

Exoribonuclease R in *Pseudomonas syringae* is essential for growth at low temperature and plays a novel role in the 3' end processing of 16S and 5S ribosomal RNA

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Running Title: Essential nature of *P. syringae* RNase R

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The (3'→5') exoribonuclease RNase R interacts with the endoribonuclease RNase E in the degradosome of the cold adapted bacterium *Pseudomonas syringae* Lz4W. We now present evidence that the RNase R is essential for growth of the organism at low temperature (4°C). Mutants of *P. syringae* with inactivated *rnr* gene (encoding RNase R) are cold sensitive and die upon incubation at 4°C, a phenotype that can be complemented by expressing RNase R in *trans*. Over expressing polyribonucleotide phosphorylase (PNPase) in the *rnr* mutant does not rescue the cold sensitivity. This is different from the situation in *Escherichia coli*, where *rnr* mutants show normal growth, but *pnp* (encoding PNPase) and *rnr* double mutants are nonviable. Interestingly, RNase R is not cold inducible in *P. syringae*. Remarkably, however, *rnr* mutants of *P. syringae* at low temperature (4°C) accumulate 16S and 5S ribosomal RNA (rRNA) that contain untrimmed extra ribonucleotide residues at the 3' ends. This suggests a novel role of RNase R in the rRNA 3' end processing. Unprocessed 16S rRNA accumulates in the polysome population, which correlates with the inefficient protein synthesis ability of mutant. An additional role of RNase R in the turnover of tmRNA was identified from our observation that the *rnr* mutant accumulates tmRNA fragments in the bacterium at 4°C. Taken together our results establish that the processive RNase R is crucial for RNA metabolism at low temperature in the cold adapted Antarctic *P. syringae*.

Regulated degradation of RNA within cell is mostly an outcome of coordinated and combined activities of endoribonucleases, exoribonucleases and RNA helicases. In bacteria, few of these enzymes interact with each other to form the RNA degradosome complex (1,2,3). The degradosome has been shown to be involved in both mRNA and rRNA degradation (4,5). In the *Escherichia coli* degradosome, RNase E (a 5'-end dependent endoribonuclease) associates with polynucleotide phosphorylase (PNPase¹, a 3'→5' exoribonuclease), RhlB (a DEAD-box RNA helicase) and enolase (an enzyme of glycolytic pathway) to constitute the 'core' complex. Additionally, DnaK, poly(A) polymerase and polyphosphate kinase have also been reported to be a part of this complex (6). A similar kind of RNA degrading complex has also been reported from *Rhodobacter capsulatus* (7). On the other hand, we have recently shown that exoribonuclease RNase R interacts with RNase E in the degradosome of the cold adapted Antarctic bacterium *Pseudomonas syringae* Lz4W (8). This bacterium does have a *pnp* gene which is expressed but does not form a part of this complex (unpublished results). PNPase and RNase R differ in their mode of action, the former exhibiting phosphorolytic activity, and the latter hydrolytic activity.

RNase R is one of the eight exoribonucleases reported in *E. coli* and distributed widely in different prokaryotes (9). Although all of these exoribonucleases display 3'→5' activity on RNA substrate, RNase R is the most highly processive enzyme among them. The enzyme was first identified in *E. coli* crude

cell extract, where RNase II contributes the bulk (98%) of the poly(A) RNA degrading activity while RNase R contributes only residual (2%) activity (10). Subsequently, it was named RNase R due to its action on rRNA and shown encoded by the *vacB* gene, which is essential for virulence in *Shigella flexneri* and *E. coli* (11). It has been proposed that RNase R along with PNPase, encoded by *rnr* and *pnp* genes, respectively, are responsible for quality control of rRNA, and that *rnr pnp* double mutants are inviable (12). Interestingly, PNPase was found important for growth at low temperature in *E. coli* and in the psychrotrophic *Yersinia enterocolitica* (13,14), although the *pnp* mutants of *P. putida* were not cold sensitive (15). RNase R, on the other hand, was shown to be cold inducible and involved in tmRNA maturation in *E. coli*, but the *rnr* mutant was not cold sensitive (16). Extensive study from Deutscher's group has now established that RNase R can degrade RNA with secondary structures without the help of helicase *in vitro*, and is proficient in degrading mRNAs with Repetitive Extragenic Palindromic (REP) sequences *in vivo* (17). The same group shows that the RNase R level is elevated in response to stress conditions including starvation and entry into stationary phase (18). The authors speculate that extensive remodeling of structured RNA occurs under stress conditions, which would require the highly processive activity of RNase R to work on the RNA substrates.

Our observation that phosphorolytic PNPase is replaced by hydrolytic RNase R in the degradosome of the psychrotrophic bacterium *P. syringae* Lz4W is puzzling (8). As a further step towards detailed characterization of the *P. syringae* degradosome machinery, knockout mutants of RNase R (*rnr*), RhIE (*rhIE*) and the C-terminal degradosome-organizing region of RNase E (*rne^C*) would be useful. We report here our results on the *rnr* knockout mutant of *P. syringae*, which was found to be severely cold sensitive. Remarkably, our data suggest that RNase R is involved in the processing of 3' ends of 16S and 5S rRNAs. The *rnr* mutants are not only defective for 16S and 5S rRNA maturation at 4°C but also accumulate unprocessed 16S rRNA in the polysomes probably making the translation machinery inefficient at low

temperature. The mutant cells also accumulate degradation intermediates of tmRNA suggesting that the important regulatory RNA is also a target of RNase R in the bacterium. We suggest that a combination of defects in RNA metabolism at low temperature in the absence of RNaseR lead to the cold sensitive phenotype of the bacterium.

EXPERIMENTAL PROCEDURES

Bacterial strains, and growth conditions. *P. syringae* Lz4W and *E. coli* cells were routinely grown in Antarctic Bacterial Medium (ABM) (5 g liter⁻¹ peptone, and 2.5 g liter⁻¹ yeast extract), and Luria-Bertani medium (LB), respectively, as reported earlier (8). When required, growth media were supplemented with antibiotics in following concentrations (μg ml⁻¹): ampicillin- 100, kanamycin- 50, tetracycline- 20, chloramphenicol- 100, and rifampicin- 400. Composition of minimal growth medium for *P. syringae* was: Na₂HPO₄- 0.12%, KH₂PO₄- 0.06%, (NH₄)₂SO₄- 0.05%, succinic acid- 1%, valine- 0.01%, isoleucine-0.01%, and 1 mM MgSO₄ (BN Sahu and MK Ray, unpublished). For growth analysis, bacterial cells from overnight culture were inoculated into fresh medium at a dilution of 1:100, and the turbidity of the cultures at 600 nm (OD₆₀₀) was measured at various time intervals. For complementation studies, plasmids were introduced into *P. syringae* strain by conjugation with *E. coli* S17-1 (19).

Recombinant DNA methods. General DNA recombinant techniques including isolation of genomic DNA, restriction analysis, polymerase chain reaction (PCR), ligation, and transformation etc were performed as described (20). All restriction enzymes, T4 DNA ligase, and Klenow enzyme used in this study were from New England Biolabs (NEB). Ominiscript RT kit (Qiagen) was used for reverse transcription and oligonucleotides were purchased from BioServe BioTech, India.

For Southern hybridization, genomic DNA was isolated from both wild type and *rnr* mutant of *P. syringae*, and digested with *SalI* enzyme. DNA fragments were separated on 1% agarose gel and then transferred onto Hybond N⁺ nylon membrane (Amersham Biosciences). PCR amplified DNA of full-length *rnr* gene (2.7 kbp)

was labeled with [α - 32 P]dATP using random primer labeling kit (Jonaki, BARC, India), and used as probe. DNA hybridization was carried out in 0.5 M sodium phosphate containing 7% SDS at 65°C for 8-10 hours. Membrane blots were washed twice with 2X and 0.1X SSC, respectively. Radioactive signals were developed using a Phosphor Imager (Fuji FLA3000).

