

## RNase Activity of Polynucleotide Phosphorylase Is Critical at Low Temperature in *Escherichia coli* and Is Complemented by RNase II<sup>∇</sup>

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**In *Escherichia coli*, the cold shock response is exerted upon a temperature change from 37°C to 15°C and is characterized by induction of several cold shock proteins, including polynucleotide phosphorylase (PNPase), during acclimation phase. In *E. coli*, PNPase is essential for growth at low temperatures; however, its exact role in this essential function has not been fully elucidated. PNPase is a 3'-to-5' exoribonuclease and promotes the processive degradation of RNA. Our screening of an *E. coli* genomic library for an in vivo counterpart of PNPase that can compensate for its absence at low temperature revealed only one protein, another 3'-to-5' exonuclease, RNase II. Here we show that the RNase PH domains 1 and 2 of PNPase are important for its cold shock function, suggesting that the RNase activity of PNPase is critical for its essential function at low temperature. We also show that its polymerization activity is dispensable in its cold shock function. Interestingly, the third 3'-to-5' processing exoribonuclease, RNase R of *E. coli*, which is cold inducible, cannot complement the cold shock function of PNPase. We further show that this difference is due to the different targets of these enzymes and stabilization of some of the PNPase-sensitive mRNAs, like *fis*, in the  $\Delta pnp$  cells has consequences, such as accumulation of ribosomal subunits in the  $\Delta pnp$  cells, which may play a role in the cold sensitivity of this strain.**

The cold shock response in *Escherichia coli* is elicited when cells which are exponentially growing at 37°C are shifted to a lower temperature, such as 15°C (for a review, see reference 38). Upon a temperature downshift, there is a transient arrest of cell growth, termed acclimation phase, during which there is a severe inhibition of general protein synthesis. However, during this phase a specific set of genes is induced which encode cold shock proteins. The highly induced cold shock proteins include CspA (20) and its homologues, such as CspB (26), CspG (36), and CspI (55), polynucleotide phosphorylase (16, 43, 44), RNA helicase CsdA (52), initiation factor IF2 (21), transcription factor NusA (17), RecA (54), histone-like protein H-NS (13), DNA gyrase (23), and ribosome-binding factor RbfA (11).

*E. coli* polynucleotide phosphorylase (PNPase) is encoded by the *pnp* gene, is induced at low temperature (24, 58), and is essential for growth at low temperatures (29, 40). However, the exact role of PNPase in this essential function has not been fully elucidated. PNPase is an exoribonuclease, promotes the processive degradation of RNA, and is also responsible for residual RNA tailing observed in *E. coli* mutants devoid of the main polyadenylating enzyme, PAP I (34, 35) (32). In vitro, PNPase catalyzes the phosphorolytic degradation of RNA, releasing nucleoside 5'-diphosphate from the 3' end of the substrate, and the reverse reaction of nucleoside 5'-diphosphate polymerization with release of phosphate (28).

PNPase is a homotrimer of a 711-amino-acid polypeptide and contains two RNA binding domains, KH and S1, located at the C terminus, and two RNase PH domains linked by an

alpha-helical domain, which are responsible for its catalytic and homotrimerization properties (19, 22, 42, 50). Systematic structure-function analysis of PNPase domains has revealed that in addition to the two core domains, the alpha-helical domain is involved in PNPase enzymatic activity (4). PNPase has three catalytic activities, phosphorolysis, polymerization, and phosphate exchange; all of these three activities reside in the core domain, consisting of the two RNase PH domains and the alpha-helical domain. Comparison of the two core domains showed that sequence conservation is indicated in the glycine and alanine residues involved in the close packing of the main elements within each core domain. The differences between the two may relate to the different functions in PNPase activity, in that the RNase PH-like phosphorolytic catalytic site may be conserved in the second domain while the first core domain is involved in the flexible binding of RNA substrate and the formation of the trimer channel structure (50). The KH and S1 domains are primarily involved in RNA binding, contribute to substrate recognition, and are dispensable. Deletion of the RNA binding domains does not abolish any of the three catalytic activities, indicating that they are contained in a domain independent of the catalytic center.

As mentioned above, during the acclimation phase, CspA homologues are highly induced. PNPase was shown to be required to repress production of CspA homologues by degrading them selectively at the end of the acclimation phase. In a *pnp* mutant, expression of CspA homologues was significantly prolonged upon cold shock (41, 56). PNPase also associates with the endonuclease RNase E and other proteins in the RNA degradosome (for a review, see reference 7).

To elucidate the cold shock function of PNPase, we screened for genes which when expressed from multicopy plasmids can complement the cold-sensitive phenotype of *pnp* deletion cells at 15°C. We observed that another 3'-to-5' processing exo-

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ribonuclease, RNase II, can complement PNPase function at low temperature. This result suggested that the RNase activity of PNPase is critical for its cold shock function. This was further supported by the observation that RNase PH domains 1 and 2 of PNPase are important for its cold shock function. We also showed that the polymerization activity of PNPase is not essential for its cold shock function. Interestingly, the third 3'-to-5' processing exoribonuclease, RNase R of *E. coli*, which is cold inducible (6), was not able to complement the cold shock function of PNPase. We further showed that this difference is due to the different targets of these enzymes and that stabilization of some of the PNPase-sensitive mRNAs, like *fis*, has consequences, such as accumulation of ribosomal subunits in the  $\Delta pnp$  cells, which may play a role in the cold sensitivity of this strain.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* strain JM83 [*F*<sup>-</sup> *ara* $\Delta$ (*lac-proAB*) *rpsL*(Str<sup>r</sup>)] (57) was used as the wild-type strain. The  $\Delta pnp$  strain, in which the entire *pnp* gene was replaced with a kanamycin cassette, was constructed according to the method described by Datsenko and Wanner (12). The deletion was confirmed by PCR analysis. The *mb*, *mr*, and *pcnB* deletion strains used in this study were obtained from the Keio collection, Japan, and the  $\Delta pnp$   $\Delta pcnB$  and  $\Delta mr$   $\Delta mb$  double deletion strains were created by P1 transduction after removing the kanamycin cassette from each strain by the method described by Datsenko and Wanner (12). The bacterial cultures were grown in Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Ampicillin (50  $\mu$ g ml<sup>-1</sup>) was supplemented as required.

The plasmids pINIII-*pnp*, pINIII-*mb*, and pINIII-*mr* were constructed by cloning the regions corresponding to *pnp*, *mb*, and *mr*, respectively, using NdeI and BamHI. The pINIII plasmids containing different domains of PNPase were created using NdeI and BamHI as per the scheme shown in Fig. 2A.

