Exoribonuclease RNase R protects Antarctic *Pseudomonas syringae* Lz4W from DNA damage and oxidative stress

Running Title: Role of RNase R in protecting against DNA damage and oxidative stress.

Pragya Mittal[1, 2,]*, Rashmi Sipani[1, 3], Apuratha Pandiyan[1, 4] Shaheen Sulthana[1, 6], Anurag K Sinha[1, 5], Ashaq Hussain[1], Malay K Ray[1] and Theetha L Pavankumar[1, 6,]*.

Affiliations:

[1] Centre for Cellular and Molecular Biology (CCMB), Council of Scientific and Industrial Research (CSIR), Uppal Road, Hyderabad-500007, India.

Present addresses

[2] MRC Human Genetics Unit, Institute of Genetics and Cancer, University of Edinburgh, EH4 2XU Edinburgh, United Kingdom.

[3] PopVax Pvt. Ltd., IV Floor, Atal Incubation Centre-CCMB Annex-II, Medical Biotechnology Complex, Hyderabad, India.

[4] Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, SAS Nagar, Punjab, India.

[5] National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark.

[6] Department of Microbiology and Molecular Genetics, and Department of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA.

***** Address correspondence to Theetha L Pavankumar, pavan@ucdavis.edu or to Pragya Mittal, pragya.mittal@ed.ac.uk. The authors have declared no conflict of interest.

Abstract

RNase R is a highly processive, 3' - 5' exoribonuclease involved in RNA degradation, maturation, and processing in bacteria. In *Pseudomonas syringae* Lz4W, RNase R interacts with the RNase E to form the RNA degradosome complex and is essential for growth at low temperature. RNase R is also implicated in general stress response in many bacteria. We show here that *rnr* gene (encoding RNase R) deletion mutant of *P. syringae* is highly sensitive to various DNA damaging agents and oxidative stress. RNase R is a multidomain protein comprised of CSD, RNB and S1 domains. We investigated the role of each domain of RNase R and its catalytic activity in nucleic acid damage and oxidative stress response. Our results revealed that RNB domain alone without its exoribonuclease activity is sufficient to protect against DNA damage and oxidative stress. We also show that association of RNase R with degradosome complex is not required for this function. Finally, based on homology modelling of RNB domain of RNase R of *P. syringae*, we propose that the presence of a tri-helix wedge domain may be involved in protection against DNA damage and oxidative stress in *P. syringae* through a putative helicase activity. Our study for the first time has discovered a hitherto unknown role of RNase R in protecting *P. syringae* Lz4W against DNA damage and oxidative stress.

Importance

Bacterial exoribonucleases play a crucial role in RNA maturation, degradation, quality control and its turnover. In this study, we have uncovered a previously unknown role of 3'- 5' exoribonuclease RNase R of *P. syringae* Lz4W in DNA damage and oxidative stress response. Here, we show that neither the catalytic exoribonuclease function of RNase R, nor its association with the RNA degradosome is essential for this function. Nevertheless, a putative helicase function of RNase R appears to have an important role in protecting against DNA damage and oxidative stress. Interestingly, in *P. syringae* Lz4W, the hydrolytic RNase R exhibits physiological roles similar to phosphorolytic 3'- 5' exoribonuclease PNPase of *E. coli*. Our data suggests that during the course of evolution, mesophilic *E. coli* and psychrotrophic *P. syringae* have apparently swapped these exoribonucleases to adapt to their respective environmental growth conditions.

Key words: *Pseudomonas syringae*, RNase R, DNA damage, Oxidative stress, RNA degradation, and RNA unwinding.

Introduction

Exoribonucleases play an important role in determining the levels of RNA in the cells (1). Some of the exoribonucleases also associate with degradosome, a multi-protein complex involved in degradation and processing of cellular RNA (2, 3). The RNR superfamily of enzymes that includes proteins like RNase II, RNase R, and the eukaryotic Rrp44/Dis3 are present in all domains of life (4). RNase R, encoded by *rnr* gene, is a 3´ to 5´ highly processive exoribonuclease involved in degradation, maturation and processing of RNA in bacteria (5-8). RNase R plays an important role in RNA quality control, such as degradation of defective tRNA and removal of aberrant fragments of the 16S and 23S rRNAs (7, 9). RNase R is capable of processing dsRNA without the help of a helicase, hence implicated in removal of mRNAs with stable stem loops, including repetitive extragenic palindromic (REPs) elements (6, 10) It is also involved in processing of tmRNA (transfer-messenger RNA) and is required for the process of trans-translation (11). Structurally, RNase R is a multidomain protein consists of RNA binding domains cold-shock domains (CSD) and S1, and the catalytic RNB domain (12). The catalytic RNB domain contains four highly conserved Aspartate residues that help to coordinate Mg^{2+} ions in the catalytic center to facilitate ribonucleolytic activity (12-14).

In *Pseudomonas syringae* Lz4W, RNase R associates with endoribonuclease RNase E, along with DEAD box helicase RhlE to form the degradosome complex (15). The *rnr* gene *P. syringae* Lz4W and *P. putida* is implicated in survival at low temperature (7, 16-18). The *P. syringae* Lz4W cells lacking RNase R accumulate unprocessed 3' ends of 16S rRNAs in polysomes, making the translation machinery inefficient at low temperature, causing cold sensitivity (7). RNase R is also involved in general cellular stress responses. In *E. coli*, RNase R levels increase under cold shock, stationary phase, and heat shock (19, 20).

In a quest to understand the role of RNase R of *P. syringae* Lz4W (RNase R^{Ps}) in general cellular stress response, we subjected the *rnr* mutant to various types of stresses and found that *rnr* mutant cells are sensitive to oxidative stress and DNA damaging agents. We show that this role of RNase R is independent of its association with the degradosome complex in *P. syringae* Lz4W. Using a series of domain deletion constructs and mutating catalytically important residues, we discovered that the catalytic RNB domain alone without its exoribonuclease function is sufficient to protect *rnr* mutant of *P. syringae* from DNA damage and oxidative stress. Our data further indicates that a putative helicase function of RNB domain is likely to play a role in protecting *P. syringae* Lz4W cells from DNA damage and oxidative stress.

Results

P. syringae rnr mutant cells are sensitive to oxidative stress and DNA damaging agents

In an attempt to understand the role of RNase R in general stress response in *P. syringae*, the *rnr* mutant was subjected to various conditions that include low and high temperatures (4 and 37 ºC), osmotic stress (1M NaCl), envelope stress (10% SDS, 100 mg/ml Lysozyme with 0.5 mM EDTA), antibiotics treatment (100 μg/ml Rifampicin and Spectinomycin), oxidative (0.25 mM Hydrogen peroxide, 15 μM Paraquat) and DNA damage stress (24 J/m^2.s ultraviolet radiation, 0.25 mM Cisplatin, 1 μg/ml Mitomycin C and 2.5 mM Hydroxyurea). As shown in Table 1, we observed that the sensitivity of *rnr* mutant against osmotic, envelope and antibiotic stresses is comparable to that of wild-type (*wt*) cells. We confirmed that the *rnr* mutant cells are cold sensitive, as shown earlier (7). Nevertheless, we found that *rnr* mutant is also sensitive to high temperature stress (Table 1).

