Exoribonuclease R in *Pseudomonas syringae* Is Essential for Growth at Low Temperature and Plays a Novel Role in the 3′ End Processing of 16 and 5 S Ribosomal RNA

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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*. Overexpressing polyribonucleotide phosphorylase 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 polyribonucleotide phosphorylase) and *rrn* 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 16 and 5 S ribosomal RNA (rRNA) that contain untrimmed extra ribonucleotide residues at the 3′ ends. This suggests a novel role for RNase R in the rRNA 3′ end processing. Unprocessed 16 S 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 transfer-messenger RNA was identified from our observation that the *rnr* mutant accumulates transfer-messenger RNA 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 cells 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 (1–3). 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 (PNPase, 2 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 that is expressed but does not form a part of this complex. 3 PNPase and RNase R differ in their mode of action, the former exhibiting phosphorolytic activity and the latter exhibiting 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, whereas RNase R contributes only residual (2%) activity (10). Subsequently, it was named RNase R because of 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 *rrn* and *pnp* genes, respectively, are responsible for quality control of rRNA and that *rrn* *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 *rrn* mutant was not cold-sensitive (16). Extensive study from Deutscher’s group (17) 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 sequences in *vivo*. The same group shows that the RNase R level is elevated in response to stress conditions including starvation and entry into the stationary phase (18).

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\[\text{The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and supplemental Figs. S1–S3.}\]

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\[\text{The abbreviations used are: PNPase, polynucleotide phosphorylase; CFU, colony forming unit; PI, propidium iodide; rRNA, ribosomal RNA; ABM, Antarctic bacterial medium; RT, reverse transcription; tmRNA, transfer-messenger RNA.}\]

\[\text{1 R. I. Purusharth and M. K. Ray, unpublished results.}\]
The authors speculate that extensive remodeling of structured RNA occurs under stress conditions, which would account for 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 toward detailed characterization of the 
P. syringae 
degradosome machinery, knock-out mutants of RNase R 
(rnr)
, RhlE 
(rhle)
, and the C-terminal degradosome-organizing region of RNase E 
(rne)
 would be useful. We report here our results on the 
rrn
 knock-out 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 16 and 5 S rRNAs. The 
rrn
 mutants are not only defective for 16 and 5 S rRNA maturation at 4 °C but also accumulate unprocessed 16 S 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 temperatures in the absence of RNase R 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, respectively, as reported earlier (8). When required, the growth media were supplemented with antibiotics in following concentrations: 100 μg ml\(^{-1}\) ampicillin, 50 μg ml\(^{-1}\) kanamycin, 20 μg ml\(^{-1}\) tetracycline, 100 μg ml\(^{-1}\) chloramphenicol, and 400 μg ml\(^{-1}\) rifampicin. Composition of minimal growth medium for 
P. syringae
 was as follows: 0.12% Na\(_2\)HPO\(_4\), 0.06% KH\(_2\)PO\(_4\), 0.05% (NH\(_4\))\(_2\)SO\(_4\), 1% succinic acid, 0.01% valine, 0.01% isoleucine, and 1 mm MgSO\(_4\).

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 (\(A_{600}\)) was measured at various time intervals. For complementation studies, the 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, PCR, ligation, and transformation etc. were performed as described (20). All of the restriction enzymes, T4 DNA ligase, and Klenow enzyme used in this study were from New England Biolabs. An Omimiscript RT kit (Qiagen) was used for reverse transcription, and oligonucleotides were purchased from BioServe BioTech (Hyderabad, India).

For Southern hybridization, genomic DNA was isolated from both wild type and 
rrn
 mutant strains of 
P. syringae
digested with Sall 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 
rrr
 gene (2.7 kilobase pairs) 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 h. The membrane blots were washed twice with 2× and 0.1× salinized sodium citrate, respectively. Radioactive signals were developed using a phosphorimaging device (Fuji FLA3000).