**Site-directed mutagenesis.** Point mutations were introduced in *pnp* through site-directed mutagenesis using PCR by the methods described by Lerner et al. and Spee et al. (27, 48). The pINIII vector carrying the wild-type *pnp* gene was used as a template for PCR. The oligonucleotides used for PCR were as follows: for R100DPNPase, 5'-TTG ACC GCC CGA TTG ATC CGC TGT TCC CGG AA-3' and 5'-TTC CGG GAA CAG CGG ATC AAT CGG GCG GTC AA-3'; for R319HPNPase, 5'-CGC GTA TCG ACG GTC ATG AAA AAG ATA TGA TC-3' and 5'-GAT CAT ATC TTT TTC ATG ACC GTC GAT ACG CG-3'; and for R398D-R399DPNPase, 5'-GGT TCT CCG AAG GAT GAT GAA ATT GGT CAG C-3' and 5'-CGT GAC CAA TTT CAT CAT CCT TCG GAG AAC C-3'. After the PCR, the template DNA was degraded by DpnI treatment. The resultant plasmids expressing R100DPNPase, R319HPNPase, or R398D-R399DPNPase were isolated and sequenced to confirm the mutant sequences.

**RNA isolation.** *E. coli* cells grown overnight in LB medium at 37°C were diluted in fresh medium. The wild-type and  $\Delta pnp$  cells carrying pINIII vector alone and the  $\Delta pnp$  cells expressing the PNPase, RNase II, or RNase R proteins were grown at 37°C to exponential phase (optical density at 600 nm [OD<sub>600</sub>] of 0.5) and then were transferred to 15°C. Samples were removed after 1 and 5 h. For primer extension to detect the *fis* mRNA level, the RNAs were isolated at 37°C before shifting to 15°C and after 72 h at 15°C. The total RNA was extracted by the hot phenol method as previously described (47). It was quantified by measuring absorbance at 260 nm. The purity of RNA was confirmed by agarose gel electrophoresis.

**Primer extension.** Primer extension was carried out by a method described previously (39). The deoxyoligonucleotide used for detection of *yfiA* was 5'-CT ACTCTTCTCAACTTCTTCGACGAAG-3', and that for detection of *fis* was 5'-TTAGTTCATGCCGTATTTTTC-3' (25). The oligonucleotides were labeled with [ $\gamma$ <sup>32</sup>P]ATP (DuPont-New England Nuclear) by using T4 polynucleotide kinase (Gibco BRL). Primer extension was carried out with 5  $\mu$ g of RNA at 42°C for 1 h in a final reaction volume of 10  $\mu$ l, with 50 mM Tris-HCl (pH 8.5), 8 mM MgCl<sub>2</sub>, 30 mM KCl, 1 mM dithiothreitol, 0.4 pmol of <sup>32</sup>P-labeled primer, 0.5 mM (each) of the deoxynucleoside triphosphates, 10 U RNase inhibitor (Boehringer Mannheim), and 6.25 U of reverse transcriptase (Boehringer Mannheim). Equal amounts of RNA samples were used for primer extension, and the products were analyzed on a 6% polyacrylamide gel under denaturing conditions.

**Isolation of polysomes and sucrose density gradient sedimentation.** Polysomes were prepared and resolved as described previously (46), with minor modifications. *E. coli* cells were grown at 37°C in LB medium supplemented to log phase. Upon reaching an appropriate culture density (OD<sub>600</sub> of 0.5; 100-ml culture), polysomes were trapped by the addition of chloramphenicol to the culture to a final concentration of 0.1 mg ml<sup>-1</sup>. After an additional 4 min of incubation, cells were harvested by centrifugation. The cell pellet was resuspended in 1 ml of buffer BP (20 mM Tris-HCl [pH 7.6], 10 mM MgCl<sub>2</sub>, 100 mM NH<sub>4</sub>Cl, and 5 mM  $\beta$ -mercaptoethanol). The cell suspension was placed in a Beckman ultracentrifuge tube. Cells were then lysed by immersing the tube into a liquid nitrogen bath for 1 min, followed by thawing them in a water bath at room temperature until no traces of ice remained. This freeze-thaw cycle was repeated two more times, and the lysate was subsequently subjected to centrifugation at 100,000  $\times$  g for 10 min in a Beckman TLA100.3 rotor. Polysomes were resolved by applying 0.2 ml of the supernatants to a 5 to 40% linear sucrose density gradient (10 ml) in buffer BP with subsequent ultracentrifugation at 4°C in a Beckman SW41 rotor for 2.5 h at 35,000 rpm.

The ribosome profile was analyzed at a 254-nm wavelength. rRNA was detected. Peaks corresponding to polysomes, and 70S, 50S, and 30S ribosomes are shown. The peak adjacent to the 30S ribosomal peak consists of small RNAs including small mRNAs, tRNAs, etc. For quantitation, the heights of individual peaks were measured from the base for each of the peaks in the profile carried out for the wild-type cells containing only the vector, and these values were considered to be 100%, and the base-to-peak values of corresponding peaks from other conditions were expressed as relative percentages.

## RESULTS AND DISCUSSION

**Genetic screening for protein counterparts of PNPase.** In order to elucidate the role of PNPase in acclimation of cells to cold, we explored genes which can complement the cold-sensitive phenotype of *pnp* deletion ( $\Delta pnp$ ) cells at 15°C. A genomic library was created in the plasmid pUC19 by partial digestion of the *E. coli* chromosomal DNA with Sau3A1. The partially digested DNA fragments were cloned into the plasmid using BamHI. The  $\Delta pnp$  cells were transformed with the genomic library. Transformants were isolated for their ability to grow at 15°C. Colony PCR analysis of the transformants showed that 147 clones contained the entire *pnp* gene. The remaining seven clones showed the presence of the *mb* gene. This gene encodes RNase II (RNase II) (53). We repeated the screening and in the second set obtained 53 colonies at 15°C. We observed that out of these, 32 candidates contained the entire *pnp* gene, 3 candidates had the *pnp* gene without the C-terminal region encoding the KH and S1 domains, and 1 candidate contained the *pnp* gene without the region encoding only the S1 domain region. The remaining 17 candidates showed the presence of the *mb* gene. Thus, in addition to PNPase, RNase II was the only other candidate protein that emerged from these studies that is able to complement the PNPase function at low temperature. To confirm if this is indeed the case, the wild-type or  $\Delta pnp$  cells harboring the pINIII plasmid as controls and the  $\Delta pnp$  cells expressing plasmid-encoded PNPase or RNase II were streaked on LB-ampicillin plates and incubated at 37°C and 15°C. As expected, all the cells were able to grow at 37°C (Fig. 1A). The wild-type cells were able to grow at 15°C, while the  $\Delta pnp$  cells showed a cold-sensitive phenotype. The growth of  $\Delta pnp$  cells was restored at 15°C by the presence of either PNPase or RNase II on a plasmid (Fig. 1A). This result confirms that RNase II can complement PNPase function at low temperature.

