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Polynucleotide Phosphorylase Protects *Escherichia coli* against Oxidative Stress[†]Jinhua Wu,[‡] Zhe Jiang,[§] Min Liu, Xin Gong, Shaohui Wu,^{||} Christopher M. Burns, and Zhongwei Li*

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ABSTRACT: *Escherichia coli* polynucleotide phosphorylase (PNPase) primarily functions in RNA degradation. It is an exoribonuclease and integral component of the multienzyme RNA degradosome complex [Carpousis et al. (1994) *Cell* 76, 889]. PNPase was previously shown to specifically bind a synthetic RNA containing the oxidative lesion 8-hydroxyguanine (8-oxoG) [Hayakawa et al. (2001) *Biochemistry* 40, 9977], suggesting a possible role in removing oxidatively damaged RNA. Here we show that PNPase binds to RNA molecules of natural sequence that were oxidatively damaged by treatment with hydrogen peroxide (H₂O₂) postsynthetically. PNPase bound oxidized RNA with higher affinity than untreated RNA of the same sequence, raising the possibility that it may act against a wide variety of lesions. The importance of such a protective role is illustrated by the observation that, under conditions known to cause oxidative damage to cytoplasmic components, PNPase-deficient cells are less viable than wild-type cells. Further, when challenged with H₂O₂, PNPase-deficient cells accumulate 8-oxoG in cellular RNA to a greater extent than wild-type cells, suggesting that this RNase functions in minimizing oxidized RNA *in vivo*. Introducing the *pnp* gene encoding PNPase rescues defects in growth and RNA quality of the *pnp* mutant cells. Our results also suggest that protection against oxidative stress is an intrinsic function of PNPase because association with the RNA degradosome or with RNA helicase B (RhlB) is not required.

RNA oxidation has recently gained attention because of its association with many diseases related to oxidative stress and aging (reviewed in refs 3 and 4). Evidence of RNA dysfunction caused by oxidation is mounting (5–8) and raises the possibility that RNA oxidative damage is a potentially important causative factor in the development of disease.

RNA damage by reactive oxygen species (ROS)¹ happens quickly, on a much shorter time scale than the half-lives of most RNA species; therefore, specific RNA surveillance mechanisms that efficiently remove oxidized RNA before cellular damage occurs are expected to exist in living organisms (9). Polynucleotide phosphorylase from *Escherichia coli* (2) and human (10) was identified in searches for activities responsible for controlling oxidized RNA because they were observed to bind synthetic RNA containing the oxidized lesion 8-hydroxyguanine (8-oxoG) with higher affinity than normal RNA. These results implicated PNPase in specifically recognizing and removing RNA molecules

containing 8-oxoG. Consistent with this model, we have previously observed that human PNPase reduces the level of 8-oxoG in RNA and protects HeLa cells under oxidative stress (11). However, a protective role for *E. coli* PNPase under oxidative stress has been controversial. PNPase-deficient cells were shown to be much more resistant than wild type to the oxidant paraquat (2). It was argued that PNPase may normally bind oxidized RNA molecules *in vivo*, making them unavailable for protein synthesis and therefore inhibiting the growth of *pnp*⁺ cells under paraquat insult (2). However, this interpretation contradicts the role for PNPase suggested by its interaction with oxidized RNA and by the observations that oxidatively damaged RNAs are dysfunctional.

E. coli PNPase is an α_3 trimer whose primary role *in vivo* is exonucleolytic degradation of RNA in the 3' → 5' direction (12, 13). A large portion of PNPase exists in the degradosome, a multienzyme complex important in RNA processing and mRNA decay (1, 14). Other members of the degradosome include an endoribonuclease, RNase E, an ATP-dependent RNA helicase, RhlB, and enolase (15). PNPase rapidly and processively degrades RNA chains on its own, while degradation of highly structured RNAs is greatly enhanced by its association with RhlB, either alone (16) or in the degradosome (17, 18). Poly(A) polymerase can also promote degradation of structured RNA molecules by PNPase by synthesizing 3' linear poly(A) tails that presumably facilitate binding of PNPase (18, 19).

Consistent with these properties, PNPase has been found to be important in many aspects of RNA metabolism. It is the major exoribonuclease for degrading mRNA decay intermediates (12) and small, noncoding RNAs (20, 21),

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¹ Abbreviations: ROS, reactive oxygen species; PNPase, polynucleotide phosphorylase; SDS, sodium dodecyl sulfate; PMS, phenazine methosulfate; cfu, colony forming units; WT, wild type; RBD, RNA-binding domains.

particularly the structured regions of these molecules (18, 20). Although *E. coli* is known to contain at least eight exoribonucleases that degrade RNA in the 3' → 5' direction with partially overlapping functions, PNPase is thought to perform a special function not readily replaced by the other exoribonucleases (13). Cells lacking PNPase alone grow slightly slower than the wild type (22) while cells lacking both PNPase and RNase II (23), or both PNPase and RNase R (24), are inviable.