Interestingly, we discovered that the *rnr* mutant cells are highly sensitive to the oxidative stress induced by H_2O_2 and Paraquat; and to DNA damage induced by ultraviolet (UV) radiation, Mitomycin C (MMC) and Hydroxyurea (HU) (Table 1). H_2O_2 and Paraquat generate reactive oxygen species (ROS), such as hydroxyl and superoxide radicals that can easily react with biological macromolecules including DNA, RNA, proteins, and lipids (21). We quantified the effect of these agents on the growth and viability of *rnr* mutant in comparison to the *wt* cells. We assessed the colony-forming ability of cells upon treatment with various doses of H_2O_2, Paraquat, UV radiation, MMC and HU. The *rnr* mutant displayed more than 100-fold higher sensitivity compared to *wt* cells to all the agents tested, suggesting a cryptic role of RNase R in protecting *P. syringae* cells from DNA damage and oxidative stress (Figure 1A). Spot assays on ABM plates containing the indicated amounts of H_2O_2, Paraquat, MMC, HU and UV irradiation, further confirmed that *rnr* mutant of *P. syringae* is highly sensitive to all the agents (Figure 1C). In contrast, we observed that the mesophilic *E. coli* cells lacking RNase R (Δ*rnr*) are refractory to oxidative stress and DNA damaging agents (Figure S1) suggesting that the protective role of RNase R against DNA damage is specific to *P. syringae*.

Furthermore, introducing the *rnr* gene encoding RNase R^{Ps} on a low copy plasmid (pGL10::*rnr*^{Ps}) rescued the growth defects of *rnr* mutant caused by DNA damage/oxidative stress confirming that the functional loss of RNase R causes the sensitivity (Figure 1A & 1C). Interestingly, when RNase R of *E. coli* (pGL*rnr*^{Ec}) is overexpressed in *P. syringae* Lz4W, it also rescued the growth defect of *rnr* mutant of *P. syringae* (Figure 1A & 1C). In *E. coli*, another

exoribonuclease PNPase has been shown to protect cells against oxidative stress and DNA damage (22-25). We examined if PNPase of *P. syringae* has similar functions and could complement the loss of *rnr* gene against oxidative and DNA damage stress. However, we observed that *rnr* mutants over-expressing PNPase could not rescue the sensitivity of *rnr* mutant to oxidative and DNA damage stress (Figure S2).

RNase R is known to upregulate under different stress conditions in *E. coli* (19, 20), hence we examined the levels of RNase R in *P. syringae* Lz4W cells by Western blotting with polyclonal anti-RNase R antibodies (15). The levels of RNase R remained unchanged after treating with stress causing agents (Figure 1B). As a control, the expression of DNA repair protein RecA was induced quickly upon treatment with UV irradiation as shown earlier (26). This is in line with a previous report that RNase R of *P. syringae* Lz4W is not induced under cold stress and in stationary phase (7). This suggests that unlike *E. coli*, the RNase R^{Ps} is not a stress inducible protein.

RNase R protects *P. syringae* cells against oxidative stress and DNA damage independent of RNA degradosome complex

RNase R associates with endonuclease RNase E and RNA helicase RhlE to form the RNA degradosome complex in *P. syringae* Lz4W (15). The C-terminal domain of RNase E acts as a scaffold for the formation of degradosome complex with RNase R and RhlE in *P. syringae* (15). Thus, we constructed a C-terminal deletion mutant of RNase E (*rne*$^{\Delta 595\text{-}1074}$) to investigate the role of RNase R associated degradosome complex in oxidative stress and DNA damage. The generation of C-terminal deleted *rne* mutant (*rne*$^{\Delta 595\text{-}1074}$) was carried out as described in materials and methods. The *rne*$^{\Delta 595\text{-}1074}$ strain was subjected to oxidative stress and DNA damage causing agents. Unlike the *rnr* mutant, the *rne*$^{\Delta 595\text{-}1074}$ strain is not sensitive to the DNA damage and oxidative stress causing agents and showed resistance similar to *wt* (Figure 2). The results suggest that the requirement of RNase R in protecting *P. syringae* cells against oxidative stress and DNA damage is an intrinsic property of RNase R and is independent of its association with the RNA degradosome complex.

The RNB domain of RNase R is sufficient in protecting *P. syringae* Lz4W against oxidative stress and DNA damage

The RNase R^{Ps} is a multidomain protein comprising of N-terminal RNA binding cold shock domain CSD (1 – 225 aa), a central nuclease domain RNB (226 – 665 aa) and the RNA binding domain S1 at the C-terminus (666 – 885 aa) (Figure 3A). To understand the role of each

domain in protecting *P. syringae* Lz4W cells against oxidative stress and DNA damage, we constructed a series of domain deletion mutants of RNase R: i) a mutant lacking CSD domain (ΔCSD), ii) a mutant lacking S1 domain (ΔS1), and iii) a mutant lacking both CSD and S1 domains (RNB) (Figure 3A). In our viability assays, the *rnr* mutants complemented with the domain deleted mutants of RNase R showed resistance to the oxidative stress and DNA damaging agents similar to *wt* cells (Figure 3B & C). This suggests that the RNB domain of RNase R alone, without the RNA binding CSD and S1 domains is sufficient to protect *P. syringae* Lz4W against oxidative stress and DNA damage.

The exoribonuclease activity of RNase R is not required for protecting *P. syringae* Lz4W against oxidative stress and DNA damage

The catalytic site of RNase R^{Ps} has four conserved aspartate residues in the active site located at positions 276, 282, 284 and 285 (Figure 4A). D276 and D285 have been proposed to be important for coordinating with Mg^{2+} ion, D282 for holding the RNA in place while D284 is known for being the residue that cleaves the phosphodiester bond (27). It was shown in *E. coli* that D280 (equivalent to the D284 residue of *P. syringae*) is the only crucial residue for the catalytic activity of RNase R without affecting binding of the RNA (14). This residue is also shown to play a crucial role in the activity of RNase II of *E. coli* and RNase R of *Legionella pneumophila* (28). Hence, we mutated the equivalent Asp284 in RNase R to alanine, overexpressed and purified the mutant protein ($RNaseR^{D284A}$) as described under materials and methods. SDS-PAGE profile of $RNaseR^{D284A}$ is shown in Fig 4B(i). RNase R^{D284A} show double protein bands on a SDS-polyacrylamide gel. The lower band is a product of C-terminally degraded RNase R protein as observed earlier (10). We couldn't separate these two bands due to marginal difference in their molecular weights. We analyzed the exoribonucleolytic activity of $RNaseR^{D284A}$ protein on Poly(A) and *malE-malF* substrates and compared with the wild-type. Wild-type RNase R degraded both poly(A) and *malE-malF* substrates within 10 mins. However, despite using high protein concentration of $RNaseR^{D284A}$ mutant (0.5 µM vs 15 µM) it failed to degrade both substrates even after prolonged incubation (Fig 4B (ii & iii)). This shows that the D284A mutation leads to complete loss of exoribonuclease activity of RNase R^{Ps}.