*E. coli* has three major 3'-to-5' processing exoribonucleases involved in mRNA turnover, namely, PNPase, RNase II, and RNase R. These enzymes are primarily involved in RNA me-

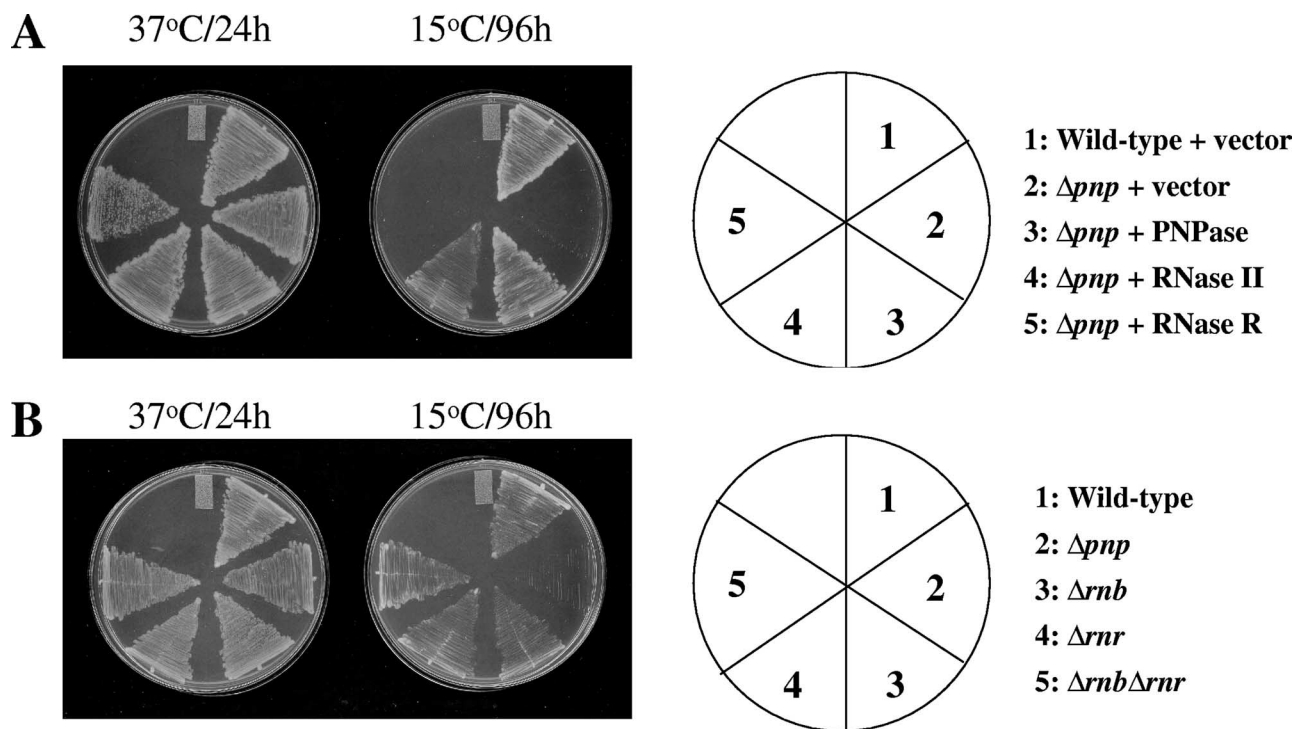


FIG. 1. (A) RNase II can complement the cold-sensitive phenotype of the  $\Delta pnp$  cells at 15°C. The *E. coli* wild-type cells were transformed with the pINIII plasmid as a control, and the  $\Delta pnp$  cells were transformed with the pINIII plasmid alone (control) or containing *pnp*, *rnb*, or *rnr*. The cells were streaked on LB plates containing ampicillin (50  $\mu\text{g ml}^{-1}$ ) and incubated at 37°C and 15°C. Results with plates incubated at 37°C for 24 h or at 15°C for 96 h are presented. (B) RNase II or RNase R is not essential for cell growth at 15°C. *E. coli* wild-type,  $\Delta pnp$ ,  $\Delta rnb$ ,  $\Delta rnr$ , and  $\Delta rnb\Delta rnr$  cells were streaked on LB plates and grown at 37 or 15°C. Results with plates incubated at 37°C for 24 h or at 15°C for 96 h are presented.

tabolism in *E. coli*, and based on the observation that double deletion of PNPase and RNase II (16) or PNPase and RNase R is lethal at 37°C (9), these may have an overlapping role(s). In spite of this, these proteins exhibit several different properties. PNPase is a phosphorylase and requires phosphate for RNA degradation, while RNase II and RNase R are hydrolases. Degradation of RNAs by PNPase results in a release of XDPs, while that by RNase II and RNase R results in a release of XMPs. RNase II and RNase R belong to the RNR family; these exhibit approximately 60% similarity in their secondary structures. However, RNase R is larger than RNase II. Both PNPase and RNase R but not RNase II are induced by cold shock. Our genetic screen showed that only RNase II can complement the cold shock function of PNPase. However, we also tested if RNase R can complement the cold shock function of PNPase in our plate assay. As seen in Fig. 1A, consistent with the results of genetic screening, RNase R failed to support growth of  $\Delta pnp$  cells at 15°C. Interestingly, deletion of RNase II either singly or in combination with RNase R does not affect cell growth at 15°C (Fig. 1B).

**Importance of RNase domains of PNPase in its cold shock function.** Our genetic screen revealed the importance of the RNase activity of PNPase. Therefore, we carried out domain analysis of PNPase to test if its RNase domains were sufficient for carrying out its cold shock function. As shown in Fig. 2, PNPase consists of two RNase PH domains linked together by an  $\alpha$ -helix domain at the N-terminal end. Its C-terminal end consists of two RNA binding domains, the KH domain and the

S1 domain. Constructs were created as depicted in Fig. 2A and cloned in the pINIII vector. The  $\Delta pnp$  cells were transformed with various plasmids as shown in Fig. 2B and grown on LB plates containing ampicillin at 37 and 15°C. Wild-type cells containing vector alone were also included as a control. As seen in Fig. 2B, cells carrying all the constructs were able to grow at 37°C. The wild-type cells containing vector alone or the  $\Delta pnp$  cells containing the entire *pnp* gene were able to grow at 15°C. The  $\Delta pnp$  cells carrying the vector alone were not able to grow at 15°C, as expected. The  $\Delta pnp$  cells carrying PNPase constructs in which the S1 domain was deleted either alone (no. 4;  $\Delta\text{S1PNPase}$ ) or together with the KH domain (no. 7;  $\Delta\text{KH}\Delta\text{S1PNPase}$ ) were able to grow at 15°C, indicating that these domains are dispensable for the function of PNPase at low temperature. This is also consistent with our observation that in our genetic screen described above, four clones, which were able to grow at 15°C, showed the presence of the *pnp* gene in which either the S1 domain alone or both the KH and S1 domains were absent. On the other hand, constructs in which either (or both) of the RNase PH domains was deleted (no. 5, 6, 8, and 9; PNP-1, PNP-2, PNP-4, and PNP-5, respectively) were not able to support growth of the  $\Delta pnp$  cells at 15°C.