Recent findings that *E. coli* PNPase is a key player in the quality control of certain RNA species are of particular interest to this work. PNPase and poly(A) polymerase are important for the degradation of a defective mutant of tRNA^{Trp} (25). The majority of this tRNA is normally degraded in wild-type cells, but degradation is impaired in cells deficient in either PNPase or poly(A) polymerase. In the absence of both enzymes, this mutant tRNA accumulates to a high level, mostly in precursor form (25). PNPase and RNase R are responsible for cleaning up rRNA fragments presumably generated from breakdown of aberrant ribosomes (26). These observations, together with the reported specific interaction with oxidized RNA, strongly suggest a role for PNPase in the degradation of oxidatively damaged RNA molecules.

In this work, we examined the role of *E. coli* PNPase in protecting cells and controlling RNA quality under oxidative challenge. Our results suggest that PNPase is indispensable under oxidative stress. Because PNPase is widely distributed and is highly conserved in all domains of life (27), our findings imply a role for this enzyme in diverse situations involving oxidative stress, from bacterial pathogenesis to cancer and age-related diseases.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* K12 strain CA244 (*lacZ*, *trp*, *relA*, *spoT*) (25) was used as wild type in most experiments. A strain lacking PNPase (*pnp*) was described previously (25). *E. coli* K12 strains AC21 (wild type) and AC24 (*rneΔ(844–1045)*) (28) were gifts from Dr. Agamemnon J. Carpousis. The *pnp* allele was transferred to AC21 and AC24 by P1 transduction. The plasmid pKAK7 harboring the *pnp* gene was provided by Dr. Sidney R. Kushner (23). Cultures from individual colonies were typically grown in M9 medium supplemented with 20 μg/mL tryptophan or in tryptone yeast extract (YT) medium. Incubation was at 37 °C with shaking.

Materials. The plasmid DNA miniprep kit was purchased from Qiagen (Valencia, CA). Catalase and guanosine were from Sigma-Aldrich (St. Louis, MO), 8-hydroxyguanosine was from Calbiochem (La Jolla, CA), [³H]poly(A) was from Amersham, SYPRO Ruby was from Bio-Rad (Hercules, CA), TRI Reagent was from Molecular Research Center (Cincinnati, OH), and the Amplex Red kit for measuring H₂O₂ content was from Molecular Probes (Invitrogen, Carlsbad, CA). A 50-mer RNA 5'-cggagaguaaaaaugaaguacguccuuccgugaaguuuuuucgau3' was chemically synthesized by Integrated DNA Technologies (Coralville, IA) and used in protein binding experiments *in vitro*. Purified *E. coli* PNPase was a gift from Drs. Yuhong Zuo and Arun Malhotra, University of Miami School of Medicine. All other chemicals were of reagent grade.

Treatment of *E. coli* Cultures with Oxidants. *E. coli* cultures were routinely grown in YT medium overnight at 37 °C with shaking. The cultures were diluted 50 times to fresh YT medium and were incubated at 37 °C with shaking to log phase.

To compare cell viability on agar plates containing oxidative reagents, exponentially growing cultures (OD_{550nm} ~ 0.5) were diluted to OD_{550nm} = 0.05 and then serially diluted. Two microliters of the serially diluted cultures was spotted on the surface of YT agar plates containing various concentrations of oxidative reagents. After incubation at 37 °C for 14–16 h, cell growth on the spots was examined and recorded.

To measure cell viability under oxidative challenge in liquid medium, H₂O₂ was added to the exponentially growing cultures (OD_{550nm} = 0.2) at various concentrations. To determine growth by cell density, aliquots of the cultures were immediately dispensed into a 96-well plate at 100 μL per well and were incubated at 37 °C with shaking at 150 rpm. The OD_{550nm} value was measured using a plate reader over a time course. To determine colony forming units (cfu), after 90 min, the cultures were diluted 1:50000 and plated on YT agar plates. After incubation overnight at 37 °C, the number of colonies was counted.

Determining H₂O₂ Content in *E. coli* Cultures Using the Amplex Red Kit. Exponentially growing cultures (OD_{550nm} = 0.2) were added to H₂O₂ at a final concentration of 0.5 mM and were incubated at 37 °C with shaking. The remaining H₂O₂ level in the cultures was determined over a time course using the Amplex Red kit as directed by the manufacturer. Briefly, 12.5 μL of H₂O₂-treated culture was mixed with 37.5 μL of reaction buffer and then with 50 μL of Amplex Red working solution in a 96-well plate. The mixture was incubated at room temperature for 30 min, and OD_{550nm} was measured in a plate reader and compared to a standard curve made with known concentrations of H₂O₂.