In our viability assays, all four single amino acid mutants protected Δ*rnr* mutant cells from oxidative and DNA damage stress, similar to *wt* cells (Figure 5). However, the D282A, D284A and D285A mutants showed marginal sensitivity to stress induced by hydroxyurea (HU) suggesting that the functional role of these residues in the activity of RNase R have a plausible

role in protecting particularly against HU induced stress. In general, the data suggest that the role of RNase R in protecting cells against oxidative stress and DNA damage is independent of exoribonuclease activity of RNase R.

To further substantiate our finding, we also combined the following mutations on full length RNase R: D284A + D285A; D276A + D284A; D276A + D284A + D285A. We examined their role in the function of RNase R in protecting against stress (Supplementary Figure 3). All the double and triple point mutants also protected Δ*rnr* cells against oxidative stress and DNA damage similar to *wt* cells. This further confirms that the exoribonuclease activity of RNase R is not essential for the protection against oxidative stress and DNA damage.

The RNB domain alone with D284A mutation can protect *P. syringae* Lz4W cells against oxidative stress and DNA damage.

RNB is the main catalytic domain responsible for the exoribonuclease activity of RNase R. As mentioned above the four conserved Aspartate residues important for the catalytic activity of RNase R are located in the motif-1 of the RNB domain (Figure 4A(ii)). To further corroborate our finding that the exoribonuclease function of RNase R^{Ps} is not required for protection against DNA damage and oxidative stress; we mutated the D284 and D284+D285 residues on catalytic RNB domain alone to alanine. RNB domain with D284A and D284A+D285A mutations was expressed in Δ*rnr* strain. The viability assays for the RNB domain with mutations show that the mutants moderately protect cells against oxidative stress and DNA damage. However, the point mutations slightly affected the ability of RNB domain to protect cells against Micromycin C, Paraquat and Hydroxyurea (Figure 5A).

In *E. coli*, RNB domain is also suggested to play an important role in RNA unwinding. It has been recently suggested that a triple helix wedge region in the RNB domain of *E. coli* RNase R is responsible for unwinding the duplex-RNA substrates (29). Our homology modelling of RNase R^{Ps} based on the crystal structure of *E. coli* RNase R, also shows the presence of a tri-helix wedge region in the RNB domain of RNase R^{Ps} (**Figure 5B**). Although we haven't examined the role of tri-helix wedge of RNase R^{Ps} in RNA unwinding function, it is possible that it may exhibit helicase activity similar to the tri-helix wedge region in the RNB domain of *E. coli* RNase R.

Discussion

Ribonucleases are important modulators involved in various cellular processes by virtue of their RNA processing and degradation activity in maintaining the dynamic transcript levels in bacterial cells. Exoribonucleases like RNase R, PNPase and RNaseJ1 are multifunctional enzymes and are implicated in various biological processes such as stress tolerance, protection from DNA damage and oxidative stress apart from their role in RNA metabolism (19, 20, 22, 24, 30, 31). RNase R is important for the RNA quality control and implicated in various stress conditions in bacteria (5, 20). In this study, we have uncovered a novel role of RNase R in protection of *P. syringae* Lz4W from oxidative stress and DNA damaging agents such as H_2O_2, Paraquat, MMC, Hydroxyurea and UV radiation.

PNPase is a phosphorolytic 3'- 5' exoribonuclease an important component of *E. coli* RNA degradosome complex. However, in *P. syringae*, instead of PNPase, the hydrolytic 3'-5' exoribonuclease RNase R interacts with the RNA degradosome complex (15). Interestingly, *pnp* mutant of *E. coli* and the *rnr* mutant of *P. syringae* exhibit similar phenotypic behaviors. The *pnp* mutant of *E. coli* and the *rnr* mutant of *P. syringae* are both sensitive to cold and both enzymes are shown to be involved in maturation of 3' end of 16S rRNA (32-35). RNase R of *Pseudomonas putida* is also implicated in survival at low temperature (7, 16-18). PNPase is also implicated in protecting *E. coli* cells against oxidative damage (31) and in DNA repair process by virtue of its 3'→5' DNase activity in *B. subtilis* and *E. coli* (22-25).

We show here that the RNase R is involved in oxidative damage and DNA repair process in *P. syringae* Lz4W. Unlike *P. syringae* RNase R, the *rnr* mutant of *E. coli* has no discernable phenotype and is refractory to these stress conditions. This implies that the protective role of RNase R is either specific to *P. syringae* Lz4W or possibly specific to *Pseudomonas* species. It is intriguing that although RNase R of *E. coli* is not involved in DNA damage and oxidative stress response in *E. coli*, yet it protects *P. syringae* Lz4W from DNA damage and oxidative stress. It is possible that the $PNPase^{Ec}$ and RNase R^{Ps} have evolutionarily swapped their functions with respect to their roles in the cellular milieu of these two bacteria. Although, we don't know why RNase R has been chosen over PNPase (unlike *E. coli)* to protect *P. syringae* Lz4W from oxidative stress and DNA damage, we speculate that the highly processive, hydrolytic RNase R is selected over the less processive, phosphorolytic PNPase to conserve the energy at low temperature.

RNA degradosome is a multi-enzyme complex that significantly contributes to the steady-state profiles of RNA transcripts in bacteria (36). Our laboratory has previously reported a novel degradosome complex comprising of the RNase E, the exoribonuclease RNase R and a DEAD-box RNA helicase RhlE in *P. syringae* Lz4W (15). By deleting of C-terminal domain of RNase E that is responsible for the assembly of degradosome complex, we showed that the association of RNase R with the degradosome complex is not needed to protect against oxidative and DNA damage stress, thus suggesting that the DNA damage resistance phenotype is an intrinsic function of RNase R.