In *E. coli*, the only 3'-5' exoribonucleases that catalyze  $\text{P}_i$ -dependent degradation of RNA, leading to release of nucleoside diphosphates, are PNPase and RNase PH (14). The primary role of PNPase is in mRNA degradation (16), while that of RNase PH is suggested to be in tRNA processing (15).

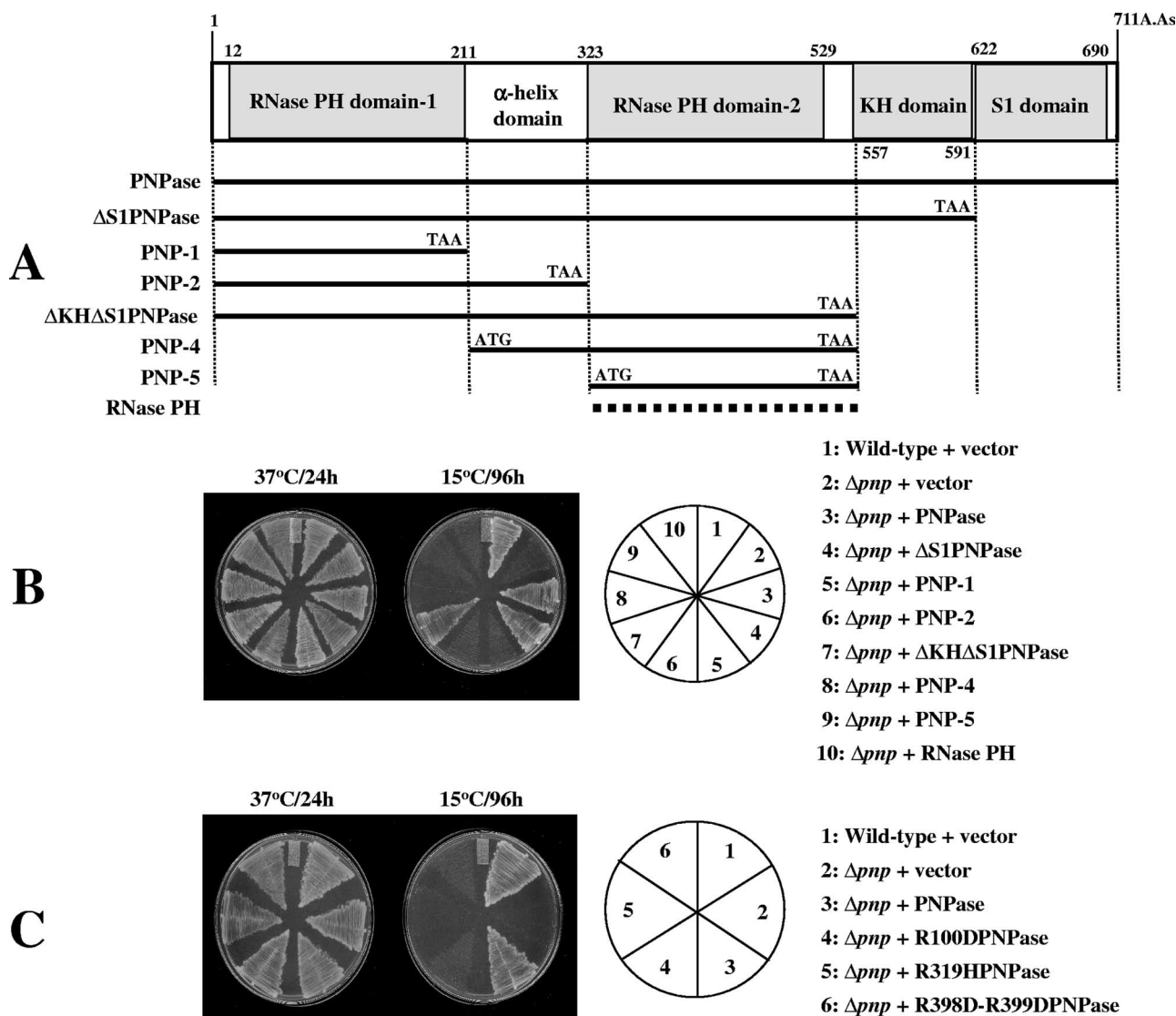


FIG. 2. RNase activity of PNPase is critical for its cold shock function. (A) Schematic representation of domains of PNPase and plasmids constructed. Different domains of PNPase were cloned in the pINIII vector using NdeI and BamHI sites, as shown. The RNase PH plasmid shown with a dashed line is the pINIII plasmid carrying *rph*, the gene encoding *E. coli* RNase PH. (B) *E. coli* wild-type cells were transformed with the pINIII plasmid as a control, and  $\Delta pnp$  cells were transformed with the pINIII plasmid alone or the pINIII plasmid containing either the entire PNPase, different domains of PNPase, or RNase PH. The plasmids carrying different domains of PNPase or RNase PH are designated as in panel A. The cells were streaked on LB plates containing ampicillin ( $50 \mu\text{g ml}^{-1}$ ) and incubated at  $37^\circ\text{C}$  or  $15^\circ\text{C}$ . Results for plates incubated at  $37^\circ\text{C}$  for 24 h or at  $15^\circ\text{C}$  for 96 h are presented. (C) *E. coli* wild-type cells were transformed with the pINIII plasmid as a control, and  $\Delta pnp$  cells were transformed with the pINIII plasmid alone or the pINIII plasmid containing either the wild-type PNPase or PNPase carrying the R100D, R319H, or R398D-R399D mutations. The cells were streaked on LB plates containing ampicillin ( $50 \mu\text{g ml}^{-1}$ ) and incubated at  $37^\circ\text{C}$  or  $15^\circ\text{C}$ . Results for plates incubated at  $37^\circ\text{C}$  for 24 h or at  $15^\circ\text{C}$  for 96 h are presented.