PNPase Polymerization Activity Assay. Cell extracts were prepared by sonication (29). PNPase was assayed by polymerization of ADP modified from a previously described method (29) in a reaction mixture (50 μL) containing 100 mM glycine–NaOH (pH 8.9), 200 mM NaCl, 0.5 mM MgCl₂, 0.5 mM [³H]ADP (203 cpm/nmol), and cell extract. After incubation for 10 min at 37 °C the reaction was stopped by the addition of 10% (w/v) trichloroacetic acid, and acid-precipitable radioactivity was determined.

Preparation of Total RNA and DNA from *E. coli* Cultures and Determination of 8-OxoG and 8-Oxo-dG Levels. *E. coli* cells were collected by centrifugation at 10000g for 1 min. Cell pellets were resuspended in 50 μL of cell lysis buffer containing 1% SDS (30), incubated for 5 min at room temperature, and then stored at –80 °C until all samples were collected. To isolate RNA, 1 mL of TRI Reagent containing 500 μM butylhydroxytoluene (BHT) and 100 μM desferol was added to each sample. RNA was extracted following the manufacturer's protocol and was dissolved in 20 μL of doubly distilled H₂O that was pretreated with Chelex-100 and stored under argon gas.

Alternatively, RNA and DNA were prepared by boiling the cell lysates for 1 min followed by phenol–chloroform extractions. For RNA isolation, 450 μL of H₂O and 500 μL of 90% liquefied phenol, pH ~4, and 10% chloroform were added to the lysates. The mixture was extracted for 10 min

by intermittent vortexing. Phenol–chloroform extraction was repeated, and RNA in the aqueous phase was precipitated by centrifugation after adding an equal volume of 2-propanol. The pellets were washed twice using 70% ethanol. Dried RNA pellets were dissolved in 20 μ L of doubly distilled H₂O. DNA was isolated by extracting the cell lysates twice with an equal volume of buffered phenol (pH 7.0):chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was pelleted, washed, and dissolved in H₂O in the same way as RNA.

The level of 8-oxoG in RNA was determined as previously described (31). Briefly, RNA was digested to nucleosides by nuclease P1 and alkaline phosphatase treatment and the mixture subjected to HPLC separation. Guanosine was detected at 254 nm using a UV detector, and 8-oxoguanosine was detected using an electrochemical detector at +600 mV. Pure nucleosides were used to identify the peaks and to calibrate the amounts of guanosine and 8-oxoguanosine in RNA samples.

The level of 8-oxo-dG in DNA was determined similarly. DNA was digested with nuclease P1 and alkaline phosphatase, and the deoxynucleosides were separated by HPLC under the same condition as for RNA nucleosides, except the resolving time was longer (70 min) and deoxyguanosine and 8-oxodeoxyguanosine were used as chemical standards.

Immobilization of RNA on Agarose Beads and Detection of the Binding of PNPase to Oxidized RNA. The 50-mer RNA was oxidized *in vitro* with H₂O₂ as described previously (31). RNA molecules were covalently linked to adipic acid dihydrazide agarose beads by a published procedure (32). Briefly, 500 pmol of RNA was treated with 5 mM sodium metaperiodate (Sigma) in the dark. Four hundred microliters of a 50% slurry of adipic acid dihydrazide agarose beads (Sigma) was washed and then mixed with the periodate-treated RNA and rotated for 12 h at 4 °C. The beads with the bound RNA were pelleted, washed, and used in reaction mixtures containing 250 μ L of *E. coli* S100 cell extracts prepared as described previously (30). The beads were pelleted by centrifugation at 106g for 3 min and washed four times with 1 mL of washing buffer (20 mM HEPES–KOH, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 5% (v/v) glycerol). After the final centrifugation, the proteins bound to the immobilized RNA were eluted by addition of 70 μ L of protein sample buffer. Eluted RNA-binding proteins were analyzed by 12% SDS–PAGE and visualized with SYPRO Ruby. A protein band corresponding to the size of PNPase was excised from stained gel, and the identity of the protein was analyzed by electrospray ionization-based mass spectroscopy using the QSTAR LC/MS facility at Florida Atlantic University.

RESULTS

PNPase Preferentially Binds to Oxidized RNA. It has been previously reported that *E. coli* PNPase binds to poly(8-oxoG•A) RNA with higher affinity than it does to poly(U•A) (2). This was the first observation that a protein specifically interacts with RNA containing an oxidative lesion. The synthetic RNA substrate used in that study contained poly(A) and ³²P-labeled poly(8-oxoG). Because the labeled probe was made with only 8-oxoG, it may adopt a structure very different from RNA of natural sequence and would not