Construction of *rnr* knockout mutant of *P. syringae*. Plasmid pBS*rnr*-tet used for knocking out *rnr* gene was constructed as follows. The *rnr* gene was first subcloned as *Kpn*I-*Xba*I fragment into pBlueScript-KS vector, taking the fragment from earlier reported plasmid pMOSBlue containing *P. syringae rnr* gene (8). The resultant plasmid pBS-*rnr* was linearized with *Hinc*II, and a tetracycline resistance gene was cloned within the *rnr* to generate pBS*rnr*-tet plasmid. The source of tetracycline resistance gene (*tet*) cassette (2.4 kbp) was pTc28 plasmid (19) from which it was cleaved out as *Xba*I-*Hind*III fragment and made blunt ended using Klenow enzyme before ligating to the *Hinc*II site of pBS-*rnr*. Plasmid construct was confirmed by sequencing and PCR analysis. For *rnr* knockout, the pBS*rnr*-tet was electroporated into *P. syringae* following denaturation of the plasmid as described earlier (21), which apparently increases homologous recombination and hence gene disruption frequency. Recombinants were selected by tetracycline resistance, and the *rnr* knockout mutation was confirmed by Southern and PCR analysis.

Plasmids for complementation analysis. For genetic complementation of *rnr* mutant, plasmids pGL*rnr*-his and pGL*pnp*-his were used. Construction of pGL*rnr*-his expressing His-tagged RNase R in *P. syringae* has been described earlier (8). For construction of pGL*pnp*-his, a similar strategy was followed. Briefly, the *P. syringae* PNPase gene (*pnp*) was amplified from the genomic DNA by PCR using the forward PnpFP1 (5'-TTCCAGTTCGGTCAGTCGACCGT-3') and reverse PnpRP2 (5'-ACGTCCTTGATC/GGACAGCTTGAT-3') primers. The 2.1 kbp PCR product was first cloned into the *Eco*RV site of the pMOSBlue plasmid (Amersham Biosciences) and

subsequently into the expression vector pET28a (Novagen). The His-tagged version of the PNPase gene was then cleaved out of pET*pnp*-his and cloned into the broad host range plasmid vector pGL10 (22) to generate pGL*pnp*-his. Reading frame and the in-frame cloning of PNPase gene were confirmed by DNA sequencing. Expression of protein was confirmed by western blot analysis, using either anti-His tag antibody or anti-PNPase antibodies.

RNA isolation and Northern hybridization analysis: Wild type and *rnr* mutant strains of *P. syringae* were grown at 22°C till the cultures attained OD₆₀₀ ~0.5. Cultures were then shifted to 4°C, and every 24 hours, 6 ml culture was removed, of which 3 ml was centrifuged immediately. To the remaining 3 ml, rifampicin was added (final concentration of 400 μ g ml⁻¹) and incubation continued at 4°C for another 60 min. Total RNA was isolated from all the samples by hot-phenol method (23), and the concentration of RNA was estimated by measuring A₂₆₀ of all the samples. RNA (10 μ g) from each sample was resolved on 1.2 % agarose gel (12) in TAE buffer (20) and transferred to Hybond N⁺ membrane by vacuum transfer using 5X SSC / 10 mM NaOH. The oligonucleotide probes (p1 through p4, Supplementary Table S1) used for Northern hybridization were 5'-end labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (NEB) following manufacturer's protocol. The 32 P-labeled oligonucleotides were separated from unincorporated γ - 32 P-ATP by gel filtration on Sephadex G50 columns. The temperatures for oligonucleotide-probe hybridization and washing of the northern blot membrane are indicated in Table S1. Probe for tmRNA was prepared by amplifying the tmRNA gene by PCR of genomic DNA using a set of forward (5'-TTAGGATTCGACGCCGGT-3') and reverse (5'-TGGTGGAGCCGGGGGATTTGAAC-3') primers, and labeled by random primer labeling method as described for Southern hybridization probe.

Circular RT-PCR for mapping of 5' and 3' ends of rRNA: The precise 5' and 3' ends of the 16S rRNA and 16S rRNA precursors were determined using circular RT-PCR method. Briefly, total RNA was circularized

using T4 RNA ligase (NEB) in presence of RNase inhibitor Rnasin (Promega). Then, reverse transcription reaction was performed with reverse transcriptase (Ominiscript RT kit, Qiagen) for 1 h at 42°C as per manufacturer's instruction, in the presence of antisense primer corresponding to a region in the 5' end of mature 16S rRNA (primer 16SRU1, same as p2 in Table S1). The resultant cDNAs spanning the junction of 5' and 3' ligated ends were then amplified by PCR using the 16SRU1 and outwardly directed 16SFU1 (5'-GGGGTGAAGTCGTAACAAGGTAGCCG-3') corresponding to a region at the 3' end of mature 16S rRNA. The amplified PCR products were separated and visualized by ethidium bromide staining on 1.5% agarose gel. DNA sequence was determined either directly following the extraction and purification of PCR products from agarose gel or following their cloning in pMOSBlue plasmid. Big-Dye™ terminator cycle sequencing kit (ABI) was used for sequencing reaction.

Western analysis: Protein blotting and immuno-detection techniques for western analysis were as described in (8). For checking low temperature induction of the RNase R, *P. syringae* cells were grown at 22°C till OD₆₀₀ reached ~0.5 and then shifted to 4°C. Culture (3 ml) was withdrawn at the indicated time and cell extracts were prepared by sonication. Protein concentration was determined using Bio-Rad protein assay kit. Equal amount of proteins were loaded in each lane for SDS-PAGE separation and blotting onto Hybond-C membrane (Amersham Biosciences). Polyclonal anti-His antibody was from Santa Cruz Biotechnology. Antibody against the *P. syringae* PNPase was raised in rabbit against the purified recombinant protein as described for the polyclonal anti-RNase R antibody (8). Alkaline phosphatase conjugated anti-rabbit goat IgG was used as secondary antibody.

Cell viability studies: Viability of cells was examined both by counting colony-forming units (CFU) in the cultures, and also by differential fluorescent staining of live and dead cells under a fluorescent microscope (Carl Zeiss, Germany). Briefly, cells were grown at 22°C till OD₆₀₀ reached ~0.5 followed by shifting to 4°C. At every 24 h interval, aliquots of culture were

withdrawn for measuring turbidity at OD₆₀₀ and spreading of cells onto ABM-agar plates for growth at 22°C. For each time point, CFUs on plates (in triplicates) were counted. Cells in cultures at each time point were also examined microscopically (19) by staining them with Syto9 and propidium iodide (PI) using LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR).

Isolation and separation of ribosome.

Ribosomes were isolated and separated on sucrose gradient in presence of 10 mM or 0.3 mM MgCl₂ depending upon the experimental requirement as described earlier (23). Ribosomes were prepared either directly from 22°C grown cells or following their shift to low temperatures for different periods. Just before harvesting, cells were treated with chloramphenicol (100 µg ml⁻¹) for 10 minutes (24). Cells were harvested and resuspended in ribosome buffer (10 mM Tris-Cl pH 7.5, 30 mM NH₄Cl, 10 mM MgCl₂ and 6 mM β-ME) containing lysozyme (1 mg ml⁻¹) and subjected to sonication. Cell debris was removed by centrifugation at 12,000 g. Supernatant was then re-centrifuged at 70,000 rpm in a Beckman table top ultracentrifuge (TLA 100.3 rotor). The ribosomal pellet was suspended in ribosome buffer and 25 A₂₆₀ unit equivalents of ribosomes were loaded onto 5 - 40% sucrose gradient for centrifugation at 35,000 rpm in a SW41 rotor (Beckman) for 2.5 h. Fractions of 500 µl each were collected from the gradient and A₂₆₀ was measured. A gradient maker (Haake-Buchler, Germany) was used for both preparation of the sucrose gradient and collection of the fractions from the tubes. When required, the pooled fractions of 30S, 50S, 70S, and polysomes were treated with Triton X-100 (1%), or with 200 µM poly(U) and pelleted by centrifugation before further analysis.

Analysis of protein synthesis. Protein synthesis was monitored by pulse-chasing the growing cells of *P. syringae* in the presence of a mixture of ³H-labeled amino acids (Amersham Life Science). Briefly, cells (OD₆₀₀ ~0.5) growing in ABM at 22°C were shifted to 4°C for incubation. At the various time points, cells were harvested and suspended in minimal growth medium supplemented with a mixture of ³H-amino acids (50 µCi ml⁻¹, specific activity 13 –

78 mCi mmol⁻¹) for 30 minutes (pulse) of labeling. The cells were then incubated (chase) for another 30 minutes in the richer medium by adding an equal volume of 2X ABM into the minimal medium. Cells were then lysed with Tris-lysozyme-EDTA buffer, and proteins were precipitated by adding equal volume of ice-cold 10% TCA. Protein pellet was spotted onto filter paper (Whatman) and radioactive counts were measured in a toluene-based scintillant (0.5% PPO and 0.03% POPOP dissolved in toluene) using a liquid scintillation counter (Packard Tricarb).