RNase PH shows similarity to RNase PH domain 2 of PNPase (50). Since our results emphasized the importance of the RNase PH domain of PNPase, we tested whether *E. coli* RNase PH (15) can complement the cold-sensitive phenotype of the  $\Delta pnp$  cells (no. 10). RNase PH is not essential for growth at low temperature (data not shown). As seen in Fig. 2, RNase PH was not able to support the growth of  $\Delta pnp$  cells at  $15^\circ\text{C}$ . This is consistent with our result that RNase PH did not come up in our genetic screen as a candidate to complement the cold-sensitive phenotype of  $\Delta pnp$  cells. Taken together, the results show that both RNase domains of PNPase are essential

for its cold shock function, and RNase PH, which has only one RNase domain, or the PNPase construct devoid of either of the RNase domains is not adequate to carry out its cold shock function. The observation that the RNA binding domains of PNPase are dispensable for its essential activity at low temperature is also consistent with a previous report that a plasmid expressing a truncated PNPase devoid of the entire S1 domain was able to complement the low-temperature growth defect of a *pnp* nonsense mutant (58). Also, it was reported that the S1 or KH domain might enhance the processivity of PNPase but they are not required for activity against single-stranded sub-

strates. PNPase lacking either the S1 or KH domain does indeed retain phosphorolytic activity against model substrates, suggesting that its catalytic activity resides within the core PH domain, also called the second core domain (49). However, this observation conflicts with a recent report by Matus-Ortega et al. (31), wherein the authors concluded that the RNA binding domains, particularly the KH domain, are vital at low temperature. One possible reason for this may be the different genetic backgrounds of the cells used. These authors used strain SK10019, in which both the *pnp* and *rph* genes are mutated or absent. It was shown that the  $\Delta pnp \Delta rph$  strain exhibits significantly slower growth (doubling time, 480 min) even at 31°C than the  $\Delta pnp$  (doubling time, 55 min) and  $\Delta rph$  (doubling time, 49 min) strains (59). In our studies, a  $\Delta pnp$  strain was used.

Previously it was reported that the mutations in RNase PH domains 1 and 2 abolish the catalytic activity of PNPase in vitro. Deletion of the KH and S1 domains, on the other hand, did not abolish the catalytic activity (22). Out of various mutations described in that study, we chose two mutations, R100D and R398D-R399D, present in the RNase PH1 and PH2 domains, respectively, to test if the mutant proteins lose their cold shock function. We also chose a mutation, R319H, described in that study, which resides in the  $\alpha$ -helical domain and abolishes the enzymatic activity of PNPase in vitro. This is consistent with an observation by another group that the  $\alpha$ -helical domain linking the two RNase PH domains of PNPase is involved in its enzymatic activity (4). The mutations were created by site-directed mutagenesis, and the pINIII plasmids carrying the mutant *pnp* constructs were transformed into  $\Delta pnp$  cells and grown on LB plates with ampicillin at 37 and 15°C. Wild-type cells and  $\Delta pnp$  cells carrying vector alone or expressing the wild-type PNPase were also included in the plate assay as controls. As seen from Fig. 2C, all cells showed growth at 37°C, while only the wild-type cells or  $\Delta pnp$  cells expressing the wild-type PNPase were able to grow at 15°C. The  $\Delta pnp$  cells did not show growth at 15°C, as expected. The cells expressing R100DPNPase, R319HPNPase, or R398D-R399DPNPase were not able to grow at 15°C. This clearly shows that the RNase activity of PNPase is essential for its cold shock function.

**Polymerization activity of PNPase is not essential for its cold shock function.** In addition to the phosphorolysis and phosphate exchange activity, PNPase also has polymerization activity (22). As described above, our genetic screen revealed that RNase II can complement the cold-sensitive phenotype of the  $\Delta pnp$  cells. However, RNase II does not have the polymerization activity of PNPase. This suggested the possibility that the polymerization activity of PNPase may not play a critical role in its cold shock function. We tested this possibility. *E. coli* has two enzymes which modify the 3' ends of RNAs, poly(A) polymerase, which is encoded by the *pcnB* gene, and PNPase. They add a poly(A) and/or poly(X) tail at the 3' end of RNA, which is important for their metabolism (33). The double deletion mutant strain devoid of these two enzymes ( $\Delta pnp \Delta pcnB$  strain) lacks poly(A) or poly(X) tails on the 3' ends of RNAs. As seen from Fig. 3, the wild-type or  $\Delta pcnB$  strain with vector alone can grow at 15°C while the  $\Delta pnp$  strain with vector alone cannot grow at this temperature, as expected. This strain showed growth at 15°C when expressing PNPase or RNase II

but not RNase R. The  $\Delta pnp \Delta pcnB$  strain showed cold sensitivity, as expected, which can be complemented by expression of PNPase or RNase II but not RNase R. The complementation of the double deletion mutant by RNase II is interesting. Since RNase II lacks polymerization activity but can complement the cold-sensitive phenotype of the  $\Delta pnp \Delta pcnB$  strain, it can be concluded that the cold-sensitive phenotype of the double deletion strain is not due to the absence of the two polymerization enzymes of *E. coli* and the polymerization activity of PNPase does not play a role in its cold shock function. The cold sensitivity of the  $\Delta pnp$  or  $\Delta pnp \Delta pcnB$  strain is thus due to the absence of the RNase activity of PNPase.

**PNPase and RNase II have different substrate specificities from that of RNase R.** The observation that the double deletion mutant of PNPase and RNase R is not viable (9) suggests that they may have some overlapping functions. However, our studies showed that RNase R cannot complement the cold shock function of PNPase. Previous studies showed that RNase R may have a different role during cold shock adaptation from that of PNPase and RNase II, in that RNase R has a more important role in maturation of SsrA/tmRNA than the other two (6, 45). RNase II and RNase R share many catalytic properties; however, certain differences are present, such as the fact that RNase R acts poorly on DNA but can degrade rRNAs quite well. On the other hand, RNase II is essentially inactive against these (8). It was proposed that these enzymes are relatively nonspecific exoribonucleases; however, our observation that RNase II but not RNase R can complement PNPase function at low temperature suggests that they may have certain differences in their specificities. In order to test this possibility, we chose one gene that was shown to be a target of PNPase based on our DNA microarray data comparing the cold shock response (15°C for 1 and 5 h) of the wild-type strain and the  $\Delta pnp$  strain (not shown) and evaluated the effect of expression of all the three enzymes on the level of its mRNA. We chose the gene, *yfiA*, encoding protein Y, for this experiment. Protein Y is induced by cold shock (1, 2). The level of mRNA for this gene was elevated in the  $\Delta pnp$  strain compared to that in the wild-type strain after 5 h at 15°C. It inhibits translation initiation during cold shock but not at normal temperatures. It reduces translation initiation during stress and quickly releases ribosomes for renewed translation initiation.