Construction of 
rrn
 Knock-out Mutant of 
P. syringae
—Plasmid pBSrrn-tet used for knocking out the 
rrn
 gene was constructed as follows. The 
rrn
 gene was first subcloned as KpnI-HindIII fragment into pBlueScript-KS vector, taking the fragment from earlier reported plasmid pMOSBlue containing 
P. syringae
 
rrn
 gene (8). The resultant plasmid pBS-rrn
 was linearized with HincII, and a tetracycline resistance gene was cloned within the 
rrn
 to generate pBSrrn-tet plasmid. The source of tetracycline resistance gene 
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with reverse transcriptase (Omniscript RT kit; Qiagen) for 1 h at 42 °C as per the manufacturer’s instructions, in the presence of antisense primer corresponding to a region in the 5′ end of mature 16 S rRNA (primer 16 SRU1, 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 16 SRU1 and outwardly directed 16 SFU1 (5′-GGG-GTGAAGTCGTAACAAGGTAGCCG-3′) corresponding to a region at the 3′ end of mature 16 S 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 pMOS-Blue plasmid. Big-Dye™ terminator cycle sequencing kit (ABI) was used for sequencing reaction.

**Western Analysis**—Protein blotting and immunodetection techniques for Western analysis were as described in Ref. 8. For checking low temperature induction of the RNase R, *P. syringae* cells were grown at 22 °C till *A*₆₀₀ reached ~0.5 and then shifted to 4 °C. Culture (3 ml) was withdrawn at the indicated time, and cell extracts were prepared by sonicating. 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 fluorescence microscope (Zeiss). Briefly, the cells were grown at 22 °C till *A*₆₀₀ reached ~0.5 followed by shifting to 4 °C. At every 24-h interval, aliquots of culture were withdrawn for measuring turbidity at *A*₆₀₀ and spreading of cells onto ABM-agar plates for growth at 22 °C. For each time point, CFUs on plates (in triplicates) were counted. The 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).

The oligonucleotide probes (supplemental Table S1, p1–p4) used for Northern hybridization were 5′ end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase (New England Biolabs) following the manufacturer’s protocol. The [γ-³²P]ATP-labeled oligonucleotides were separated from unincorporated [γ-³²P]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′-TTAGGATTCGACGCCGTTGAAC-3′) and reverse (5′-GGG-TAGCCG-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 16 S rRNA and 16 S rRNA precursors were determined using circular RT-PCR method. Briefly, total RNA was circularized using T4 RNA ligase (New England Biolabs) in the presence of RNase inhibitor RNasin (Promega). Then reverse transcription reaction was performed
Essential Nature of *P. syringae* RNase R

**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, the cells were treated with chloramphenicol (100 μg/ml) for 15 min (24). The 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-mercaptoethanol) containing lysozyme (1 mg/ml) and subjected to sonication. Cell debris was removed by centrifugation at 12,000 × g. Supernatant was then recentrifuged at 70,000 rpm in a Beckman table top ultracentrifuge (TLA 100.3 rotor). The ribosomal pellet was suspended in ribofuged at 70,000 rpm in a Beckman table top ultracentrifuge 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 Biosciences). Briefly, cells (A₆₅₀ ~ 0.5) growing in ABM at 22 °C were shifted to 4°C for incubation. At the various time points, the 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 min (pulse) of labeling. The cells were then incubated (chase) for another 30 min in the richer medium by adding an equal volume of 2× ABM into the minimal medium. The cells were then lysed with Tris-lysosome-EDTA buffer, and proteins were precipitated by adding equal volume of ice-cold 10% trichloroacetic acid. Protein pellet was spotted onto filter paper (Whatman), and radioactive counts were measured in a toluene-based scintillant (0.5% 2,5-diphenyl oxazole and 0.03% 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl) benzene 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 *P. syringae* metabolism of the cold-adapted *P. syringae* Lz4W, an *rnr* knock-out mutant strain (*rnr*Δtet) 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 because of the location of a single SalI recognition site within the tet cassette (Fig. 1B). The lack of RNase R production because of 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. 1, D and E) reveals that the mutant, although 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. To confirm that the observed phenotype was only due to absence of functional RNase R, the *rnr* mutant was transformed with plasmid pGLmr-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* knock-out mutant.

Viability of *rnr* Mutants Decreases at 4 °C—The cold-sensitive phenotype of the *rnr* mutant can be either due to lack of growth or...
due to cell death. 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 A_{600} of the culture of rnr mutant increased during the first 24 h 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 24 h but then gradually declined, and at 144 h ~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 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 because of cell death and lysis (Fig. 2C). In wild type the number of living cells (green) expectedly increased because of 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 ~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^{+} 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, because 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 whether 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 that 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 with 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 (supplemental 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—Because the rnr mutant of P. syringae is cold-sensitive, we checked whether RNase R is up-regulated at low temperature in this bacterium. Western analysis using polyclonal anti-RNase R antibodies sug-