We further investigated the possible role of each domain of multi-domain RNase R in protecting *P. syringae* cells from oxidative and DNA damage stress. Interestingly, we found the catalytic RNB domain of RNase R^{Ps} alone is sufficient for protecting *P. syringae* cells from oxidative and DNA damage stress, while the CSD and S1 domains are dispensable. In *E. coli*, RNB domain alone degrades structured RNA (12). Therefore, we envisaged that the degradation of structured RNAs either by full-length RNase R^{Ps} or by the catalytic RNB domain itself could play a protective role against oxidative and DNA damage stress in *P. syringae* Lz4W. We replaced the highly conserved catalytically important aspartate residues of RNase R^{Ps} with alanine. These mutants showed no apparent sensitivity to oxidative stress and DNA damaging agents, indicating the RNA degradation function of RNase R is not required. Further, the double- and triple-point mutations of the aspartate residues in combinations also resulted in similar results, confirming that the ribonuclease function of RNase R^{Ps} is not essential. Thus, the ability of RNase R to degrade highly stabilized RNA secondary structures does not have a role in protecting *P. syrin*gae cells against DNA damage and oxidative stress. Intriguingly, our data shows that the catalytically inactive RNase R^{Ps} and RNB domain itself can protect cells from oxidative and DNA damage stress, implying the importance of RNB domain alone with no exoribonuclease activity in this scenario.

If the association of RNase R with degradosome complex and its exoribonuclease activity is not essential, then how does RNase R protect *P. syringae* cells from DNA damage and oxidative stress? How does a catalytically inactive RNB domain itself without CSD and S1 domains could rescue *P. syringae* cells from oxidative and DNA damage stress? However, our data suggests that the RNB domain without catalytic function has a cryptic functional role which helps in protecting Lz4W cells from these stress conditions.

RNase R is known to process structured dsRNA without the help of a helicase and is implicated in removal of mRNAs with stable stem loops (6). It was earlier shown that cold sensitivity of *csdA* mutant of *E. coli* can be complemented by overexpressing RNase R, possibly through its helicase function (37). Importantly, the RNB domain of RNase R is also shown to possess helicase activity in *E. coli* (32, 38, 39). A recent study showed the presence of a winged tri-helix domain in RNB domain of *E. coli* that is responsible for the helicase function (29). This prompted us to investigate the presence of a similar tri- helix wedged domain in RNase R of *P. syringae*. Our homology modelling of *P. syringae* RNase R based on the RNase R of *E. coli*, shows a similar tri-helix wedge in RNB domain. Hence, we hypothesize that RNase R could participate in protection of *P. syringae* Lz4W against oxidative and DNA damage stress via its helicase activity. This is further supported by a recent observation in *P. syringae* Lz4W that the DEAD box helicases CsdA and SrmB are involved in protection against DNA damage and oxidative stress (40).

This study uncovered a hitherto unknown role of RNase R in the physiology of cold adapted bacterium *P. syringae* Lz4W. Here, we report a novel role of the RNase R in protecting *P. syringae* Lz4W against DNA damage and oxidative stress. Our study indicate that the protective role of RNase R is apparently specific to *P. syringae* Lz4W. Our structural homology modelling provides an insight into the presence of a tri-helix wedge region in the RNB domain of RNase R. which might play a role in protecting against oxidative and DNA damage stress by virtue of its putative helicase activity. Although our data biochemically doesn't not establish the helicase activity of RNB domain of RNase R, the genetic evidence provided here is comprehendible. In future it would be of relevance to elucidate the mechanism by which RNase R protects *P. syringae* Lz4W cells from DNA damage and oxidative stress.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are described in Table 2. *Pseudomonas syrinage* Lz4W cells were grown in Antarctic Bacterial medium (ABM: 5g liter^{-1} peptone and 2g liter^{-1} yeast extract) at both 22ºC and 4ºC. *E. coli* strains were routinely grown in Luria-Bertani medium at 37ºC. The growth media, when required, were supplemented with Antibiotics as follows: 100 µg ml^{-1} Ampicillin, 50 µg ml^{-1} Kanamycin, 20µg ml^{-1} tetracycline, and 400 µg ml^{-1} rifampicin. For complementation studies, plasmids were mobilized into *P. syringae* strain by conjugation using *E. coli* S17-1 helper strain. For growth analysis, cultures were grown in ABM

overnight and were inoculated in a fresh media in 1:100 dilutions and OD_{600} (O.D at 600 nm) was measured at various time intervals as indicated.

Spot assays and quantitation of sensitivity to DNA damaging agents

Cells were grown overnight at 22 ºC in AB media containing appropriate antibiotics and inoculated into fresh AB media with 1:100 dilutions. To measure the sensitivity to NaCl (1M), SDS (10% w/v), Lysozyme (100 mg/ml) + EDTA (500 mM), Ampicillin (100 µg), Rifampicin (100 µg), Spectinomycin (100 µg), Paraquat (20 µM), Mitomycin C (1000 ng), H_2O_2 (0.2 mM), Hydroxyurea (2.5 mM), Cisplatin (0.2 mM); up to 5-fold serial dilutions of exponentially grown cells at 22 ºC were made and spotted on ABM agar plates containing stress inducing agents as indicated. Plates were incubated at 22 ºC for 48 h. For UV sensitivity, up to 5-fold serially diluted exponentially grown cultures were spotted on ABM plates and exposed to UV radiations at indicated doses at the rate of 3 J/m^2/sec and plates were incubated in dark at 22 ºC for 48 h.

For quantitative analysis, exponentially growing cells were incubated for 30 mins with oxidative stress/DNA damaging agents at indicated concentrations, washed, then serially diluted, and plated on ABM-Agar plates or LB agar plates (for *E. coli* strains). Plates were incubated at 22 ºC for 48 h and the colony forming units (cfu) were scored. The percentage of cell survival was calculated by considering the cfu from untreated cells as 100%.

Reagents and general DNA recombinant methods

Routine molecular biology techniques like Genomic DNA isolation, restriction enzyme digestion, PCR, ligation, transformation, conjugation, Southern hybridization, Western analysis and RNA isolation were performed as described earlier (41). All restriction enzymes, T4 DNA ligase, T4 Polynucleotide kinase were purchased from New England Biolabs. Oligos used in the study were from Bioserve India (Hyderabad, India). PCR was carried out using proof reading Pfx DNA polymerase from Invitrogen (San Deigo, CA, USA). DNA sequencing was performed using ABI Prism dye terminator cycle sequencing method (Perkin-Elmer) and analyzed on an automated DNA sequencer (ABI model 3700; Applied Biosystems). Antibodies were purchased from Santa Cruz Biotechnology.

For Southern hybridization, genomic DNA was isolated from WT and *ΔC-rne* mutant cells, digested with PstI enzyme. The products of restriction digest were separated on a 1% w/v Agarose gel and transferred onto Hybond N+ membrane (Amersham Biosciences). For the probe, PCR amplicon of full-length *rne* gene was labeled with [α-^{32}P] dATP using random primer

labeling kit (Jonaki, BARC, India). Radioactive signals were detected and quantified by phosphorimager (Fuji FLA 3000).