The wild-type and  $\Delta pnp$  cells carrying the pINIII vector alone and the  $\Delta pnp$  cells expressing the PNPase, RNase II, or RNase R protein were grown at 37°C to exponential phase (OD<sub>600</sub> of 0.5) and then were transferred to a 15°C environment. Samples were removed after 1 and 5 h for RNA extraction. Primer extension was carried out to detect the *yfiA* mRNA level. Results of the primer extension are shown in Fig. 4. In the wild-type strain, the *yfiA* mRNA level goes down after 5 h at 15°C (compare lanes 1 and 2), while in the  $\Delta pnp$  strain, the *yfiA* mRNA level is significantly higher (lane 4). As shown later in Fig. 7, this high level of the *yfiA* transcript is due to its stabilization in  $\Delta pnp$  cells. This stabilization is lost in the  $\Delta pnp$  strain expressing plasmid-encoded PNPase (lane 6) and also in cells expressing plasmid-encoded RNase II (lane 8). However, in the cells expressing plasmid-encoded RNase R, the *yfiA* mRNA remains stabilized (lane 10). This result suggests that some of the targets of PNPase which are important for cold shock adaptation

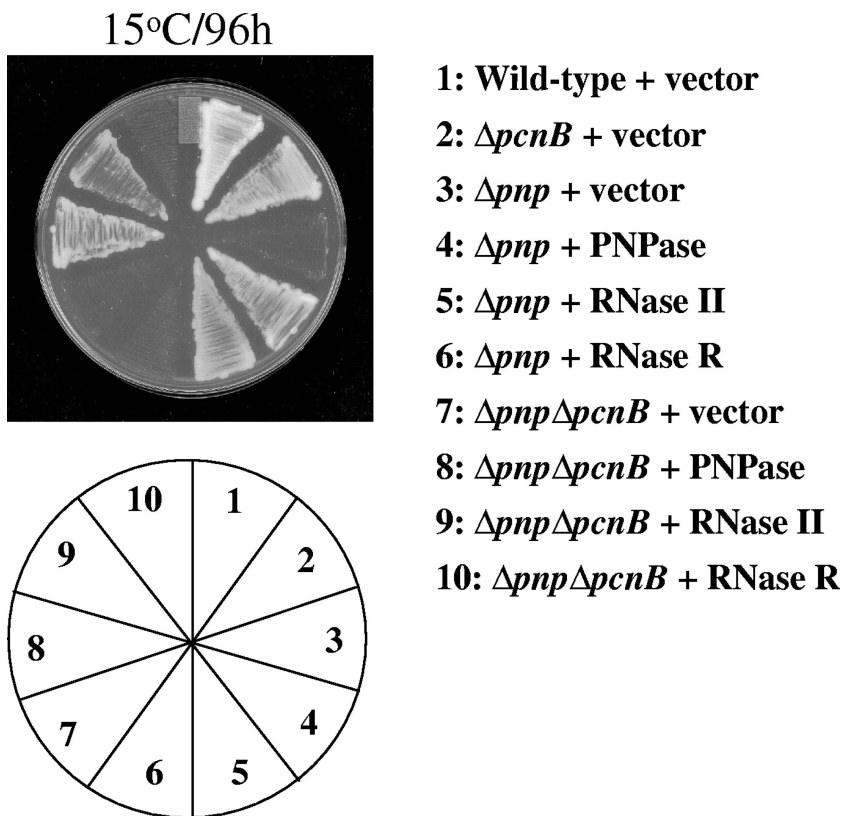


FIG. 3. The polymerization activity of PNPase is not essential for its cold shock function. *E. coli* wild-type,  $\Delta pnp$ ,  $\Delta pcnB$ , and  $\Delta pnp \Delta pcnB$  cells were transformed with the pINIII plasmid alone as a control. The  $\Delta pnp$  and  $\Delta pnp\Delta pcnB$  cells were also transformed with the pINIII plasmid containing PNPase, RNase II, or RNase R. The cells were streaked on LB plates containing ampicillin ( $50 \mu\text{g ml}^{-1}$ ) and incubated at 15°C. Results for plates incubated for 96 h are presented.

of cells are different from those of RNase R and thus RNase R cannot complement the cold shock function of PNPase.

**Deletion of PNPase causes 30S, 50S, and 70S ribosomal subunits to accumulate.** Next, we tested the possibility of the

ribosome profile in the  $\Delta pnp$  cells being affected, since several mRNAs are stabilized by deletion of PNPase and also since deletion of PNPase renders the cells sensitive to low temperatures and it is well documented that the cold-sensitive mu-

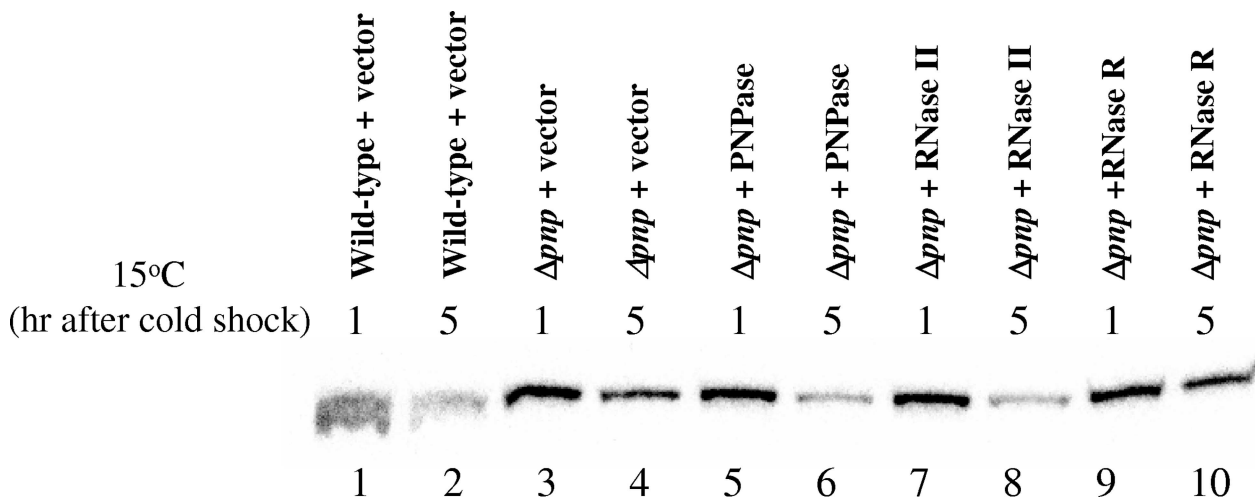


FIG. 4. PNPase has different targets from those of RNase R. Cells from which total RNAs were isolated by the hot phenol method are indicated above the respective lanes. The exponentially growing cells were cold shocked at 15°C for 1 h and 5 h, and samples were collected for RNA isolation. Equal amounts of RNA samples were used for primer extension with deoxyoligonucleotide corresponding to the *yfiA* gene. The products were analyzed by 6% polyacrylamide gel electrophoresis under denaturing conditions.