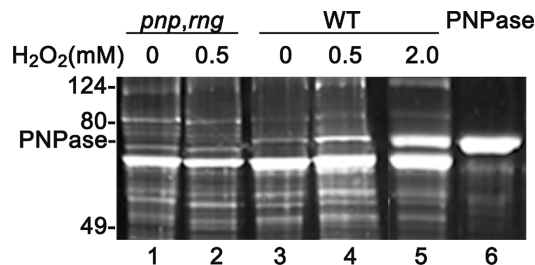


FIGURE 1: PNPase binds oxidized RNA with high affinity. Crude cell extracts were incubated with RNA beads. After washing, bead-bound proteins were eluted, separated by SDS–PAGE, and visualized with SYPRO Ruby. The S100 extracts from wild type (WT) and a mutant lacking PNPase (77.1 kDa) and RNase G (55.4 kDa) were used. The concentrations of H₂O₂ used for the treatment of RNA are shown on top of the lanes. Migration of protein size markers is shown on the left. Purified PNPase protein was run in lane 6.

contain any other oxidative damages. For these reasons, PNPase binding to that RNA may not reflect its interaction with natural RNA sequences containing oxidative damages that may exist in cells.

In this work, we have carried out RNA-binding analysis using a chemically synthesized 50-mer RNA oligonucleotide containing part of the sequence of the *rpmJ* mRNA which encodes the ribosomal protein L36 in *E. coli*. Oxidized RNA was generated by incubating this oligoribonucleotide with H₂O₂. Treatment with H₂O₂ generates oxidative damages (as measured by 8-oxoG content) to this RNA depending on the concentration of the oxidant. The untreated RNA contains ~1 8-oxoG/10³ G. Treatment with H₂O₂ increases the level of 8-oxoG nearly 20-fold at 0.5 mM and at least 100-fold at 2 mM. The oxidized and untreated RNA samples were linked to agarose beads and used to identify proteins that interact preferentially with the oxidized RNA.

As shown in Figure 1, one of the bands that is more abundant when protein is purified using beads containing oxidized RNA (lanes 4 and 5) than control beads with untreated RNA (lane 3) comigrates with purified PNPase (lane 6). Furthermore, the abundance of this band was greater when RNA was oxidized with 2 mM H₂O₂ (lane 5) than with 0.5 mM H₂O₂ (lane 4). The untreated RNA in lane 3 contains a significant amount of 8-oxoG so it is likely that at least part of the protein in this band is due to binding the basal level of oxidized RNA. Although comigration with purified PNPase supports the possibility that the band is composed primarily of PNPase, additional studies show this to be the case: (1) the protein band was absent when the extract was prepared from cells lacking PNPase (lanes 1 and 2), and (2) electrospray ionization-based mass spectroscopy analysis using the QSTAR LC/MS facility at Florida Atlantic University identified PNPase (with a positive protein score of 616) in the extracted band. Our result agrees with the previous finding that PNPase interacts with 8-oxoG RNA with high affinity (2) and also proves that it binds more tightly to oxidized RNA than to untreated RNA of natural sequence and which may contain a variety of oxidative lesions.

PNPase-Deficient Mutant Is Hypersensitive to Oxidative Challenges. Because a *pnp* mutant strain was previously shown to be hyperresistant to paraquat (2), we first compared the growth of our wild-type and *pnp* mutant cells in the presence of paraquat. The null *pnp* mutation was verified

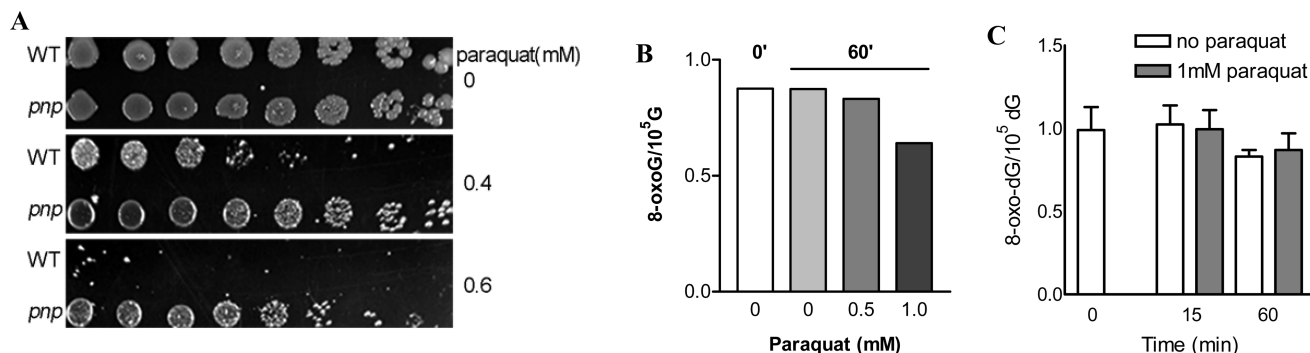


FIGURE 2: Cell viability and RNA oxidation upon treatment with paraquat. (A) Exponentially grown cultures of wild-type and *pnp* mutant strains were normalized to OD_{550nm} = 0.002 and then serially diluted 1:5. The diluted cultures were inoculated on YT agar plates containing 0, 0.4, and 0.6 mM paraquat. (B) The level of 8-oxoG was determined in total RNA isolated from exponentially grown wild-type cultures after treatment with paraquat at the indicated concentration and time. (C) The level of 8-oxo-dG was determined in total DNA isolated from exponentially grown wild-type cultures after treatment with 1 mM paraquat at the indicated time.