![Figure 3](image3.png)

**FIGURE 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 the 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 the right panel, the blot was probed with anti-PNPase antibodies. Each lane contained 15 μg of proteins of cell lysate. B, growth of rnr mutant expressing His-PNPase in trans at 4 °C. A_{600} was noted every 24 h. Both mutant and the mutant expressing His-PNPase failed to grow at 4 °C.
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FIGURE 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 are shown as boxes on the rRNA operon. Relative locations of the oligonucleotide probes (p1-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 (rrr). Probe p1 corresponds to sequence complementary to unprocessed residues of 3’ end of 16 S rRNA. 22 °C grown cells were shifted to 4 °C, and RNAs were prepared from 0-h (in respect to 4 °C), 24-h, 48-h, and 72-h time points directly, or following 60 min of rifampicin treatment (marked as 60 on the lanes). 10 μg of 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 D. D, Northern blot hybridization with the p2 oligonucleotide probe comprising residues complementary to mature 16 S rRNA. Experimental conditions were as in B. E, p1 probe hybridization result with RNAs prepared from the Rrnr R complemented mutant strain (rrrPLgRnr-his). The experimental conditions were same as in B, F, p4 oligonucleotide probe hybridization results showing accumulation of unprocessed 5 S rRNA in the rrr mutant of P. syringae. Northern hybridization conditions were as in B.

gests 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 °C) and high (22 °C) temperatures (Fig. 4B), which was different from the observation in E. coli. RNase R was reported to be stress-inducible, especially under low temperature and at the stationary phase in the mesophilic E. coli (16, 18).

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

The p1 probe corresponding to the nucleotide sequence lying 11 bases downstream of the mature 3’ end of 16 S rRNA, hybridized to the 16 S rRNA of the rrr mutant at low temperature. Fig. 5B shows accumulation of the incompletely processed 16 S rRNA population at various time points only in the rrr 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 16 S 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 min after rifampicin treatment in both (lanes C, 60 min, Fig. 5B). This suggests that some other enzyme is capable of 16 S 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 an increased degradation of 16 S 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 16 S rRNA at 4 °C (see below). The processing of 16 S rRNA in the complemented rrr mutant, as shown in Fig. 5E, was normal.

In a control experiment when p2 oligonucleotide sequence corresponding to a matured region of 16 S rRNA was used as probe for hybridization, the 16 S rRNA profiles of the mutant and wild type were similar, except for one additional defect noticed in the mutant. This defect in rrr mutant...
16 S rRNA, processing of the 5 S rRNA is also defective in the \( rnr \) mutant of \( P. \ syringae \) at low temperature.

**Unprocessed 16 and 5 S rRNAs Are Present in the Ribosome Fractions of \( rnr \) Mutants—**Unprocessed rRNAs are normally incorporated into preribosomal 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 16 S rRNA in the ribosome particles. For this, rRNAs prepared from the sucrose gradient separated ribosomal fractions (Fig. 6A) were probed with \( ^{32} \)P-labeled p1 oligonucleotide. Remarkably, 3’-unprocessed 16 S rRNA was found mainly in the polysomes (Fig. 6B, lower panel, lanes 15–22), 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 5 S rRNA, the unprocessed 5 S rRNA precursors were found mainly in the 50 S ribosomal particles (Fig. 6C). 5 S rRNA signals in the polysome fractions were hardly visible (Fig. 6B, lanes 17–22). This suggests that although immature 5 S rRNA is not defective for assembly into 50 S particles, these large ribosomal particles are hardly distributed among the polysomes.

**Nature of Unprocessed 16 S rRNA in the \( rnr \) – Mutant—**To further analyze the nature of unprocessed regions in the 16 S rRNAs of \( rnr \) null mutant, nucleotide sequences of the circular RT-PCR products of rRNAs was determined as described under “Experimental Procedures.” Although the 16 S rRNAs from wild type produced one prominent product of \( \sim 100 \) bp, the mutant strain produced an additional major product of \( \sim 200 \) bp (supplemental Fig. S2). DNA sequence analysis of the cloned PCR products established that two types of unprocessed 16 S rRNAs (type I and II) accumulate in the \( rnr \) – mutant strain, which have 36 additional residues beyond the 3’ matured end (Fig. 7A and supplemental Fig. S3). The unprocessed 3’ tail of 16 S rRNA in case of \( E. \ coli \) is 33 residues long (25, 26). The type I unprocessed 16 S rRNA surprisingly contained nucleotide extensions at the 5’ end too (Fig. 7 and supplemental 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 16 S 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 the processing defect did not lead to any apparent assembly defect of ribosomal particle, accumulation of 3’-unprocessed 16 S 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 a mixture of \( ^{3} \)H-labeled amino acids in growth medium. We found that, after 48 h 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 16 S rRNA are perhaps inefficient in protein synthesis. The reason why it takes \( \sim 24 \) 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 cells get 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. Although only a trace amount of precursor tmRNA was detected in cells after 24 h, 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 is 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 repetitive extragenic palindromic 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 16 and 5 S 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 16, 23, and 5 S rRNAs, which sometimes contain tRNAs in the spacer region between the 16 and 23 S, and after 5S, is processed by a series of cleavage and trimming reactions to gen-

