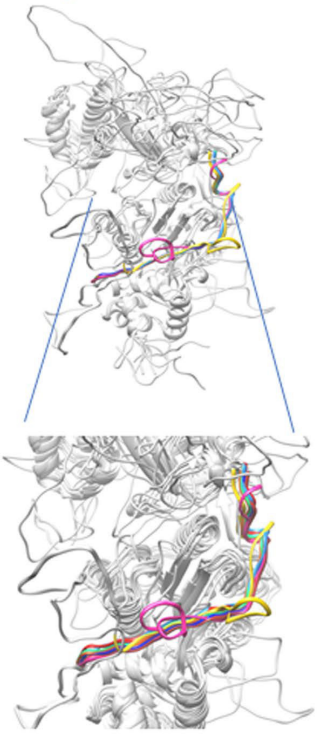

Figure 1

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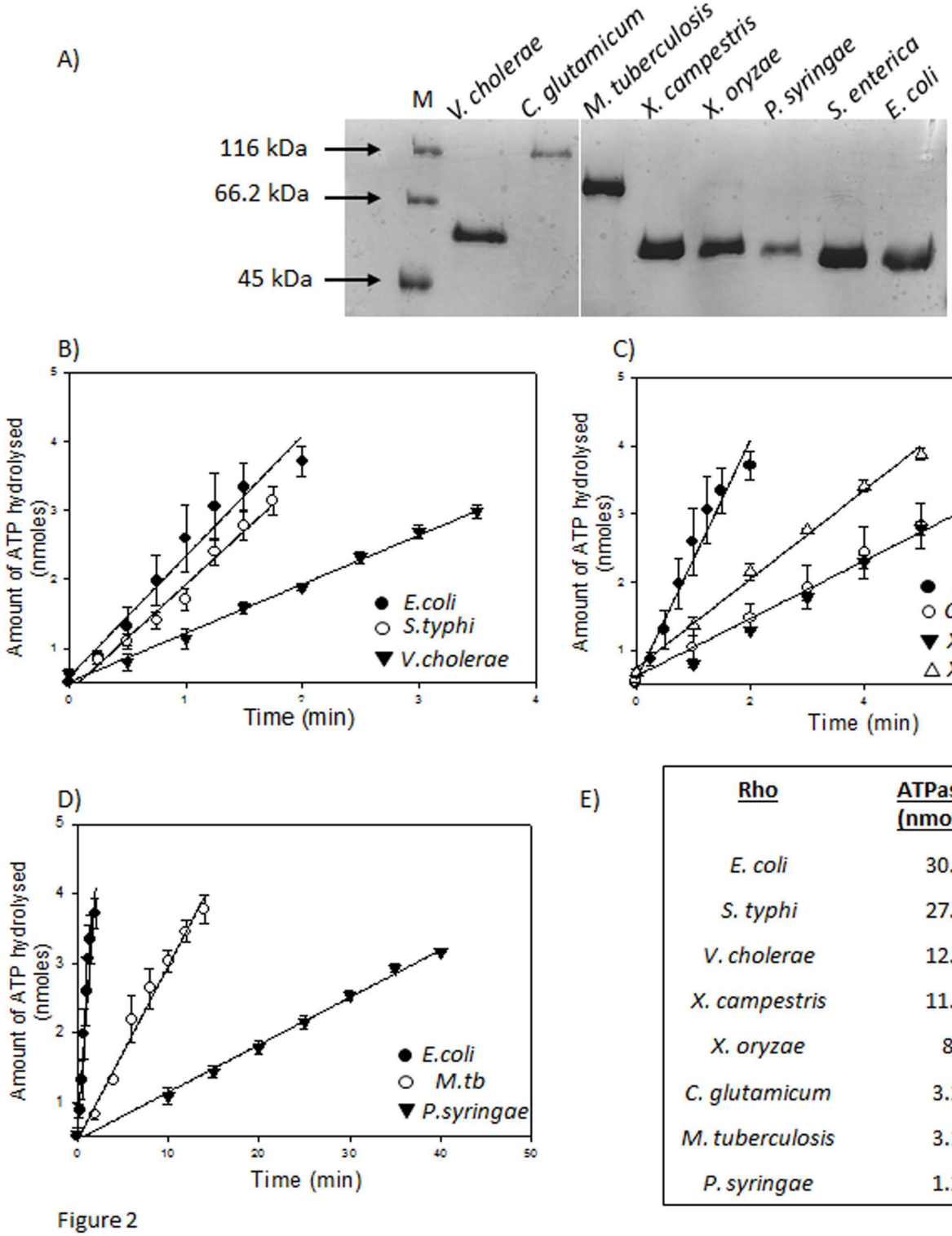