genetically by PCR and phenotypically by observing lack of PNPase-mediated poly(A) degradation (data not shown). Cultures were serially diluted and plated as spots onto YT agar with or without paraquat, and growth was recorded after incubation overnight. As shown in Figure 2A, growth of wild-type cells is inhibited by paraquat partially at 0.4 mM and almost completely at 0.6 mM. In contrast, growth of the *pnp* mutant was reproducibly better at all concentrations of paraquat in complete agreement with the previous observations (2).

We examined the level of oxidative damage in RNA and DNA under paraquat treatment. As shown in Figure 2B, treatment with paraquat did not increase the level of 8-oxoG in cellular RNA and may even have caused a slight reduction. Paraquat at 1 mM also did not affect the level of 8-oxo-dG in DNA (Figure 2C). Therefore, we suspect paraquat caused reduction of cell viability in *E. coli* by mechanism(s) unrelated to nucleic acid oxidative damage (see discussion below). We therefore tested other oxidants.

Using the same plating method, we studied the relative sensitivity of wild-type and *pnp* mutant cells to H₂O₂ and phenazine methosulfate (PMS), oxidative reagents known to damage cytoplasmic components (Figure 3A,B). Wild-type and *pnp* mutant cells show no apparent difference in growth in the absence of an added oxidant; however, in the presence of 0.6 and 0.8 mM H₂O₂, or 50 μM PMS, the *pnp* mutant grows much more poorly than the wild type. These results contradict those obtained with paraquat treatment, suggesting that PNPase does in fact play an important role protecting cells under oxidative stress.

Hydrogen peroxide has been widely used in experiments to generate oxidative damage to cellular DNA and RNA. We further studied cell viability in liquid cultures in response to H₂O₂ treatment by measuring cell density at OD_{550nm} and colony forming units (cfu). Figure 3C shows representative responses of the wild-type and *pnp* mutant strains after 90 min incubation with or without H₂O₂. In the absence of H₂O₂, little difference in cell density was observed, and density of both wild-type and *pnp* mutant cultures reduced depending on H₂O₂ dosage. However, the reduction is much more pronounced for the *pnp* mutant. Similarly, exposure to H₂O₂ reduced cfu in a dosage-dependent manner (Figure 3D) with the *pnp* mutant strain being much more sensitive. This result confirms that H₂O₂-induced cell density decrease is due to increased cell death. The hypersensitivity of *pnp* mutant cells

to H₂O₂ was also observed when the cultures were treated under various conditions in minimum and rich media (S. Wu, X. Gong, and Z. Li, data not shown). Taken together, these results suggest that PNPase plays an important role protecting *E. coli* cells from oxidative challenge.

PNPase Activity Is Only Slightly Affected by Treatment with Chemicals. Oxidative stress may cause dramatic changes in gene expression by affecting posttranscriptional processes including translation and mRNA decay (33, 34). Viability of wild-type cells in the presence of the chemicals could be supported by regulating the activity of PNPase, a process missing in the *pnp* cells. To examine this possibility, we have measured PNPase activity under various treatment conditions. As shown in Figure 4, untreated cells show a small (~15%) reduction in ADP incorporation in stationary cultures (at 60 and 120 min). The activity was further decreased by treatment with paraquat at 120 min and stayed higher after addition of H₂O₂ or PMS. The changes in activity caused by the treatments are so moderate that altered PNPase expression cannot be a major cause for the differences in the viability of wild-type and *pnp* cells under oxidative stress.

H₂O₂ Induces Accumulation of Higher Levels of 8-OxoG in RNA from PNPase-Deficient Cells. The facts that PNPase binds specifically to oxidized RNA and that mutant cells lacking PNPase are hypersensitive to oxidative challenge suggest that PNPase may be involved in controlling the level of oxidized RNA in the cell. To see if this might be the case, we determined if 8-oxoG level in RNA is affected *in vivo* by PNPase deficiency. As shown in Figure 5A where RNA was isolated by TRI Reagent, in the absence of H₂O₂ treatment, the level of 8-oxoG in the *pnp* mutant cells is not significantly different from that in the wild type. Treatment with 1 mM H₂O₂ increased 8-oxoG level in the wild-type cells about 2-fold in 15–60 min. However, a higher increase in 8-oxoG level was observed in the *pnp* mutant cells, about 3–4-fold in 15–60 min after addition of H₂O₂. Similar increases in 8-oxoG level were observed in RNA prepared by phenol–chloroform extraction after boiling the cell lysates in the presence of SDS (Figure 5B). This result demonstrates that PNPase is required to minimize the level of 8-oxoG in RNA following oxidative challenge.