FIGURE 7. Nucleotide sequence and the putative secondary structures around the 3' and 5' termini of precursor 16 S rRNA. The junction region of ligated 5' and 3' ends of 16 S rRNAs from wild type and rnr mutant of P. syringae were amplified by circular RT-PCR using appropriate primers and analyzed by nucleotide sequencing (supplemental Figs. S2 and S3). A depicts the two types of unprocessed precursor molecules (types I and II) with untrimmed 3' tails 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 16 S 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 the 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 16 S rRNA determined in this study has also been indicated.
erate individual mature rRNAs. In *E. coli*, RNase III cleaves the 30 S 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. Although 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 16 S rRNA (26). Maturation of 5 S 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 23 and 5 S rRNA (34). Enzyme involved in 3' end maturation of 16 S rRNA has still not been elucidated in *E. coli* (26). Therefore, our observation that the *rnr* mutant of *P. syringae* accumulates immature 16 S rRNA molecules with unprocessed 3' end residues, most probably because of the lack of trimming by RNase-dependent 3'→5' exoribonuclease activity, is significant. By expressing RNase R in *trans* from a plasmid in the mutant, unprocessed 3' end nucleotides of 16 and 5 S 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 16 S 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 16 S 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 16 S rRNA that accumulates in the polysomes failed because of high degradation activity of purified RNase R on all rRNAs in the polysome. It is also unknown whether RNase R in *vivo* works in association with other proteins for trimming the 3' ends of 16 and 5 S rRNAs in *P. syringae*. This is important for the fact that no 16 S 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 23, 16, and 5 S 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 5 S rRNAs in *P. syringae*.

Unprocessed 16 S rRNA accumulates in polysomes of the *rnr* mutant of *P. syringae*, and the cells are

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**FIGURE 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 zero time point was taken as control for normal protein synthesis ability. The values obtained for controls in wild type (80,000 cpm/10^6 cells) and mutant (75,000 ± 4000 cpm/10^6 cells) were taken as 100% for calculation and plotted as a bar diagram.

**FIGURE 9.** Defective tmRNA degradation in *rnr* mutant. RNAs (10 µg in each lane) were separated on 6% polyacrylamide, 8 M urea gel in 1× Tris-borate-EDTA buffer (20) and electroblotted onto Hybond N+ membrane. The blots were probed with 32P-labeled full-length tmRNA gene of *P. syringae*. Shown are phosphorimaging results 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 in the extreme left of panels A and B contain 32P-labeled RNA precursor marker (Ambion), and lanes 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 gel used for Northern blotting to indicate the equal loading of RNA samples. RNA was quantified by measuring A(260) values of the samples.
defective in protein synthesis. This is consistent with the long standing view that 30 S ribosomes containing precursor 16 S rRNA are not biologically active and that maturation of 16 S 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 16 S 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 16 S rRNAs are trapped in the translation-defective polysomes of the *rnr* mutant.

The highly defective tmRNA degradation observed in the *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 implicate 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 defects in the stalked cells of *C. crescentus* *rnr* mutant are interesting (16, 29). Because 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 toward cell death at low temperature. Additionally, metabolic defects might arise in the mutant cells of *P. syringae* because of 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? Is it 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 temperatures, is probably not correct because 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 with temperature changes. It is, however, to be noted that similar levels of expression of any protein do not ensure similar levels of activity. For example, RNA degradation activity of *P. syringae* RNase R at 22 °C is higher than at 4 °C under *in vitro* condition, 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 16 and 5 S 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 are due to a defect in the activity of degradosomal complex in the bacterium.

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