E)

Rho	ATPase activity (nmol/min/µg)
E. coli	30.3 ± 0.3
S. typhi	27.6 ± 0.2
V. cholerae	12.6 ± 0.1
X. campestris	11.2 ± 0.1
X. oryzae	8 ± 0.1
C. glutamicum	3.2 ± 0.3
M. tuberculosis	3.1 ± 0.2
P. syringae	1.2 ± 0.1

Figure 2

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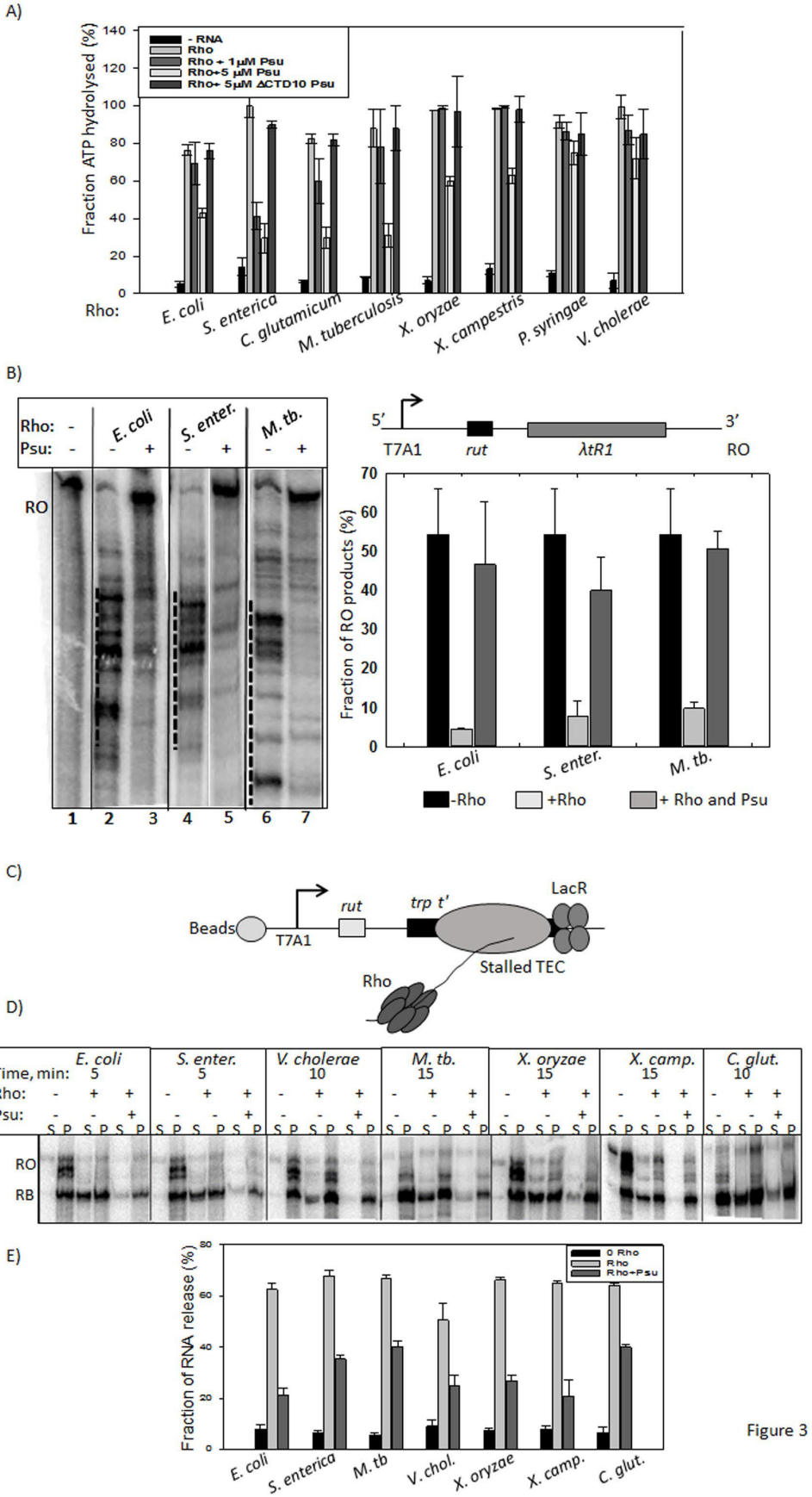