Construction of mutants of RNase R and RNB by site directed mutagenesis and genetic complementation studies

The *rnr*^{Ec}, *rnr*^{Ps} genes and the domain deletion mutants of *rnr*^{Ps} were amplified from the genomic DNA of Lz4W using the primers listed in Table 3. All constructs contain 6x-Histidine tag at the N-terminal end of the protein. For D276A, D282A, D284A and D285A point-mutations in RNase R^{Ps}, site-directed mutagenesis of pGL*rnr* (7) was carried out using self-complementary primers harboring indicated mutations (listed in Table 3). Subsequently, the double D284A + D285A, D276A + D284 and triple D276A+D284A+D285A mutations were generated by using respective complementary primers using pGLrnr plasmid with preexisting mutations. Similarly, the D284A and D284A+D285A mutations were introduced into pGLRNB vector. Mutations and integrity of full-length genes were confirmed by DNA sequencing.

Purification of RNase R D284A **mutant and biochemical assays**

The wild-type RNase R and D284A mutant derivatives were purified by expressing these proteins in *rnr* null mutant of *P. syringae* Lz4W as escribed earlier (10). RNase R activity was assayed *in vitro* by its ability to degrade ssRNA substrate Poly(A) and structured RNA substrate *malE-malF* as described earlier (10). Briefly, Poly(A) was labelled at 5'-end using [γ^{32}P] and the *malE-malF* substrate was internally labelled using (α^{32}P). Assays were carried out in 20-µl reaction mixtures containing 25 mM Tris-HCl (pH 7.0), 100 mM KCl, 0.25 mM $MgCl_2$, 5 mM DTT. 10 pmol of labelled substrate was treated with 0.5 µM RNase R^{WT} and 15 µM RNase R^{D284A} at the indicated time-points at room temperature, and stopped by adding 10mM EDTA, 0.2% SDS and 1 mg/ml of proteinase K. RNA degradation products were resolved on 8% denaturing 8 M urea PAGE and degradation products were analysed by Phosphoimager (Fuji).

Western analysis

For examining the induction of RNase R, the exponentially grown wild-type *P. syringae* cells were incubated with DNA damaging agents. 1 ml aliquot was collected before treating cells with DNA damaging agents. 5 ml of cells were treated separately with Paraquat (15 µM), MMC (1000 ng), UV (18 J/m^2), and incubated at 22ºC. 1 ml of aliquot was collected at 30, 60 and 120 min intervals, Cells were further lysed by sonication in presence of Protease inhibitor cocktail (Roche, USA). Protein concentration from cell extracts was measured using Bio-rad Protein

Assay kit, also by the absorbance at 280 nm (A_{280}) using the Nanodrop spectrophotometer. Equal amount of protein was loaded and resolved on a 10 % w/v SDS-PAGE. The resolved proteins were later electro-blotted onto Hybond C-Extra membrane (Amersham Biosciences) and immunodetection was carried with polyclonal rabbit Anti-RNase R antibodies as described earlier (15). For detecting the levels of RecA expression, cells were treated with UV (18 J/m^2) and incubated at 22 ºC in dark. Culture aliquots were collected after 30 minutes of treatment and immuno-detected by polyclonal anti-RecA rabbit antibodies as described earlier (26).

Generation of C-terminal deletion of *rne* (RNase E gene)

For generating C-terminal deletion of *rne* gene, a 784 bp fragment from N-terminal of *rne* gene was first amplified using primer set Rne_Nterm_FP_BamHI and Rne_Nterm_RP_HindIII having the restriction enzyme sites for BamHI and HindIII respectively **(Table 2).** A second fragment from C-terminal of *rne* gene was PCR amplified using the primer set Rne_Cterm_FP_HindIII and Rne_Nterm_RP_SalI having the sites for restriction enzymes HindIII and SalI (Table 2). Both fragments were initially digested with HindIII and ligated. The ligated product was further digested with BamHI and SalI and cloned into pBlueScript-KS vector. The resultant plasmid pBS(N+C)*rne* was linearized by HindIII, and a tetracycline resistance gene was cloned to obtain the pBS(N+C)*rne*-tet plasmid. The tetracycline resistance gene (*tet*) cassette (~2.4 KB) was obtained from the pMOS^{tet} (42) as a HindIII fragment. Plasmid construct was confirmed by sequencing and PCR analysis. Plasmid pBS(N+C)*rne*-tet plasmid was alkaline denatured as described earlier (42). Alkali denatured plasmid DNA was electroporated into the wild-type *P. syringae* cells. Recombinants were selected on ABM plates containing tetracycline and the knock-out was confirmed by Southern and Western analysis.

Modeling of RNase R^{Ps} protein

The amino acid sequence of Lz4W RNase R was taken from NCBI (GenBank: AUB77091.1). A model of RNase R^{Ps} was constructed using the SWISS-MODEL (43). The closest template identified was PDB: 5xgu.1, which refers to the RNase R structure from *Escherichia coli*. The RMSD between these two structures was then calculated using the Matchmaker tool of Chimera (44). The RMSD value was calculated between 613 pruned atom pairs is 0.248 angstroms; (across all 625 pairs: 0.930). The two structures were superimposed as shown in the figure 5B.

Statistical analysis

Data was calculated and analyzed by using Prism 5 (GraphPad Software Inc, USA).

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Figure legends

Figure 1 *S*ensitivity of *P. syringae* of Δ*rnr* and complemented strains to DNA damage and oxidative stress causing agents. A, Percentage cell survival of WT, Δ*rnr*-pGL10, Δ*rnr*-pGL*rnr*^{Ps} and Δ*rnr*- pGL*rnr*^{Ec} upon treating with DNA damage and oxidative stress causing agents at indicated concentrations. Data are from the three independent experiments with mean ± SE. **B,** Expression levels of RNase R of *P. syringae* upon treating with DNA damage and oxidative stress causing agents, determined by Western analysis using polyclonal RNase R specific antibodies. The bottom panel shown the expression level of RecA protein upon UV irradiation as a positive control. **C,** Qualitative assessment of cell viability by spot assays. WT, Δ*rnr*-pGL10, Δ*rnr*- pGL*rnr*^{Ps} and Δ*rnr*- pGL*rnr*^{Ec} treated with DNA damage and oxidative stress causing agents at indicated concentrations.

Figure 2 *S*ensitivity of C-terminal domain deletion mutant of *rne* gene (*rne* $^{\Delta 595 - 1074}$) encoding RNase E compared to Δ*rnr P. syringae* cells. Qualitative assessment of cell viability by spot assays. WT, Δ*rnr* and Δ*rne* $^{\Delta 595 - 1074}$ were exposed to DNA damage and oxidative stress causing agents at indicated concentrations.