In order to determine if the reduced cell viability and increased RNA damage are attributable to an indirect deficiency of the *pnp* mutant cells in hydrolyzing H₂O₂ causing prolonged oxidative stress, we measured the disap-

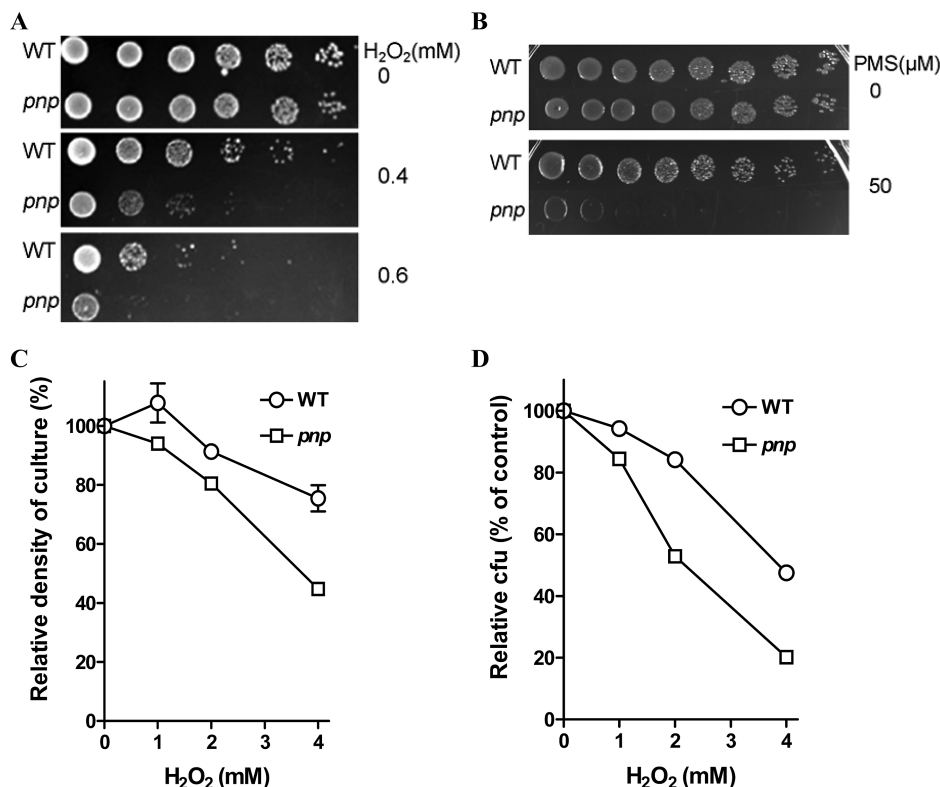


FIGURE 3: PNPase-deficient cells are hypersensitive to H_2O_2 and PMS. (A) Sensitivity of wild-type (WT) and *pnp* mutant cells to H_2O_2 . Cultures were grown, diluted, and inoculated as described in Figure 2A. (B) Sensitivity of wild-type and *pnp* mutant cells to phenazine methosulfate (PMS). (C) Sensitivity of wild-type and *pnp* mutant cells to H_2O_2 determined by cell density. Cultures were grown to exponential phase in liquid YT medium, treated with indicated concentrations of H_2O_2 for 90 min in a 96-well plate ($n = 4$), and incubated at 37 °C with shaking at 150 rpm. Cell density was measured in a plate reader at OD_{550nm} and is expressed as the percent of an untreated control. (D) Sensitivity to H_2O_2 determined by colony forming units (cfu). Exponentially growing cultures were challenged with the indicated concentrations of H_2O_2 for 90 min. The cultures were then diluted, plated on YT agar plates, and incubated overnight at 37 °C. The data are expressed as the percent of an untreated control.

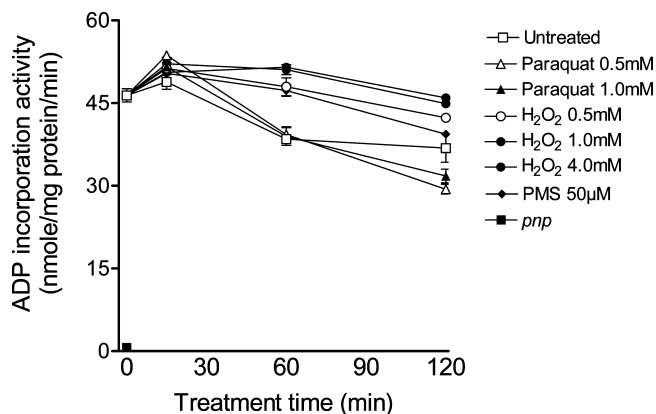


FIGURE 4: Activity of PNPase under various treatment conditions. *E. coli* cultures were grown in YT medium. At $OD_{550nm} = 0.5$, the cultures of the wild-type strain were treated with various chemicals at concentrations shown in the legend. Cells were collected at the indicated time points. PNPase polymerization activity was measured by ADP incorporation into RNA as described in Materials and Methods using 18 μ g of protein from cell extracts in a 50 μ L reaction. The averages of two measurements and standard errors are shown.