Figure 3

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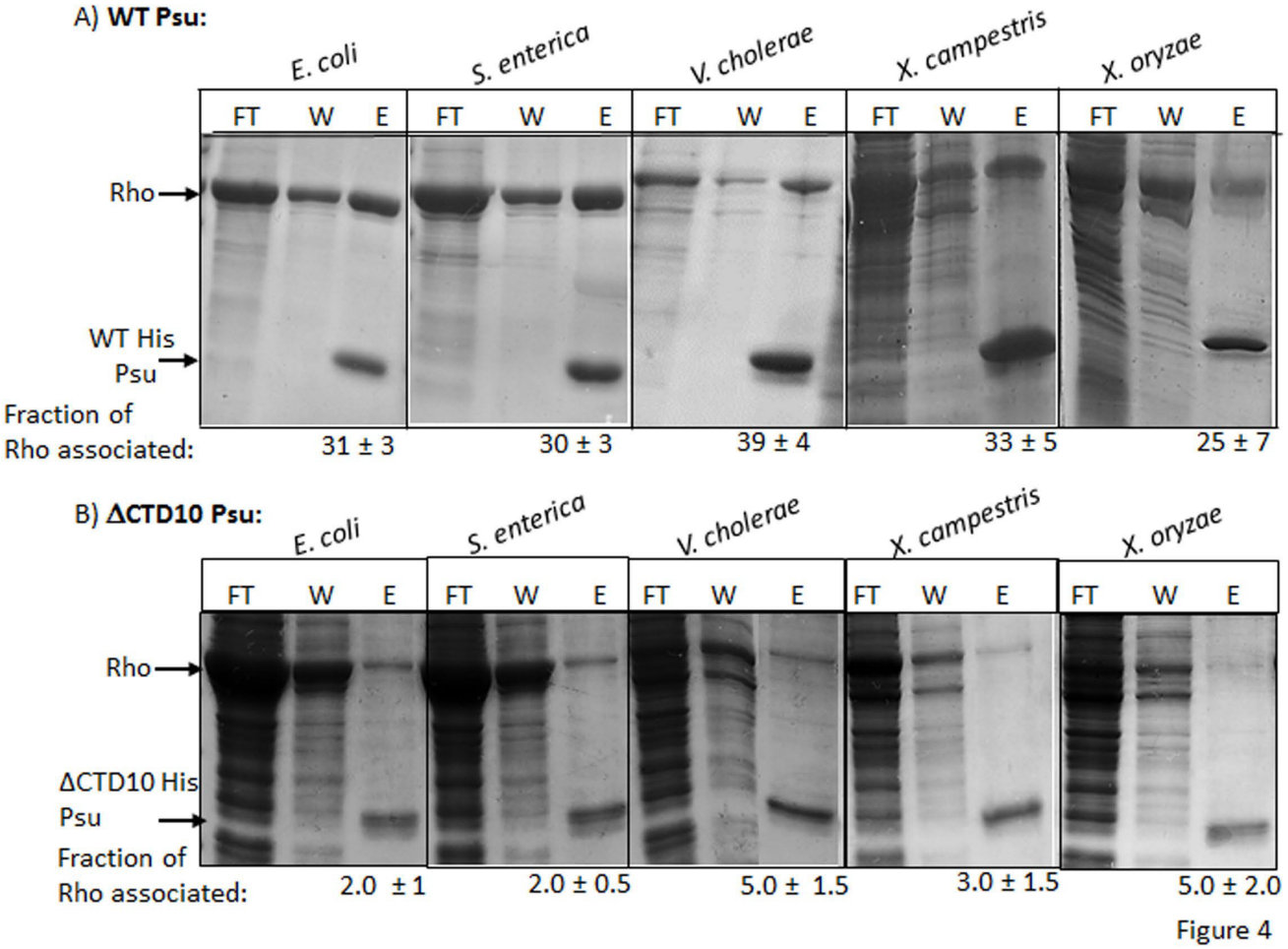