Figure 3 *S*ensitivity of Δ*rnr P. syringae* strain complemented with domain/s deleted mutants of RNase R. A, Schematic representation of structural domains of *P. syringae* RNase R. The domain/s deleted RNase R constructs; cold shock domain (CSD, 1 – 225 aa), catalytic RNB domain (226 – 665 aa), and S1 domain (666 – 885 aa) are shown. **B**, Percentage of cell survival of WT, Δ*rnr*-pGL10, Δ*rnr*- pGL*rnr-ΔCSD*, Δ*rnr*- pGL*rnr-ΔS1* and Δ*rnr*- pGLRNB*(ΔCSD +ΔS1*) upon treating with DNA damage and oxidative stress causing agents at indicated concentrations. Data are from the three independent experiments with mean ± SE. **C**, Qualitative assessment of cell viability by spot assays. WT, Δ*rnr*-pGL10, Δ*rnr*- pGL*rnr-ΔCSD*, Δ*rnr*- pGL*rnr-ΔS1* and Δ*rnr*- pGLRNB*(ΔCSD +ΔS1*) were treated with DNA damage and oxidative stress causing agents at indicated concentrations.

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Figure 5 *S*ensitivity of Δ*rnr P. syringae* strain complemented with catalytic site mutants of RNB domain of RNase R. A, Qualitative assessment of cell viability by spot assays. WT, Δ*rnr*-pGL10, Δ*rnr*- pGLRNB, Δ*rnr*- pGLRNB-D284A and Δ*rnr*- pGLRNB-D284+D285A were treated with DNA damage and oxidative stress causing agents at indicated concentrations. **B,** Structural model of *P. syringae* RNase R constructed by superimposing on the structure of *E. coli* RNase R (PDB:5xgu.1) using SWISS-MODEL. The tri-helix wedge region of RNB domain of *P. syringae* RNase R is shown in black color.

Figure S1 *S*ensitivity of Δ*rnr E. coli* strain to DNA damage and oxidative stress causing agents. Qualitative assessment of cell viability by spot assays of WT, Δ*rnr* and Δ*recB* mutant of *E. coli*. RecB mutant was used as a positive control against DNA damaging agents. Cells were exposed to DNA damage and oxidative stress causing agents at indicated concentrations.

Figure S2 *S*ensitivity of Δ*rnr P. syringae* strain complemented with PNPase of *P. syringae*. Qualitative assessment of cell viability by spot assays. WT, Δ*rnr*-pGL10 and Δ*rnr*-pGL*pnp* were exposed to DNA damage and oxidative stress causing agents at indicated concentrations.

Figure S3 *S*ensitivity of Δ*rnr P. syringae* strain complemented with double- and triple-mutants of catalytic site residues of RNase R. Qualitative assessment of cell viability by spot assays. WT, Δ*rnr*-pGL10, *rnr*- pGL*rnr*-(D276A+D284A), *rnr*- pGL*rnr*-(D276A+D285A) and *rnr*-pGL*rnr*-(D276A+D284A+D285A) treated with DNA damage and oxidative stress causing agents at indicated concentrations.