RESULTS

***rnr* knock out mutants of *P. syringae* are cold sensitive:** To assess the importance of RNase R in RNA metabolism of the cold adapted *P. syringae* Lz4W, an *rnr* knock out mutant strain (*rnr::tet*) was created. The strategy used for knocking out *rnr* is shown schematically in Fig. 1A. Integration of the *tet*-cassette by homologous recombination and disruption of the *rnr* gene in mutant was confirmed by Southern hybridization (Fig. 1B) and by PCR analysis of genomic DNA (data not shown). Expectedly, the *rnr* gene specific probe hybridized to only one *SalI* restriction fragment in the wild type but to two fragments in the mutant owing to the location of a single *SalI* recognition site within the *tet*-cassette (Fig. 1B). The lack of RNase R production due to inactivation of the *rnr* was confirmed by western analysis of cell extract using anti-RNase R antibodies (Fig. 1C).

We then examined growth of the *rnr* mutant at 22°C and at 4°C. Growth analysis (Fig. 1D & E) reveals that the mutant, while marginally affected at room temperature (22°C), is severely defective for growth at 4°C. The mutant strain failed to grow even after 15 days of incubation at low temperature. In order to confirm that the observed phenotype was only due to absence of functional RNase R the *rnr* mutant was transformed with plasmid pGLrnr-his for expression of RNase R in *trans*. The mutant cells produced recombinant His-tagged RNase R and grew well at 4°C, similar to wild type, but a mutant strain carrying pGL10 (empty vector) remained cold sensitive. This confirmed that only the lack of RNase R is responsible for

cold sensitive phenotype of the *rnr* knockout mutant.

Viability of *rnr* mutants decreases at 4°C: The cold sensitive phenotype of the *rnr* mutant can either be due to lack of growth or due to cell death. In order to test this, we checked the viability of cells by two different methods following a temperature downshift (22° to 4°C) of the cultures. Fig. 2A represents the growth curve of the *rnr* mutant and wild type Lz4W after the shift of cells to 4°C. The OD₆₀₀ of the culture of *rnr* mutant increased during the first 24h of incubation but subsequently declined slowly but continuously, suggesting a possible cell lysis as a result of cell death at 4°C. CFU measurements of the cultures were also consistent with this, as shown in Fig. 2B. The CFU in the cultures increased in the first 24h but then gradually declined, and at 144h around 95% of the mutant cells were dead. On the other hand, cultures of wild type or the complemented mutant i.e., *rnr*⁺(pGLrnr-his) strain did not show any decrease of CFU.

Cell death of *rnr* mutants at 4°C was also confirmed by staining cells with vital dye Syto9 and propidium iodide (PI) that stain nucleic acids. With increase in the time of incubation at 4°C not only did the number of live cells (Syto9 stained, green) decrease but also the total number of cells in case of the *rnr* mutant, probably due to cell death and lysis (Fig. 2C). In the wild type the number of living cells (green) expectedly increased due to cell division, but the frequency of PI stained dead cells (red) within the cell population appears to remain constant even upon reaching the stationary phase. We also noticed that the size of wild type cells of *P. syringae* as reported earlier (19), decreases (from ~2.8 μm length to about 1.3 μm) when incubated at 4°C but cell size of the *rnr* mutant does not alter much. This makes mutant cells appear 2-3 fold bigger (~3 μm) than the wild type cells at 4°C under similar conditions. However, few cells (~1%) in the mutant were 3-5 fold longer than the rest. Interestingly, cell elongation occurred in the *pnp*^{ts} *rnr* double mutants of *E. coli* defective for both PNPase and RNase R at the nonpermissive temperature (42°C) but not in the single mutants of *pnp* and *rnr* (12). It was speculated in the report that cell

elongation of the mutants could be due to defective cell division, which might be true of *rnr* mutant of *P. syringae* at low temperature too.

Increased amount of PNPase does not rescue cold sensitivity of *rnr* mutant. In *E. coli* RNase R and PNPase have overlapping function, since each of the single mutants are viable but the double mutation is lethal (12). Neither of the single mutants shows any defect in rRNA turnover. The fact that *rnr* mutants of *P. syringae* are cold sensitive suggests that PNPase does not complement the RNase R function in the bacterium at 4°C. However, we wanted to check if increased amount of PNPase is capable of complementing the cold sensitive phenotype of *rnr* mutant to any extent. For this, the *rnr* mutant strain was transformed with pGLpnp-his for expression of His-PNPase in *trans*. Expression of the recombinant PNPase was confirmed by western analysis using anti-His antibodies which cross-reacted to the protein with expected 75 kDa molecular mass (Fig. 3A). The level of PNPase in this strain, as estimated from the western blot analysis using anti-PNPase antibodies, was ~ 2.5 - 3 fold higher compared to the wild type (Fig. 3A, right panel). Growth of the *rnr*(pGLpnp-his) strain was then compared with the wild type and *rnr* mutant carrying empty pGL10 plasmid at 4°C. The *rnr* mutant expressing recombinant PNPase was equally growth defective as was the *rnr* mutant (Fig. 3B). The recombinant His-PNPase was confirmed to be enzymatically active (supplementary Fig. S1). This suggests that increased amount PNPase is unable to complement the RNase R function in the bacterium at 4°C.

RNase R is not cold inducible in *P. syringae*. Since the *rnr* mutant of *P. syringae* is cold sensitive, we checked whether RNase R is upregulated at low temperature in this bacterium. Western analysis using polyclonal anti-RNase R antibodies suggests that this protein is not cold inducible (Fig. 4A). Neither did the amount of RNase R decrease in cells at the stationary phase of both low (4°) and high (22°C) temperatures (Fig. 4B), in difference to the observation in *E. coli*. RNase R was reported to be stress inducible especially under low

temperature and at stationary phase in the mesophilic *E. coli* (16, 18).

***rnr* mutants are defective in 16S and 5S rRNA processing:** RNase R along with PNPase has been implicated in rRNA turnover in *E. coli* (12). The death of *P. syringae rnr* cells at low temperature therefore prompted us to look at the status of different rRNA species in the mutant. We examined the status of 23S, 16S and 5S rRNA species by Northern analysis. Positions of various oligonucleotides used for northern hybridizations have been shown schematically in Fig. 5A.

The p1 probe corresponding to the nucleotide sequence lying 11 bases down stream of the mature 3' end of 16S rRNA, hybridized to the 16S rRNA of the *rnr* mutant at low temperature. Fig. 5B shows accumulation of the incompletely processed 16S rRNA population at various time points only in the *rnr* mutant after shifting to 4°C. Fig. 5C shows the ethidium bromide stained gel for RNA loading control. Although a certain amount of incompletely processed 16S rRNA was noticeable at 22°C at the zero time point of both wild type and the mutant (lanes C with 0 min samples, Fig. 5B), it disappeared within 60 minutes after rifampicin treatment in both (lanes C - 60 min, Fig. 5B). This suggests that some other enzyme is capable of 16S rRNA maturation at the higher temperature (22°C) but it fails to do so at 4°C. Interestingly, Fig. 5B also reveals that there is a increased degradation of 16S rRNA precursors (smearing of the RNA on Northern blot) with time in the absence of RNase R, which occurs much earlier than the 72 h noted in Fig. 5D for the degradation of matured 16S rRNA at 4°C (see below). The processing of 16S rRNA in the complemented *rnr* mutant, as shown in Fig. 5E, was normal.

In a control experiment when p2 oligonucleotide sequence corresponding to a matured region of 16S rRNA was used as probe for hybridization, the 16S rRNA profiles of the mutant and wild type were similar, except one additional defect noticed in the mutant. This defect in *rnr* mutant was manifested by accumulation of the degradation intermediates of 16S rRNA only at a later stage (after 72h) of incubation at 4°C (Fig. 5D). Thus, RNase R might also be involved in general turnover

and/or quality control of 16S rRNA. Accumulation of similar rRNA degradation intermediates was reported for the temperature sensitive *rnr pnp* double mutants of *E. coli* when they were grown at non-permissive temperatures (12). Interestingly, we did not observe any accumulation of unprocessed and/or degradation intermediates of 23S rRNA in the *rnr* mutant of *P. syringae* when the probe p3 was used for northern hybridization (data not shown). This suggests that 3' end processing of 23S rRNA does not involve RNase R.