Figure 4

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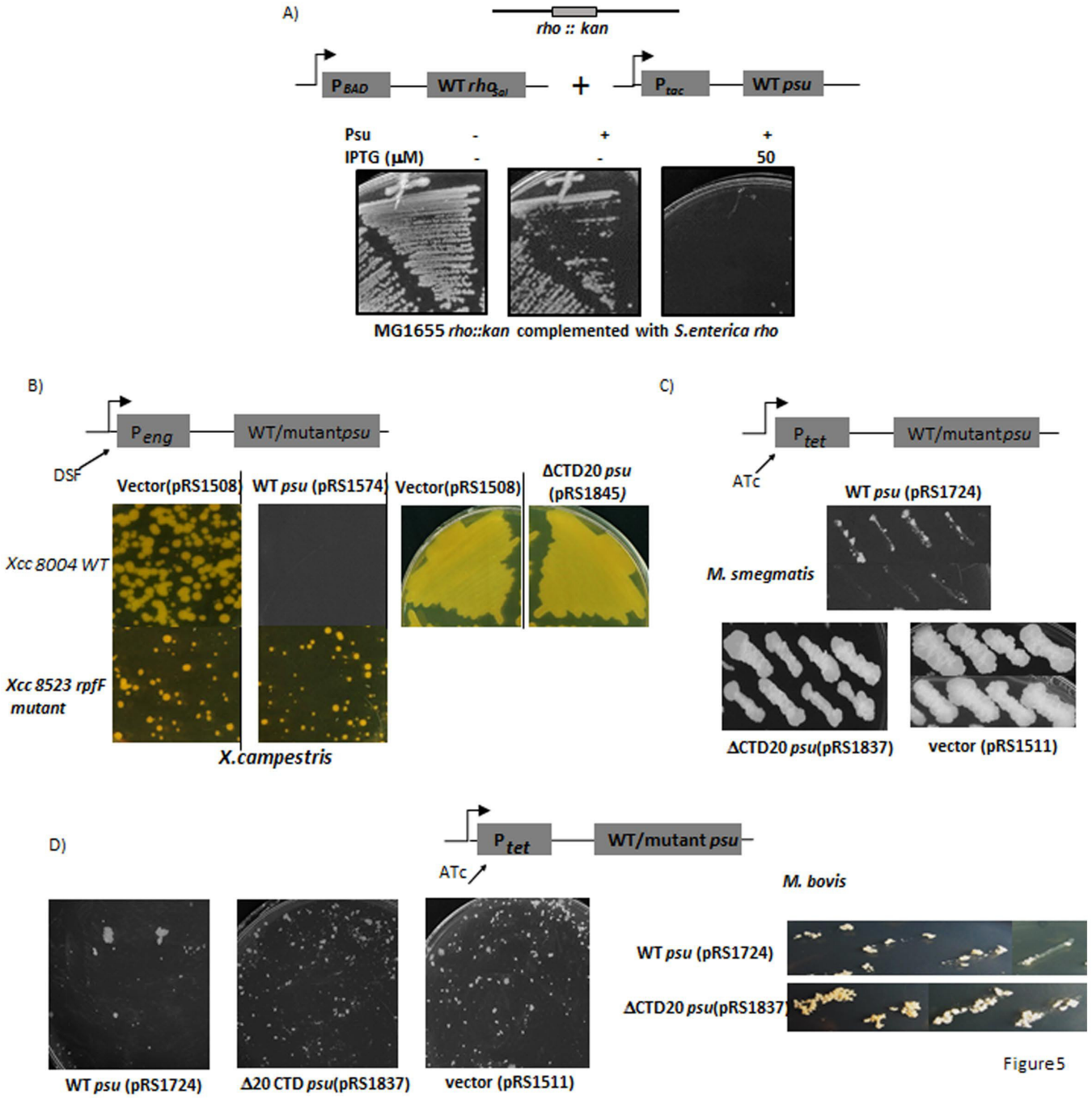

Figure 5

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