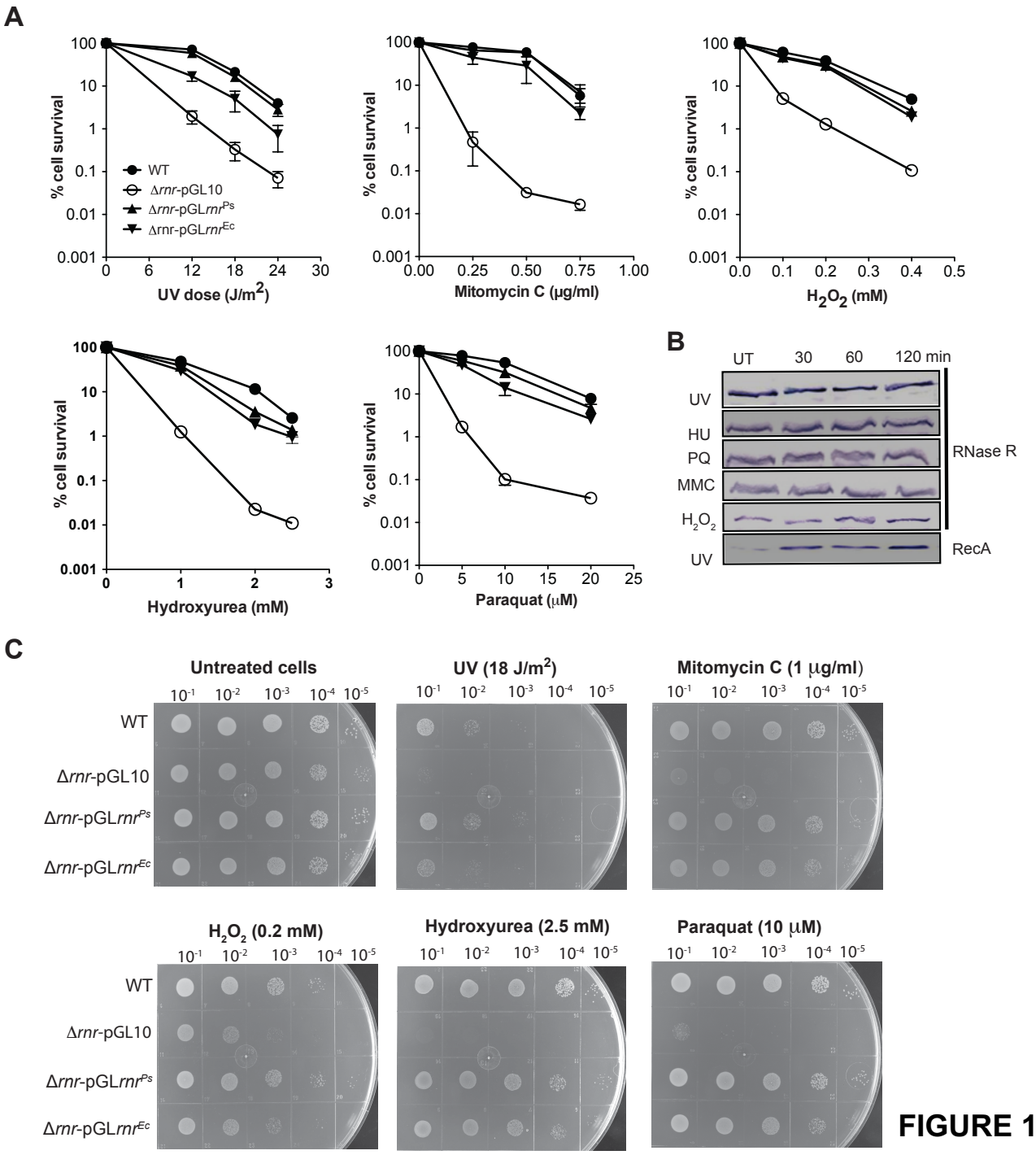

FIGURE 1

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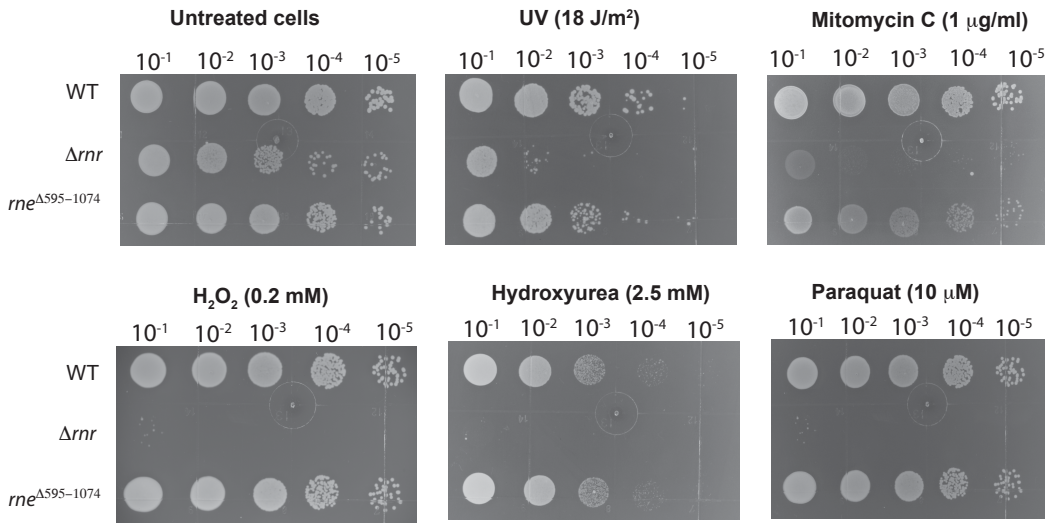

FIGURE 2

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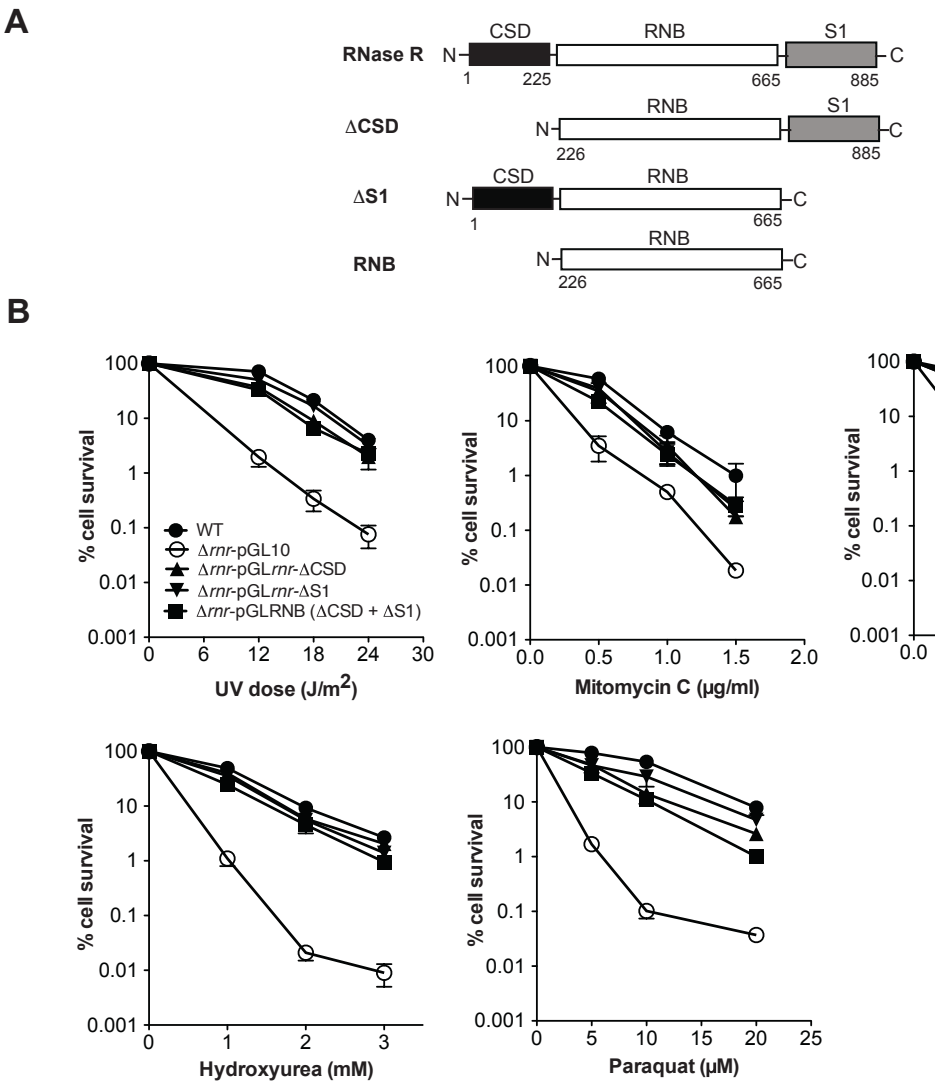