We also examined status of the 3' end of 5S rRNA in the *rnr* mutant at 4°C. The probe p4 containing nucleotides beyond the matured 3' end of 5S rRNA was used for hybridization analysis. It appears that the 5S rRNA population in the mutant cells hybridize to p4 probe (Fig. 5F). Thus, in addition to 16S rRNA, processing of the 5S rRNA is also defective in the *rnr* mutant of *P. syringae* at low temperature.

Unprocessed 16S and 5S rRNAs are present in the ribosome fractions of *rnr* mutants. Unprocessed rRNAs are normally incorporated into pre-ribosomal particles and show altered ribosomal profiles. Hence we analyzed the ribosome profile of the *rnr* mutant. Surprisingly, ribosomal profiles of the *rnr* mutant appeared normal, similar to wild type, at 4°C. Next we checked the distribution of incompletely processed 16S rRNA in the ribosome particles. For this, rRNAs prepared from the sucrose gradient separated ribosomal fractions (Fig. 6A) were probed with ³²P-labeled p1 oligonucleotide. Remarkably, 3'-unprocessed 16S rRNA was found mainly in the polysomes (lanes 15-22 of lower panel, Fig. 6B) suggesting their assembly into ribosomal particles and protein synthetic machinery. On the other hand, when ribosomal fractions were probed with p4 corresponding to the unprocessed 3' end sequence of 5S rRNA, the unprocessed 5S rRNA precursors were found mainly in the 50S ribosomal particles (Fig. 6C). 5S rRNA signals in the polysome fractions were hardly visible (lanes 17-22, Fig. 6B). This suggests that immature 5S rRNA is although not defective for assembly into 50S particles, these large ribosomal particles are hardly distributed among the polysomes.

Nature of unprocessed 16S rRNA in the *rnr* mutant: To further analyze the nature of unprocessed regions in the 16S rRNAs of *rnr* null mutant, nucleotide sequences of the circular RT-PCR (cRT-PCR) products of rRNAs was determined as described under the Materials and Methods. While the 16S rRNAs from wild type produced one prominent product of ~100 bp, the mutant strain produced additional major product of ~200 bp (Supplementary Fig. S2). DNA sequence analysis of the cloned PCR products established that two types of unprocessed 16S rRNAs (type I and II) accumulate in *rnr* mutant strain, which have additional 36 residues beyond the 3' matured end (Fig. 7A and Supplementary Fig. S3). The unprocessed 3' tail of 16S rRNA in case of *E. coli* is 33 residues long (25, 26). The type II unprocessed 16S rRNA surprisingly contained nucleotide extensions at the 5' end too (Fig. 7 and Fig. S3). The 65 nucleotide 5'-extended leader was similar to a 66 nucleotide leader sequence produced by the RNase E processing cleavage in the case of *E. coli* RNase G⁻ mutant (26). It is interesting to note that the 5' end unprocessed 16S precursor that accumulates in *E. coli* RNase E⁻ and G⁻ double mutants also gets incorporated into ribosomes.

***rnr* mutants are defective in protein synthesis at 4°C.** Although rRNA processing defect did not lead to any apparent assembly defect of ribosomal particle, accumulation of 3'-unprocessed 16S rRNA in polysomes is known to affect translation processes (25). To test this, we examined the protein synthesis activity of wild type and *rnr* mutant cells at 4°C in a pulse chase experiment using mixture of ³H-labeled amino acids in growth medium. We found that, after 48h of incubation at 4°C, protein synthesis in *rnr* mutant cells is reduced to 36% of the wild type (Fig. 8). This suggests that *rnr* mutants with polysomes containing unprocessed 16S rRNA are perhaps inefficient in protein synthesis. The reason why it takes about 24 h before an effect on protein synthesis is evident in the mutant cells might be related to the time taken by the inactive ribosomes containing unprocessed rRNAs to critically outnumber the normal ribosomes already existing in the cytoplasm.

tmRNA defect in absence of RNase R. tmRNA, also known as SsrA, plays an important role in rescuing ribosomes that are stalled on

mRNAs lacking stop codons (28). In the process, it tags the nascent peptides, which are then identified for degradation. This mechanism ensures that cell gets rid of defective proteins and the ribosomes become available for fresh rounds of translation. It has been shown in *E. coli* that processing of tmRNA becomes defective in absence of RNase R at the low temperature (16). RNase R was also implicated in degradation of tmRNA in *Caulobacter crescentus* (29). Therefore, we examined the status of tmRNA in the *rnr* mutant of *P. syringae*. We found that the processing of tmRNA was only mildly affected but there was a strong defect in the degradation of tmRNA in the *rnr* mutant at 4°C. While only a trace amount of precursor tmRNA was detected in cells after 24h, numerous degradation intermediates accumulated in all the time points of 4°C incubated cells following the downshift of temperature (Fig. 9A). The complemented mutant however displayed normal turnover of tmRNA (Fig. 9B).

DISCUSSION

Most information on RNA metabolism and RNA degrading machinery in bacteria comes from studies with *E. coli*. Although the general nature of the conclusions have been found to be true in most bacteria the exploration of other bacterial systems are providing us with novel information (6, 8, 30). In the present study we specifically examined the importance of RNase R, which is unique to the *P. syringae* degradosome. Earlier reports had shown that the highly processive 3'→5' exoribonuclease RNase R has the ability to degrade structured RNAs, both *in vitro* and *in vivo*. Most significantly, the enzyme was proposed to play a significant role in the quality control of ribosomal RNAs, and degradation of REP sequences of mRNA in *E. coli* (12, 17). A role of RNase R in the maturation of tmRNA in *E. coli* during cold shock, and in the degradation of tmRNA in *C. crescentus* during cell cycle has also been reported (16, 29). However, there is no report on the role of this enzyme in rRNA maturation in bacteria. This study provides evidence for a novel role of RNase R in the maturation of 16S and 5S rRNAs in the cold adapted *P. syringae*.

A lot is known about rRNA maturation steps and ribosome biogenesis in bacteria, with data mostly from *E. coli* (25). The primary transcript of rRNA including 16S, 23S, and 5S rRNAs, which sometimes contain tRNAs in the spacer region between the 16S and 23S, and after 5S, is processed by a series of cleavage and trimming reactions to generate individual mature rRNAs. In *E. coli*, RNase III cleaves the 30S primary transcript to produce the individual rRNA precursors, which undergo secondary processing reactions subsequently to generate the mature 5' and 3' termini of each rRNA. Apart from RNase III, three more endoribonuclease RNase E, RNase G (Caf A), and RNase P are involved in the maturation process. While RNase P generates the mature 5' end of tRNAs (31), RNase E and RNase G are responsible for generation of the 5' ends of mature 16S rRNA (26). Maturation of 5S rRNA on the other hand involves the activities of both RNase E and the exoribonuclease RNase T (32, 33). RNase T is responsible for maturation of the 3' ends of both 23S and 5S rRNA (34). Enzyme involved in 3' end maturation of 16S rRNA has still not been elucidated in *E. coli* (26). Therefore, our observation that the *rnr* mutant of *P. syringae* accumulates immature 16S rRNA molecules with unprocessed 3'-end residues, most probably due to the lack of trimming by RNase R dependent 3'→5' exoribonuclease activity, is significant. By expressing RNase R *in trans* from a plasmid in the mutant, unprocessed 3'-end nucleotides of 16S and 5S rRNAs can be removed (Fig. 5E).