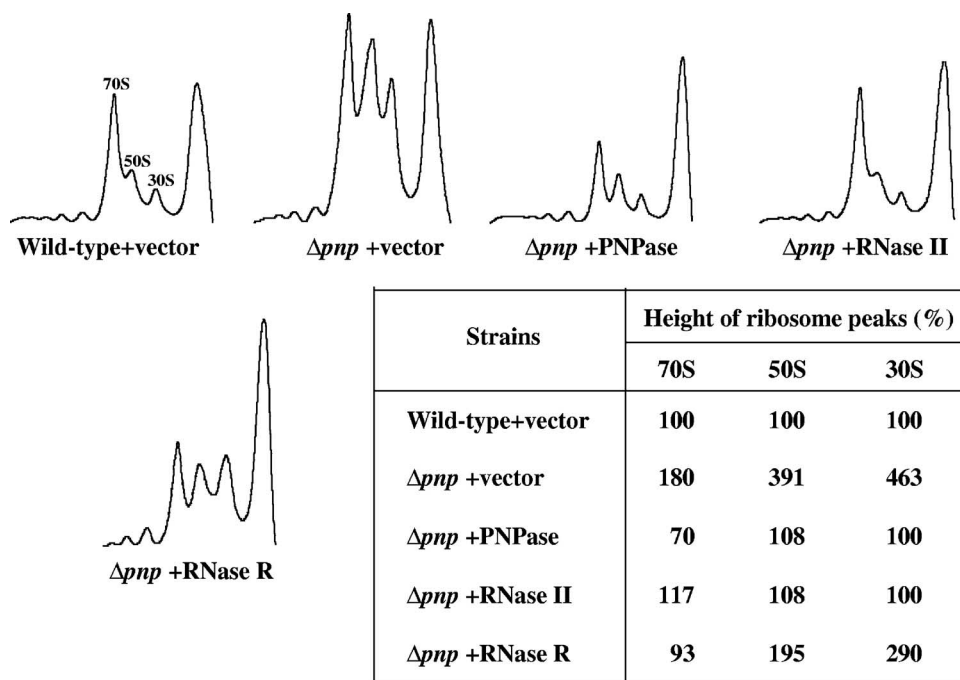


FIG. 5. Deletion of PNPase leads to accumulation of 30S, 50S, and 70S ribosomal subunits. The *E. coli* wild-type and  $\Delta pnp$  cells were transformed with the pINIII plasmid alone as a control. The  $\Delta pnp$  cells were also transformed with the pINIII plasmid containing PNPase, RNase II, or RNase R. The cells growing exponentially at 37°C were cold shocked at 15°C, and samples were collected 72 h after the cold shock. Polysomes were isolated and resolved as described in Materials and Methods.

tants of *E. coli* often are defective in ribosome metabolism (5, 10, 18, 37, 51). A defective ribosomal profile would affect the translation, leading to growth retardation.

Wild-type and  $\Delta pnp$  cells containing pINIII vector alone and  $\Delta pnp$  cells expressing PNPase, RNase II, or RNase R were cold shocked at 15°C for 72 h, and samples were removed for polysome profile analysis. Cell lysates were subjected to 5 to 40% sucrose density gradient fractionation, as described in Materials and Methods. As shown in Fig. 5, in the  $\Delta pnp$  cells carrying the pINIII vector alone, the 30S (>4.5 times), 50S (~4 times), and 70S (~2 times) ribosomal subunits were accumulated. Plasmid-based expression of PNPase or RNase II was able to counteract this accumulation, restoring the normal ribosome profile. The table included in Fig. 5 shows quantitation of the ribosome profile values for easy comparison. In the cells expressing RNase R, the 50S ribosomal subunits (~2 times) and the 30S ribosomal subunits (~3 times) were still accumulated, although the accumulation was less than that in the  $\Delta pnp$  cells carrying the pINIII vector alone. The 70S ribosomal subunits were not accumulated in this strain.

We next tested if this accumulation is a consequence of stabilization of *fis* in the  $\Delta pnp$  cells. Fis is a small, 11.2-kDa DNA binding protein that serves as a transcription regulator of many genes, including rRNA genes (3, 30). We carried out primer extension to detect *fis* mRNA levels in the cells from which the ribosome profile was isolated. Isolation of mRNA and primer extension was carried out as described in Materials and Methods. As shown in Fig. 6, a significantly higher level of *fis* mRNA was detected after 72 h at 15°C in the  $\Delta pnp$  cells than in the wild-type cells (compare lanes 2 and 4). As shown later in Fig. 7, this high level of *fis* transcript is due to its

stabilization in  $\Delta pnp$  cells. This stabilization was lost in the  $\Delta pnp$  cells overexpressing PNPase (lane 6) or RNase II (lane 8) but not RNase R (lane 10). This result suggests that the unusual accumulation of ribosomal subunits seen in Fig. 5 may be a consequence of stabilization of *fis* mRNA in the  $\Delta pnp$  cells. It is interesting that the *fis* mRNA is significantly stabilized in the  $\Delta pnp$  cells expressing RNase R (lane 10), but the accumulation of ribosomal subunits in this strain is less than that in the  $\Delta pnp$  cells carrying vector alone. This may be due to the fact that RNase R but not RNase II can efficiently degrade rRNA (8). However, as seen in Fig. 5, this degradation is not complete, and the cells retain a significant amount of accumulated ribosomal subunits. It is possible that this accumulation results in the cold sensitivity of the  $\Delta pnp$  cells. One cannot exclude the possibility that other mRNAs which are stabilized in the  $\Delta pnp$  cells may also influence the ribosome profile in this strain. A time-dependent analysis of genes affected by PNPase deletion upon cold shock would shed light on this aspect.