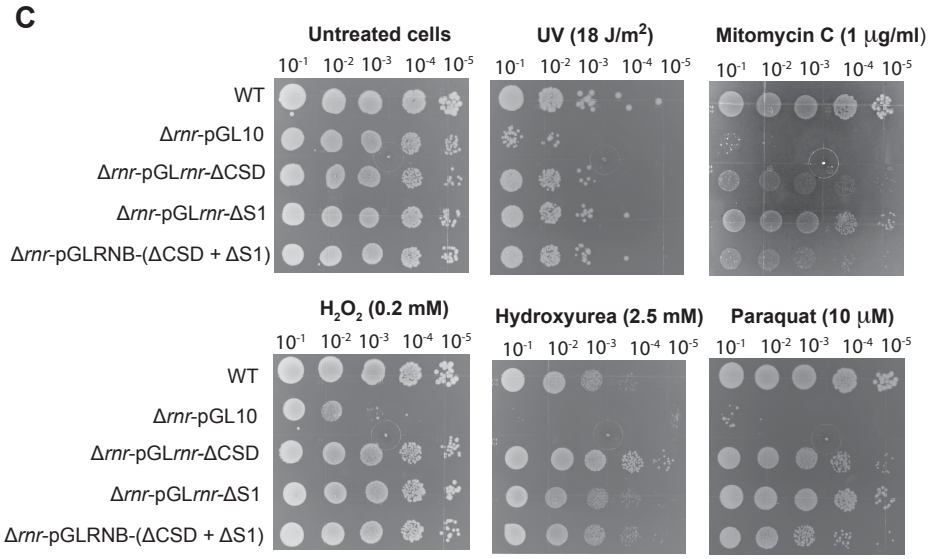

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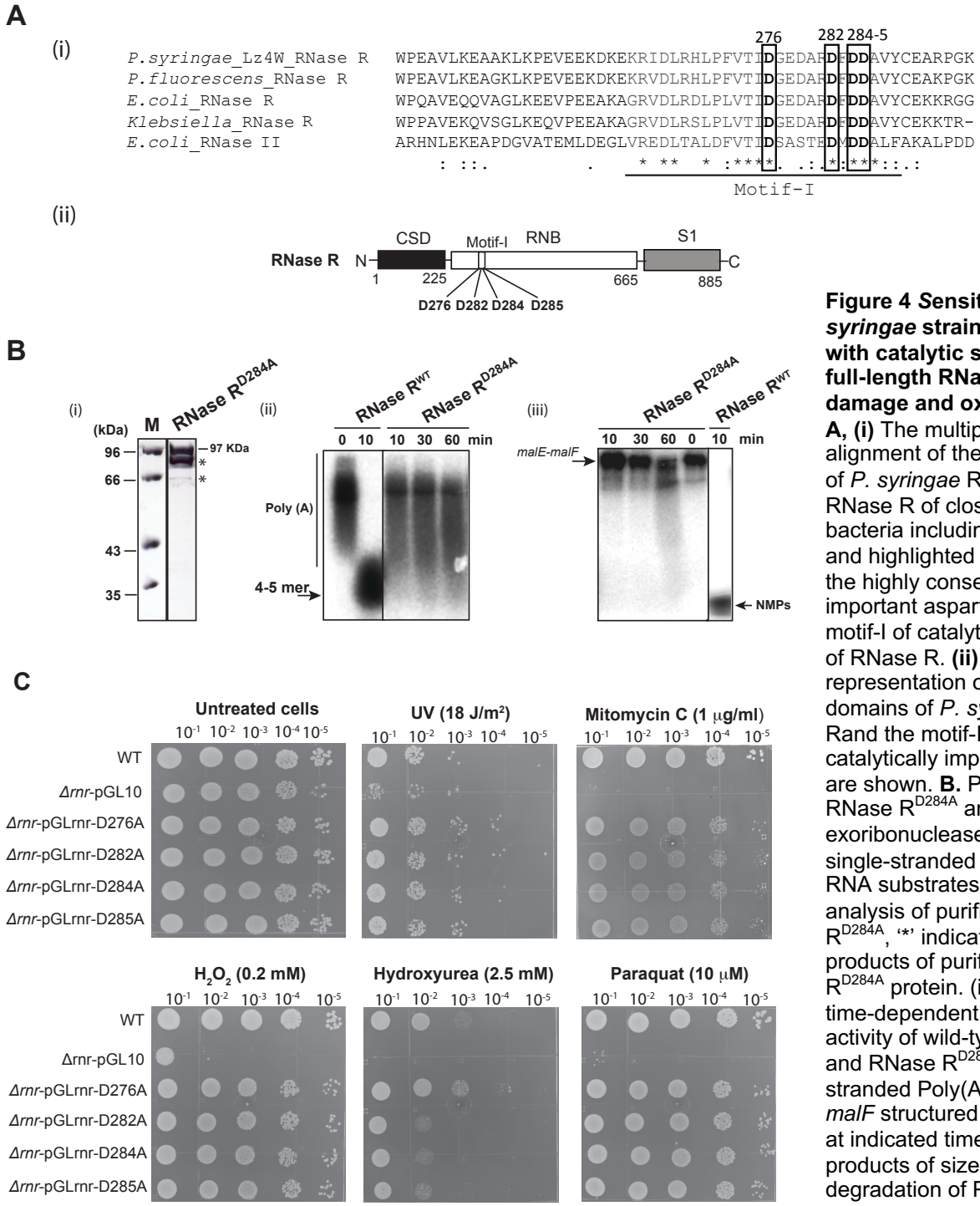
FIGURE 3



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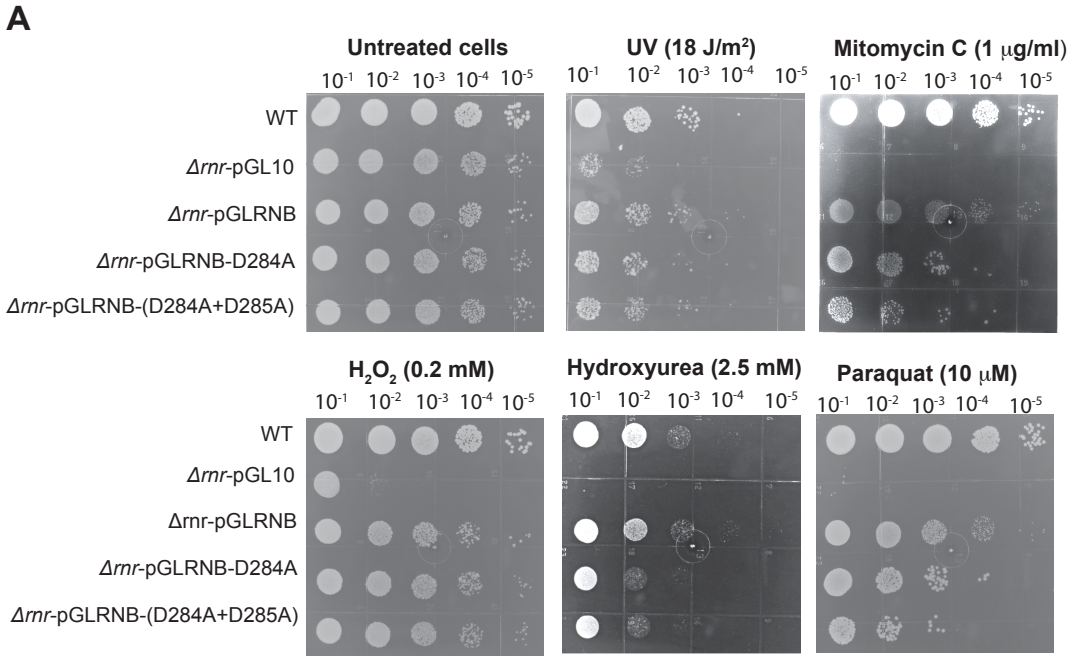

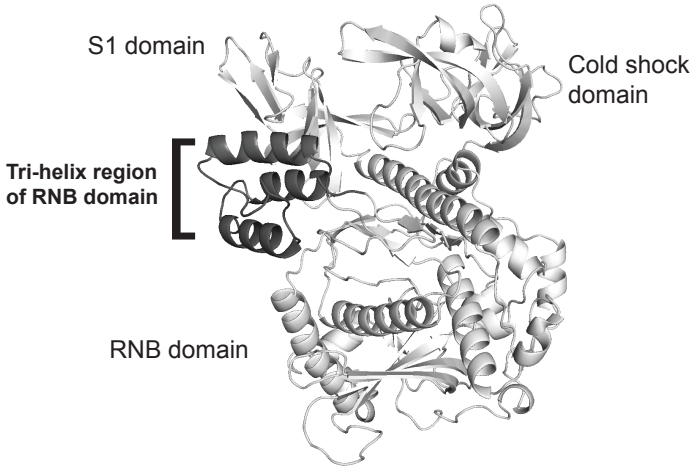

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