The 3' nucleotide of the 36 residues tail of unprocessed rRNA molecules in the *rnr* mutant expectedly coincided with the putative RNase III cleavage site on the double stranded stem of rRNA precursor molecule (Fig. 7B). However, the observation that some unprocessed molecules (type II) would also contain 5' extended leader sequence (Fig. 7A) was unexpected. It is not clear at present whether the removal of the 5' unprocessed sequence is dependent on the trimming of 3' unprocessed tail of precursor 16S rRNA, or how are the removal of the extra residues from both 5' and 3' ends are dependent on each other. Taking into account the data on processing steps and the nucleotide sequences of different precursor

rRNAs in *E. coli*, it appears that the 5' end of the type II precursor molecules represent the RNase E cleavage site (Fig 7B). It also appears that the RNase G cleavage step, which generates the mature 5' end of 16S rRNA (26), has presumably been affected by the lack of trimming of 3' end residues in the *rnr* strain. It must be noted here that our initial attempts to remove the extra residues *in vitro* from the 3' end of unprocessed 16S rRNA that accumulates in the polysomes failed due to high degradation activity of purified RNase R on all rRNAs in the polysome (unpublished). It is also unknown whether RNase R *in vivo* works in association with other proteins for trimming the 3' ends of 16S and 5S rRNAs in *P. syringae*. This is important for the fact that no 16S intermediates with multiple discrete 3' ends has ever been detected, especially in *E. coli*. Thus, use of an exoribonuclease for production of precise 3' end is likely to require coordinated activities of other proteins, including perhaps ribosomal proteins. Interestingly, while this study was in progress a report appeared to show that the RNR1, an RNase R homologue of *Arabidopsis thaliana* is also involved in the maturation of 3' ends of 23S, 16S, and 5S rRNAs in chloroplasts (35). This suggests that RNase R homologues in other systems might play a similar role in rRNA maturation. We are however yet to determine the nature of unprocessed residues in the defective 5S rRNAs in *P. syringae*.

Unprocessed 16S rRNA accumulates in polysomes of the *rnr* mutant of *P. syringae* and the cells are defective in protein synthesis. This is consistent with the long standing view that 30S ribosomes containing precursor 16S rRNA are not biologically active, and that maturation of 16S rRNA is essential for protein synthesis in *E. coli* (36, 37). We propose that defective protein synthesis is one of the important factors causing death of *P. syringae rnr* mutants at low temperature. However, the intriguing aspect that needs to be investigated in future is to find out the significance of the accumulation of unprocessed 16S rRNA in polysomes (Fig. 6). It is known in the case of *E. coli* that the assembly of rRNAs into ribosomal particles takes place so fast that most maturation processes take place in the ribosomes, and the process is more efficient under translating conditions (25). It is therefore

likely that ribosomal particles containing 3'-untrimmed 16S rRNAs are trapped in the translation-defective polysomes of the *rnr* mutant.

The highly defective tmRNA degradation observed in *rnr* mutant of *P. syringae* is also significant. Although the mutant cells show accumulation of a precursor form of tmRNA at the early time point during low temperature shift, our data implicates the role of RNase R mostly in tmRNA degradation rather than in tmRNA processing. In this respect, tmRNA-processing defects seen in *rnr* mutants of *E. coli* during cold shock, or tmRNA-degradation defect in the stalked cells of *C. crescentus rnr* mutant are interesting (16, 29). Since tmRNA plays a pivotal role in not only rescuing stalled ribosomes on damaged mRNA but also in tagging the truncated defective peptides for proteolytic degradation (28), the defect of tmRNA metabolism in the *rnr* mutant of *P. syringae* might cause a physiological imbalance in the cell, perhaps contributing towards cell death at low temperature. Additionally, metabolic defects might arise in the mutant cells of *P. syringae* due to the known role of RNase R in mRNA degradation, which has not been examined in this study.

Low temperature specific defects in growth and RNA metabolism observed in the *rnr* mutant of *P. syringae* underscore the fact that RNase R enzyme activity is essential at low temperature. But why does not the lack of RNase R cause much defect at higher temperature (22°C) in the bacterium? It is possible that another enzyme carries out the RNase R dependent functions at higher temperature. At 4°C, RNAs might have more extensive and highly stabilized structures, and the organism needs a highly efficient and processive exoribonuclease like RNase R. At higher temperature (22°C), a less processive enzyme might be sufficient to process/degrade RNAs with less extensive and less stabilized structures, and hence cells without RNase R would grow almost normally. There is of course a second possibility that the enzyme, which complements the RNase R activity at 22°C, but fails to express at a lower temperature (4°C), has not been tested in the present study. However, our study clearly establishes that PNPase does

not substitute the RNase R function in *P. syringae*. The third possibility, that RNase R works only at low temperature is probably not correct for the reasons that RNase R expression levels remain similar at both low and high temperatures, and the ability of RNase R to associate with ribosomal particles, or the interaction with RNase E in degradosome, does not alter due to temperature change (unpublished observation). It is, however, to be noted that similar levels of expression of any protein does not ensure similar levels of activity. For example, RNA degradation activity of *P. syringae* RNase R at 22° is higher than at 4°C under *in vitro* condition (unpublished observation), and therefore, a similar level of RNase R at 4°C might be critical for RNA metabolism at lower growth temperatures but not at higher temperatures. This explanation needs further investigation.

In conclusion, a significant finding of this study is that RNase R activity in the cold adapted *P. syringae* is singularly important for growth at low temperature. Equally significant is our observation that the RNase R activity is required for 3'end processing of 16S and 5S

rRNAs, suggesting a previously unidentified role of RNase R, in addition to its role in tmRNA degradation in the cold adapted bacterium. Our future aim is to understand why the deprivation of RNase R in cells shows only a low temperature specific defect, how many of the RNA metabolic defects are due to the sole function of RNase R and how many of them due to defect in the activity of degradosomal complex in the bacterium.

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FOOTNOTES

¹The abbreviations used are: PNPase, polynucleotide phosphorylase; CFU, colony forming unit; PI, propidium iodide; PPO, 2,5-diphenyl oxazole; POPOP, 1,4-bis(4-methyl-5-phenyl-2-oxazolyl) benzene; SSC, salinated sodium citrate; TAE, Tris-acetate-EDTA; TBE, Tris-borate-EDTA; TCA, trichloroacetic acid.

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FIGURE LEGENDS

Fig. 1. Generation of *rnr* knockout mutant of *P. syringae* and growth analysis. (A) Schematic representation of the construct pBS*rnr*-tet used for *rnr* disruption. A few important restriction sites have been marked. Numbers below the *rnr* gene refer to the nucleotide position of the RNaseR open reading frame. (B) Southern analysis using *rnr* specific probe as described under Materials and Methods. The probe hybridizes to a 7 kbp DNA fragment in wild type, which is missing in the *rnr* mutant. Due to a single *SalI* site present in the *tet*-cassette two DNA bands of smaller size are visible instead of one in the mutant lane. (C) Western analysis of the wild type and mutant cells using polyclonal anti-RNase R antibody. Note that RNase R is absent in the *rnr* mutant. (D) Growth of the bacterial strains at 22°C. OD₆₀₀ of the cultures was measured every two hours. (E) Growth of the bacterial strains at 4°C. OD₆₀₀ was taken every 24 hours.

Fig. 2. Analysis of cell death at low temperature. (A) Growth of the indicated strains was followed as a function of optical density (OD₆₀₀) of the cultures. Cells grown at 22 °C till 0.5 OD₆₀₀ were shifted to 4°C, and OD₆₀₀ was measured every 24h. (B) Loss of cell viability in *rnr* mutant as measured by counting CFU. The counting was done every 24h following the low temperature (4°C) shift and the data were plotted. (C) LIVE-DEAD staining of cells after shifting of cultures from 22°C to 4°C. Cells were stained with LIVE/DEAD BacLight bacterial viability kit which comprises of Syto9 and Propidium Iodide. Syto9 stains live cells green and PI stains dead cells red.

Fig. 3. Inability of PNPase overexpression to complement the cold sensitivity of *rnr* mutant. (A) Western analysis showing expression of His-PNPase in the *rnr* mutant. In left panel, the blot was probed with anti-His antibody. Lane M shows low molecular weight protein markers. Lane 1 was loaded with 15 µg of total cell lysate protein. In right panel, the blot was probed with anti-PNPase antibodies. Each lane contained 15 µg proteins of cell lysate. (B) Growth of *rnr* mutant expressing His-PNPase in *trans* at 4°C. OD₆₀₀ was noted every 24h. Both mutant and the mutant expressing His-PNPase failed to grow at 4°C.

Fig. 4. Western analysis of RNase R expression. Immunoblots were probed with anti-RNase R polyclonal antibodies. (A) Testing for cold induction. Bacterial culture (OD₆₀₀ ~0.5) grown at 22°C was shifted to 4°, and cell aliquots were analyzed for RNase R expression at the indicated time. Each lane contains cell extracts with 10 µg of protein. Lane 'C' contains the sample from cells at the 0 time point, immediately before the shift. (B) Testing growth phase specific expression. Cells at different growth phases corresponding to culture OD₆₀₀ values of about 0.3, 0.7, 1.2 and 2.1 were harvested and analyzed in lanes 1, 2, 3, and 4, respectively. Two temperature (4° and 22°C) grown cells were tested. Each lane contains cell extracts with 15 µg of protein.