pearance of H_2O_2 from the cultures. As shown in Figure 5C, H_2O_2 is degraded rapidly by both wild-type and *pnp* mutant cells without significant difference, revealing that PNPase deficiency does not reduce H_2O_2 hydrolysis activities (e.g.,

catalases and peroxidase), and the enzyme and likely plays a direct role controlling the quality of RNA under oxidative stress.

The pnp Gene Complements the Phenotype of pnp Cells in Response to H_2O_2 . To examine if the response of *pnp* mutant cells to oxidative challenges was due to loss of PNPase, we investigated cell viability and RNA damage level using cells containing the *pnp* gene on the plasmid pKAK7, a medium copy number plasmid derived from pBR322 (23). The wild-type and *pnp* mutant cells were transformed with pKAK7 or the control vector pBR322. The presence of chromosomal and plasmid-borne *pnp* alleles in these four strains was confirmed by PCR amplification of genomic DNA from wild-type and mutant *pnp* cells and by identification of the plasmid DNA (data not shown).

We first analyzed the activity of PNPase in crude cell lysates made from these cultures to confirm PNPase expression (Figure 6C). The activity of the extract from *pnp*/pBR322 was very low as expected, and that of wild type/pBR322 should come from PNPase. Introduction of pKAK7 increased PNPase activity in both wild-type and *pnp* cultures to approximately 2.5-fold, respectively. The limited increase in PNPase activity above wild-type level is likely caused by the known posttranscriptional autoregulation of *pnp* expression through an RNase III-dependent mechanism, because the region involved in *pnp* autoregulation is included in pKAK7 (23, 35).

As expected, if lack of PNPase is responsible for the sensitivity of growth to H_2O_2 , pKAK7 restored growth of

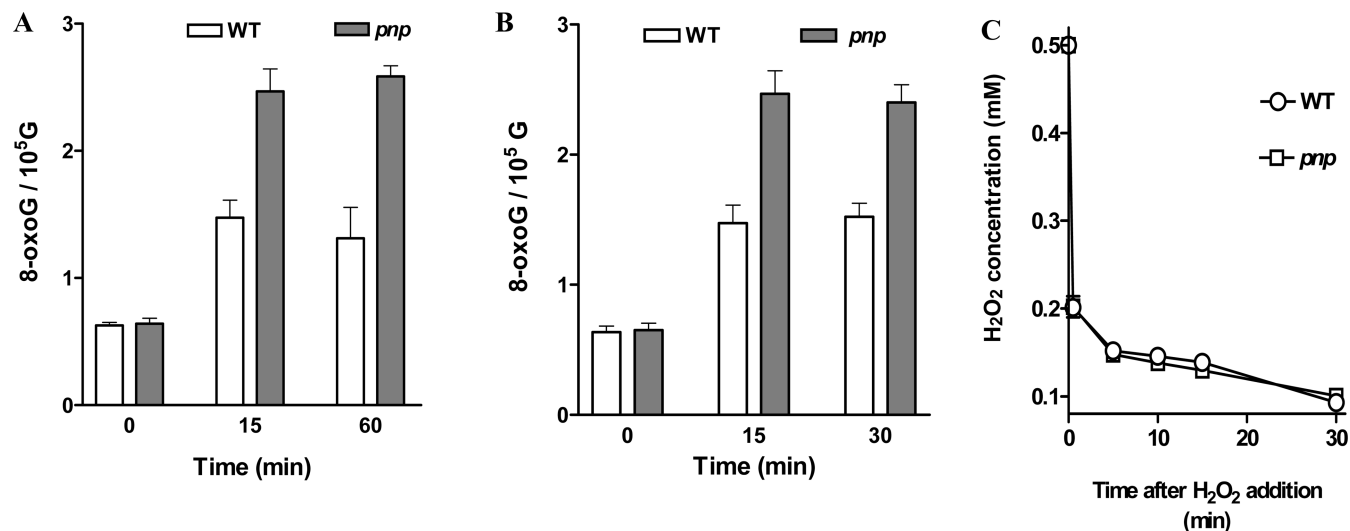


FIGURE 5: H_2O_2 -induced RNA damage is increased by PNPase deficiency, but H_2O_2 hydrolysis is not. Exponentially grown wild-type and *pnp* mutant cultures were treated with or without 1 mM H_2O_2 . Total RNA was isolated by TRI Reagent in (A) and by phenol–chloroform extraction in (B) at the indicated time points. The level of 8-oxoG in RNA was determined by HPLC. Means of two measurements and standard errors are shown. (C) Wild-type and *pnp* mutant cultures were grown to $OD_{550nm} = 0.5$ in YT medium, and H_2O_2 was added to a final concentration of 0.5 mM. Samples were taken at the indicated time points and assayed for H_2O_2 concentration using the Amplex Red kit.