In order to confirm that the high levels of *yfiA* and *fis* mRNAs seen in the  $\Delta pnp$  cells compared to those in the wild-type cells upon cold shock are due to their stabilization and not to their increased transcription, we analyzed stability of these mRNAs in these two strains. Cells were cold shocked as described above, rifampin was added to stop the transcription, and RNA samples were isolated at various time points. Primer extension was then carried out to detect the respective mRNAs. As seen in Fig. 7, both *yfiA* and *fis* mRNAs were stabilized in the  $\Delta pnp$  cells. In the case of *yfiA* mRNA, the half-life is less than 1 min (Fig. 7A, lane 2) in the wild-type cells while a significant amount of mRNA is detected even after 5 min in the  $\Delta pnp$  cells (lane 8). Similarly, in the wild-type cells,

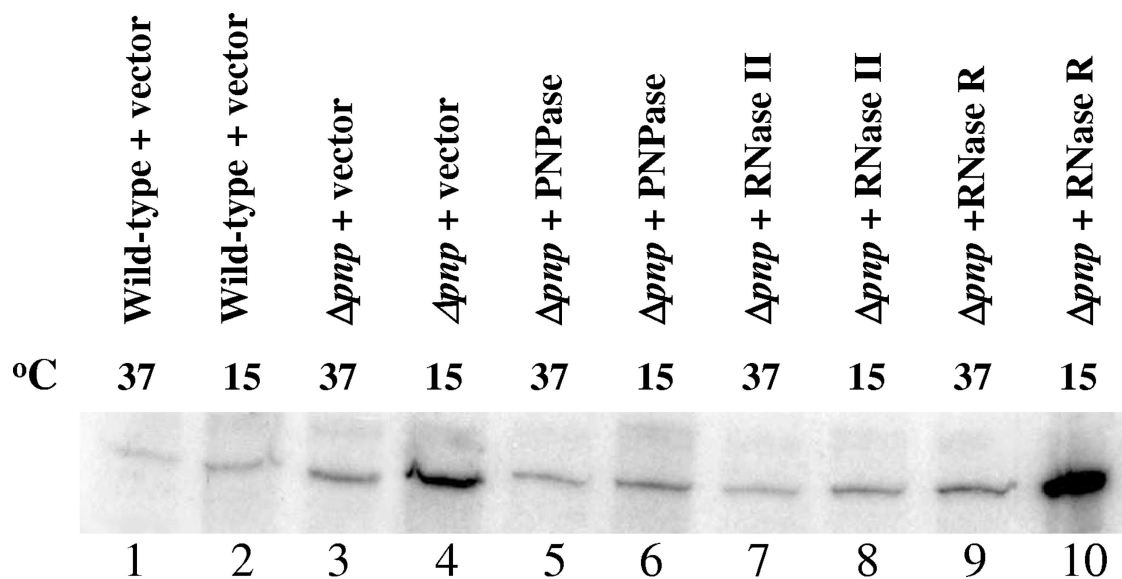


FIG. 6. *fis* mRNA is stabilized in the absence of PNPase. Cells from which total RNAs were isolated by the hot phenol method are indicated above the respective lanes. The cells growing exponentially at 37°C were cold shocked at 15°C, and samples were collected for RNA isolation before cold shock and 72 h after cold shock. Equal amounts of RNA samples were used for primer extension with deoxyoligonucleotide corresponding to the *fis* gene. The products were analyzed by 6% polyacrylamide gel electrophoresis under denaturing conditions.

the *fis* mRNA rapidly disappears and is barely detectable even at 1 min (Fig. 7B, lane 2), while in the  $\Delta pnp$  cells, it is stable even after 20 min following rifampin addition (lane 12). This result proves that indeed these two genes are targets of the RNase activity of PNPase at low temperature and are stabilized in the  $\Delta pnp$  cells.

**Conclusion.** The RNase activity of PNPase is critical for its cold shock function, while its polymerization activity is dispens-

able. Another 3'-5' processing exonuclease, RNase II, can complement cold shock function of PNPase, but RNase R cannot. This is attributed to the difference in the substrate specificities of these enzymes. Further analysis of targets of PNPase which can be acted upon by RNase II but not by RNase R can shed light on the essential role of PNPase during cold shock adaptation of cells. Stabilization of some of the PNPase-sensitive mRNAs, such as *fis*, has consequences such

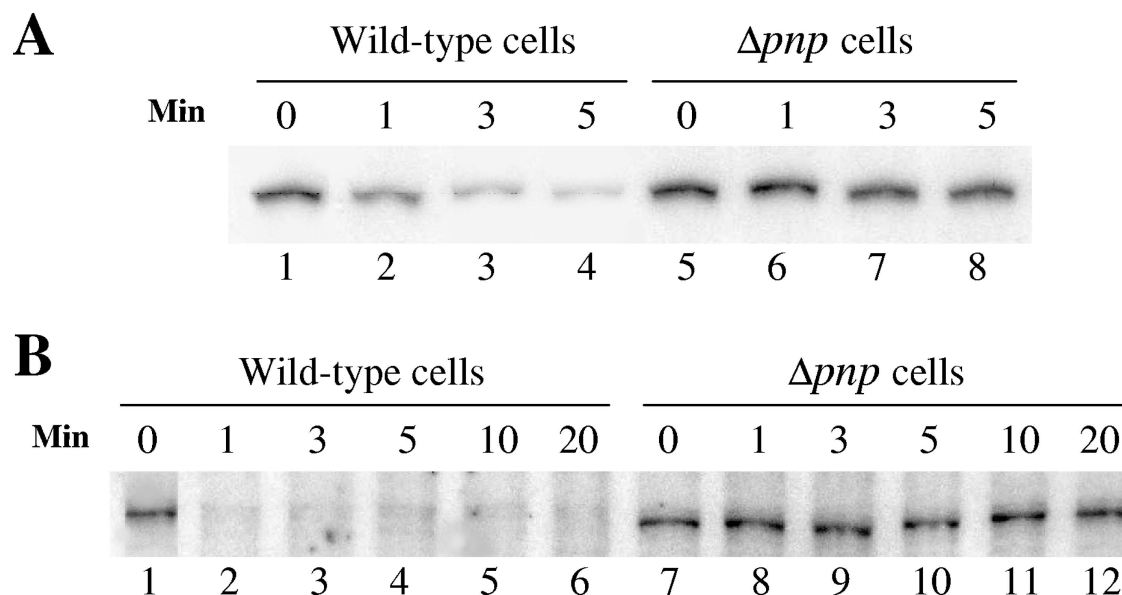


FIG. 7. An increase in transcript levels of *yfiA* (A) and *fis* (B) in the  $\Delta pnp$  cells is due to stabilization of the respective mRNAs. Total RNAs were isolated from wild-type and  $\Delta pnp$  cells by the hot phenol method. The exponentially growing cells were cold shocked at 15°C for 1 h (for the *yfiA* gene) or 72 h (for the *fis* gene), the transcription was stopped by adding rifampin, and samples were collected for RNA isolation at the times indicated in the figure. Equal amounts of RNA samples were used for primer extension with deoxyoligonucleotides corresponding to the *yfiA* or *fis* gene. The products were analyzed by 6% polyacrylamide gel electrophoresis under denaturing conditions.



as accumulation of ribosomal subunits in the  $\Delta pnp$  cells, which may play a role in the cold sensitivity of this strain.

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