Fig. 5. Northern analysis of rRNA. (A) Schematic representation of rRNA operon of *P. syringae* to indicate the position of oligonucleotide probes used for northern hybridization. Mature region of rRNAs and tRNAs have been shown as boxes on the rRNA operon. Relative locations of the oligonucleotide probes (p1 through p4) used in the northern analysis have been shown as short thick lines below the operon. (B) Result of p1 oligonucleotide probe hybridization to RNAs from wild type (wt) and RNase R inactivated mutant (*rnr*). Probe p1 corresponds to sequence complementary to unprocessed residues of 3' end of 16S rRNA. 22°C grown cells were shifted to 4°C and RNAs were prepared from 0h (in respect to 4°C), 24h, 48h, and 72h time points directly, or following a 60 minutes of rifampicin treatment (marked as 60 on the lanes). 10 µg RNA was loaded in each lane and separated by electrophoresis on 1.2% agarose gel. (C) Ethidium bromide stained gel used for Northern blot shown in panel B. (D) Northern blot hybridization with the p2 oligonucleotide probe comprising residues complementary to mature 16S rRNA. Experimental conditions were as in (B). (E) p1 probe hybridization result with RNAs prepared

from the RNase R complemented mutant strain (*rnr*(pGL*rnr*-his)). Experimental conditions were same as in (B). (F) p4 oligonucleotide probe hybridization result showing accumulation of unprocessed 5S rRNA in the *rnr* mutant of *P. syringae*. Northern hybridization conditions were as in (B).

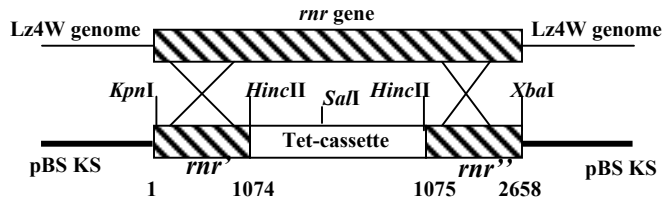
Fig. 6. Distribution of unprocessed 16S and 5S rRNAs in ribosome fractions. Ribosomes were isolated from both wild type and *rnr* mutant cells grown at 22°C and then shifted to 4°C for 24h, and separated on sucrose gradient. (A) Fractions (~500 µl each) were collected from the gradient, and A₂₆₀ were measured for the plotting of ribosomal profile (upper panel). 40 µl aliquots from alternative fractions were then examined for distribution of rRNAs (lower panel). Since the ribosomal profiles were similar in the wild type, and mutants, only the wild type profile is shown here. (B) & (C) Northern hybridization of RNA in the ribosomal fractions (23 in case of wt and 22 in case of *rnr* mutant). Lanes from left to right represent fractions from top to bottom of the gradient. The last lanes in both wt and mutant panel contain ribosome pellet. Alternate fractions were used for northern analysis. Probes were p1 (B) and p4 (C) specific for unprocessed 3' end residues of 16S and 5S rRNAs, respectively. Negative hybridization result for only p1 but not for p4 probe in the fractions from wild type has been shown (top panel in B).

Fig. 7. Nucleotide sequence and the putative secondary structures around the 3' and 5' termini of precursor 16S rRNA. The junction region of ligated 5' and 3' ends of 16S rRNAs from wild type and *rnr* mutant of *P. syringae* were amplified by cRT-PCR using appropriate primers and analyzed by nucleotide sequencing (supplementary Figs. S2 and S3). (A) depicts the two types of unprocessed precursor molecules (type I and type II) with untrimmed 3' tail of 36 nucleotides that accumulate in *rnr* mutant. Type II molecule has an additional 65 nucleotides leader sequence at the 5' end. Mature 3' end and the putative RNase E cleavage site have been marked on the sequence by vertical arrows. (B) shows the putative folded structures around the 5' and 3' terminal regions of precursor 16S rRNA. Both mature and the unprocessed 5' and 3' ends observed in the study have been marked by arrows. A smaller arrow near the mature 5' end indicates the heterogeneity observed in some sequence. Residues shown beyond the unprocessed 5' end and the conserved box-C sequence are taken from DNA sequence of the rRNA operon to depict the duplex secondary structure of the region. Conserved box-C sequence where the RNase III cleavage occurs has been marked as a rectangular box. The number of residues at the 3' extended tail (36 bases) and the 5' leader region (65 bases) of the defective 16S rRNA determined in this study has also been indicated.

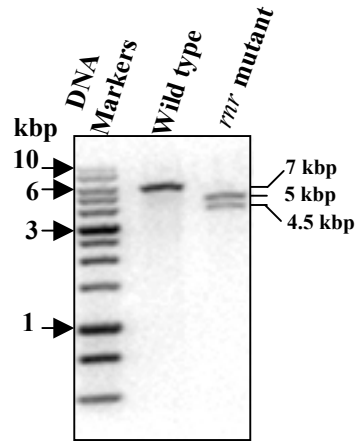
Fig. 8. Protein synthesis ability of the *rnr* mutant at 4°C. Protein synthesis at 4°C was measured at different time points by pulse chasing the cells with a mixture of ³H-labeled amino acids as described under Experimental procedures. Incorporation of radioactive amino acids into cells at 0 time point was taken as control for normal protein synthesis ability. The values obtained for controls in wild type (80,000 ± 5000 cpm per 10⁶ cells) and mutant (75,000 ± 4000 cpm per 10⁶ cells) were taken as 100% for calculation and plotted as bar diagram.

Fig. 9. Defective tmRNA degradation in *rnr* mutant. RNAs (10 µg in each lane) were separated on 6% polyacrylamide-8M urea gel in 1XTBE buffer (20) and electroblotted onto Hybond N⁺ membrane. Blots were probed with ³²P-labeled full-length tmRNA gene of *P. syringae*. Shown are phosphor Images of samples from wild type and *rnr* mutant that were incubated at 24, 48, and 72 h at 4°C (A), and those from the RNase R complemented mutant (B). Lanes marked with 'M' contain ³²P-labeled RNA century marker (Ambion), and 'C' contain RNA samples from cells at 22°C just before the shift to 4°C. Lower panels in (A) and (B) show portions of the ethidium bromide stained gels used for Northern blotting to indicate the equal loading of RNA samples. RNA was quantified by measuring OD₂₆₀ values of the samples.

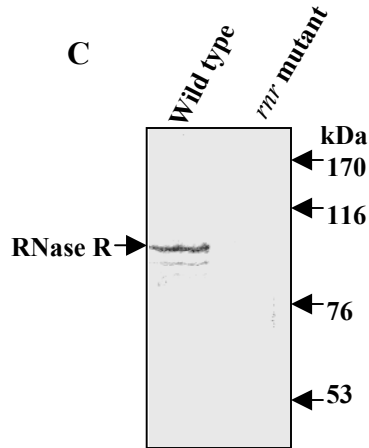
A



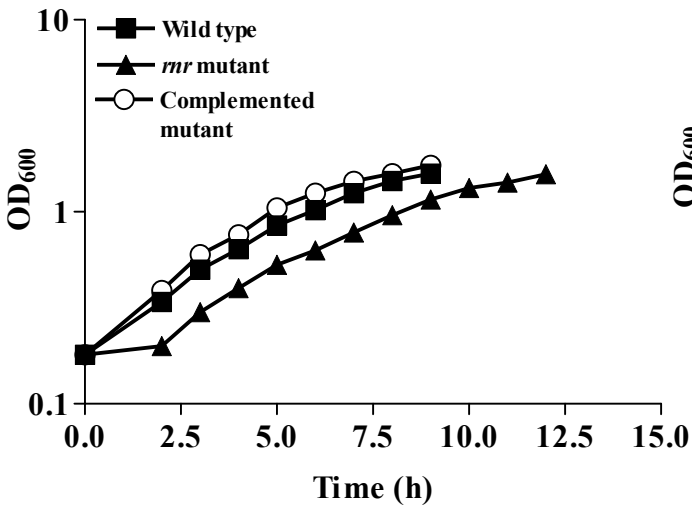
B



C



D



E

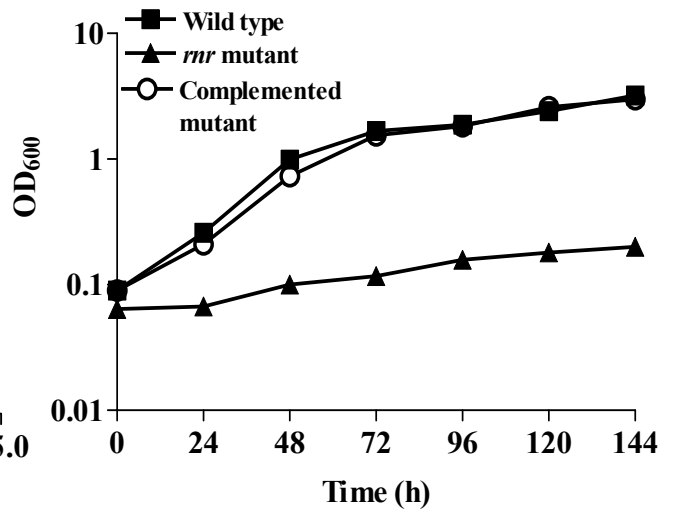


Figure - 1

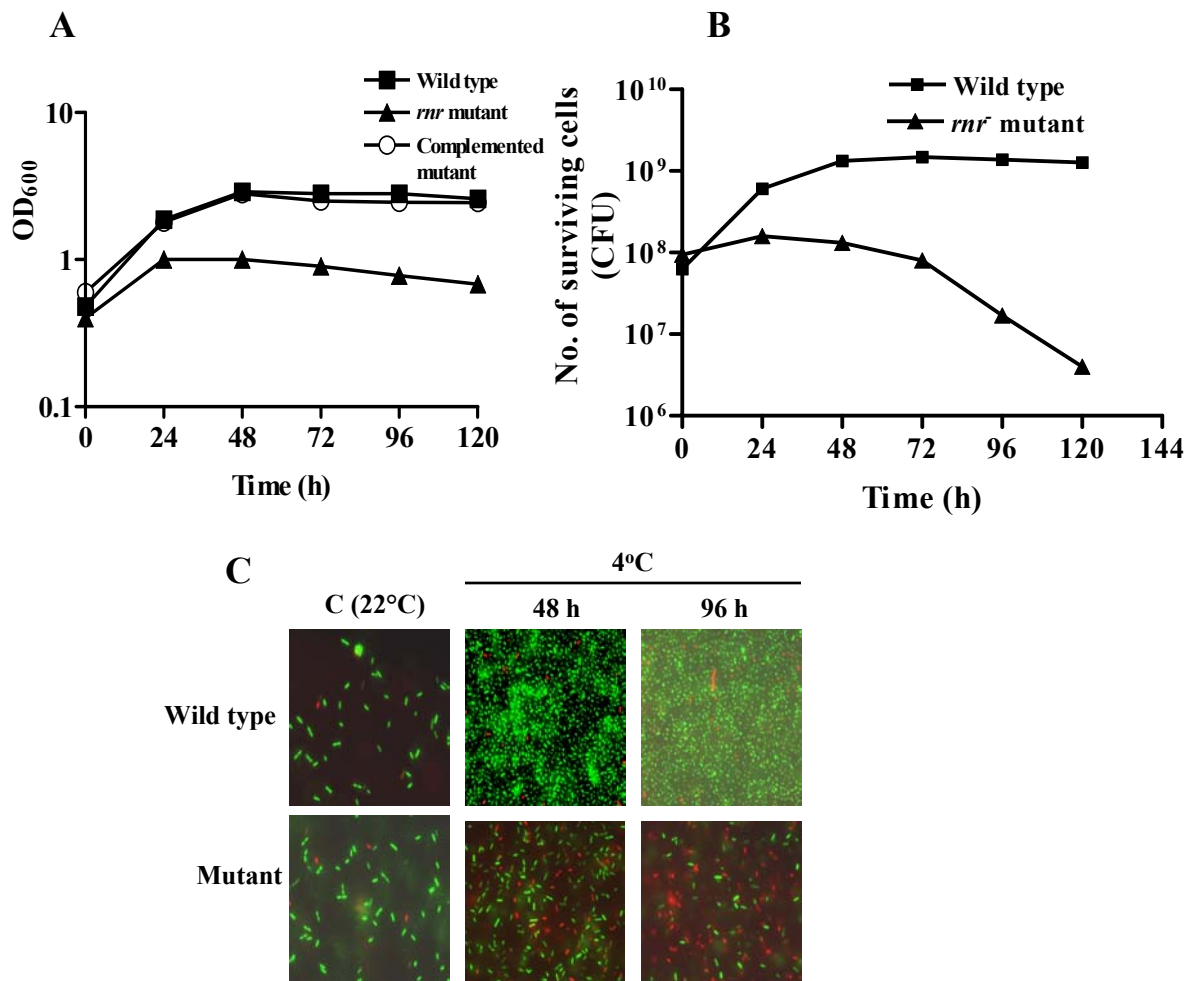


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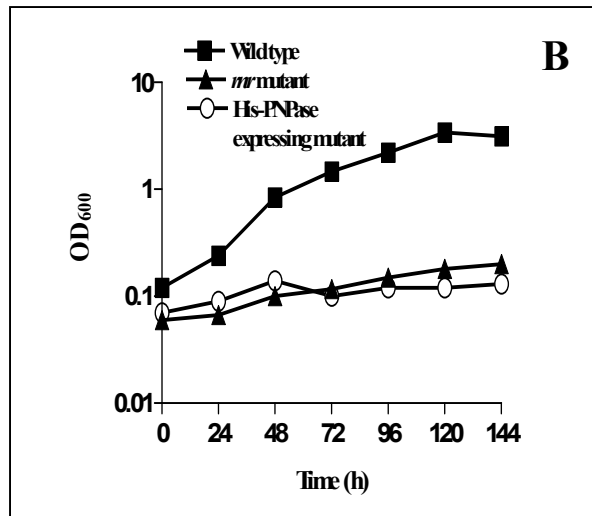
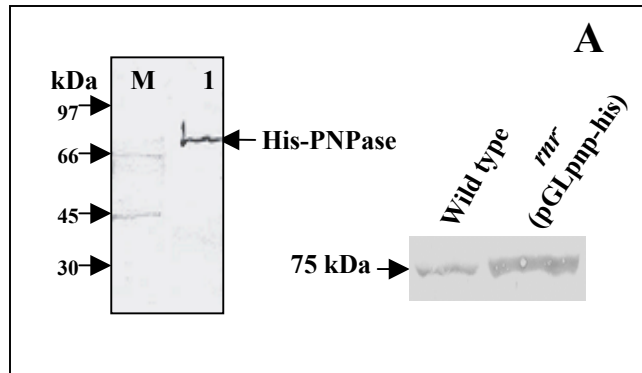


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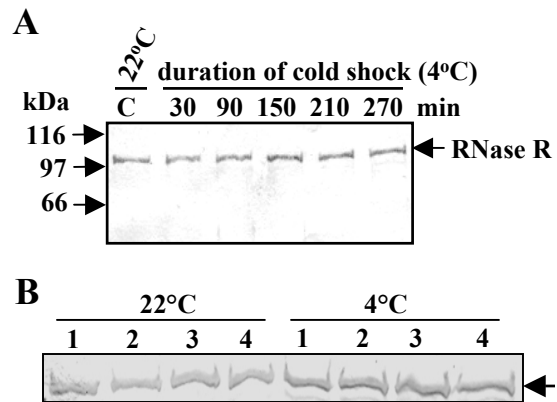
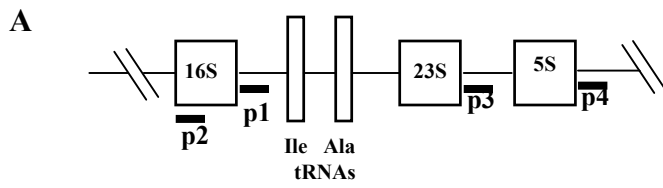
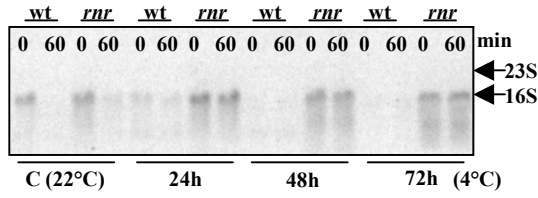


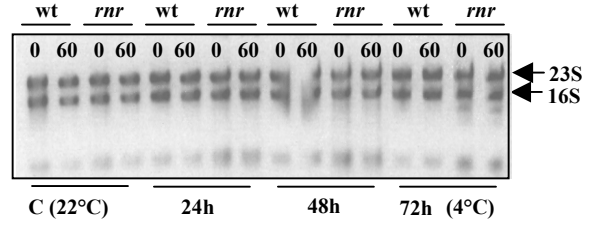
Figure - 4



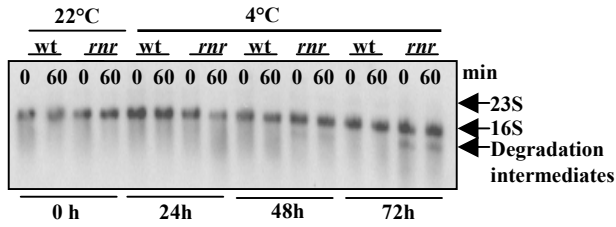
B Northern: Probe – p1



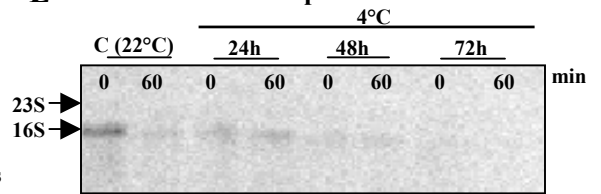
C Ethidium bromide stained gel



D Northern: Probe – p2



E Northern: Probe – p1



F Northern: Probe – p4

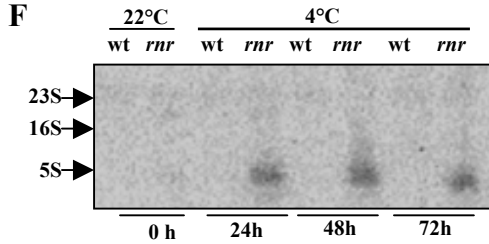


Figure - 5

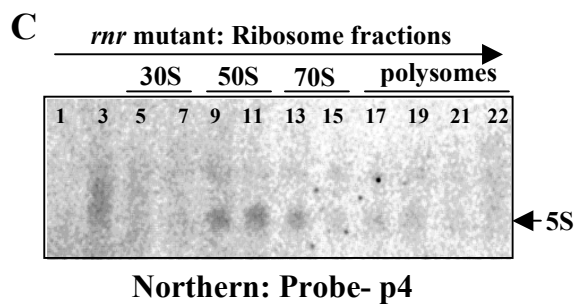
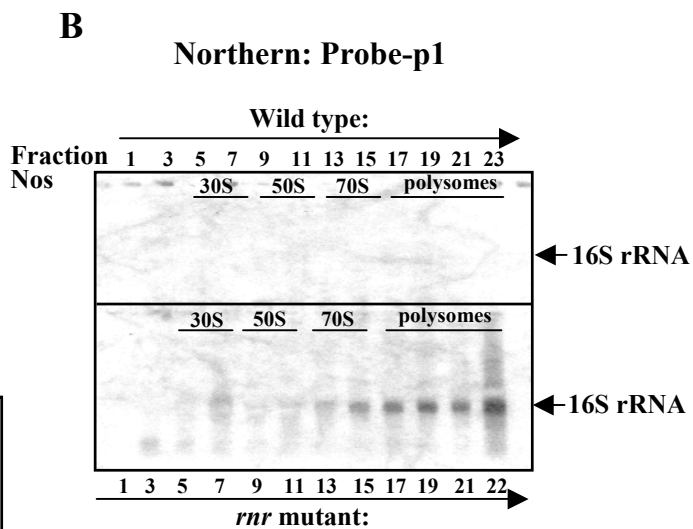
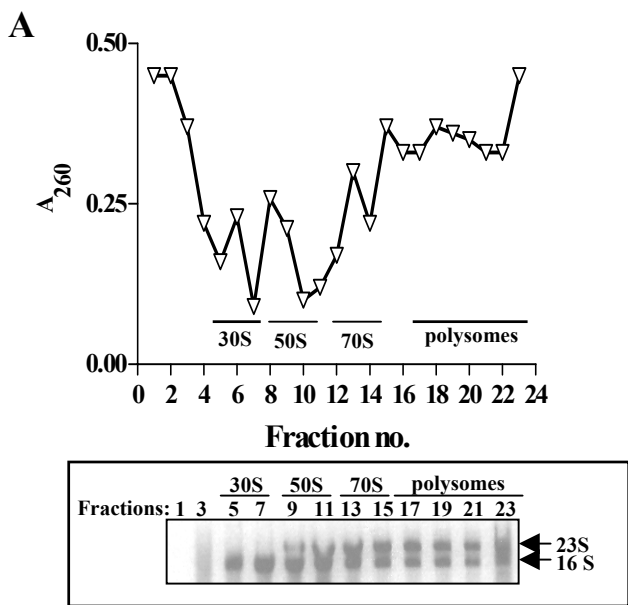


Figure - 6

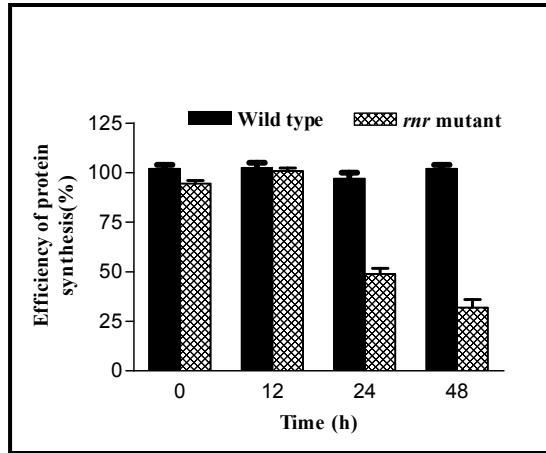


Figure-8

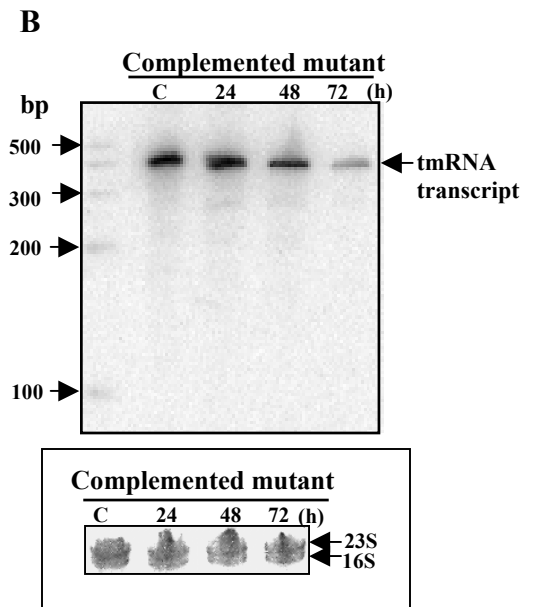
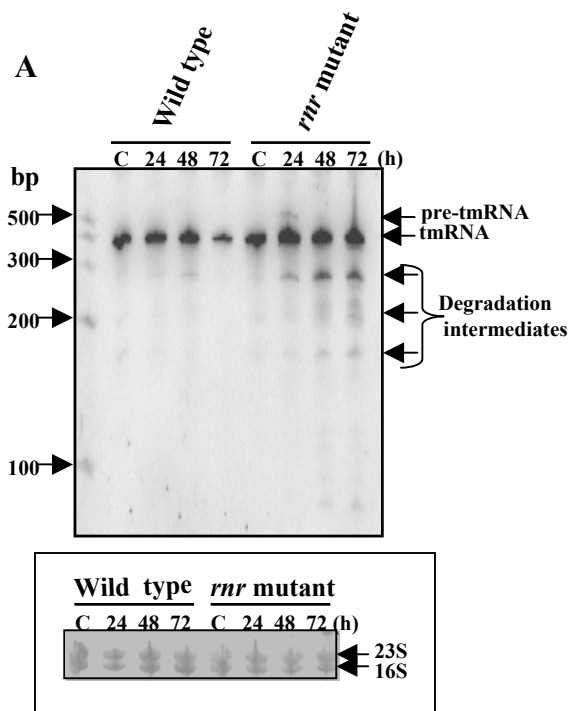


Figure- 9