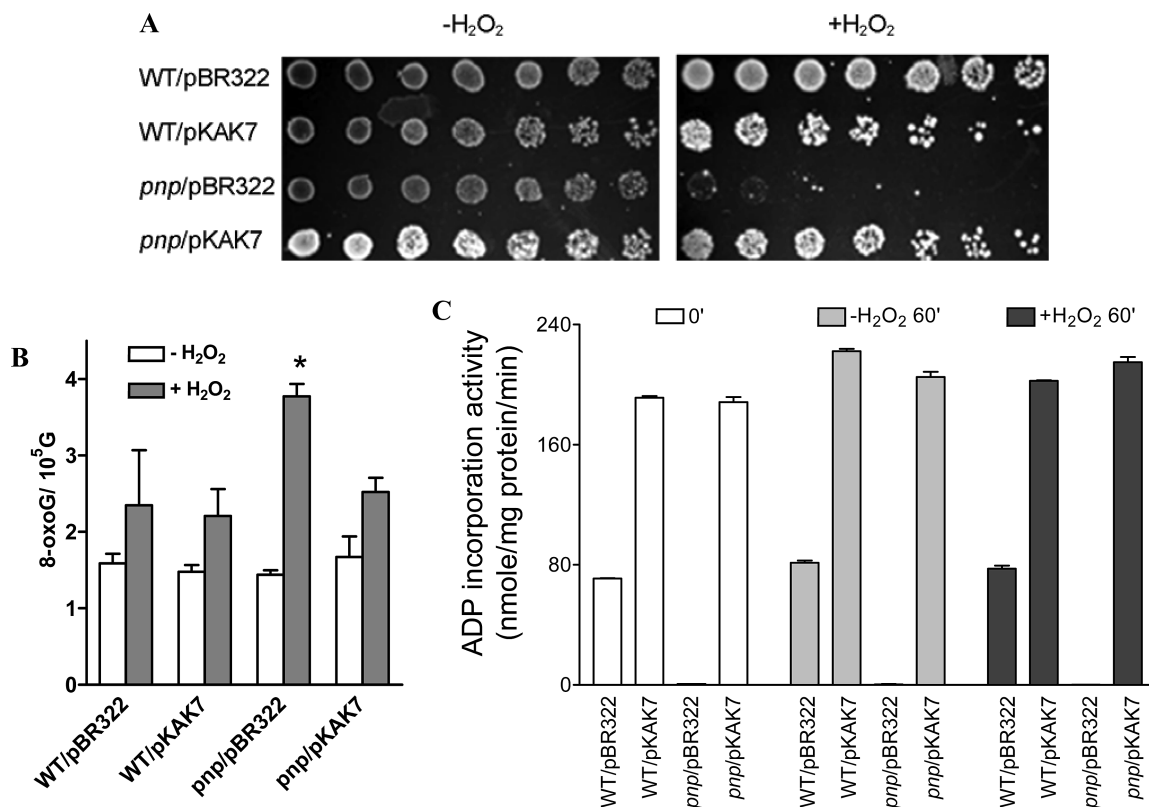


FIGURE 6: Introduction of *pnp* gene rescue response to H_2O_2 insult. (A) pKAK7 harboring the *pnp* gene (23) and the control plasmid pBR322 were introduced into the wild-type and *pnp* mutant cells, respectively. Midlog cultures containing 50 μ g/mL ampicillin were serially diluted and inoculated on YT agar plates with or without 0.5 mM H_2O_2 to determine H_2O_2 sensitivity. (B) Total RNA was isolated 15 min after treatment with or without 0.5 mM H_2O_2 , and the level of 8-oxoG in RNA was determined by HPLC. The mean of duplicate experiments and standard errors are shown. (C) Cultures at $OD_{550nm} = 0.5$ (0 min) were treated for 1 h with or without 1 mM H_2O_2 . PNPase activity was measured by ADP incorporation into RNA using 6 μ g of protein in each 50 μ L reaction. The averages of two measurements and standard errors are shown.

pnp cells in the presence of H_2O_2 to wild-type level, but pBR322 did not (Figure 6A). Similarly, pKAK7, but not pBR322, reduced the level of 8-oxoG in *pnp* cells following H_2O_2 treatment to wild-type level (Figure 6B). These results show that PNPase is necessary *in vivo* for reduction of lesions from RNA and for cell survival under

oxidative challenge. Unexpectedly, wild type/pKAK7 gave slightly fewer colonies than wild type/pBR322 on plates with or without H_2O_2 although all cultures were inoculated at the same OD_{550nm} . We do not know the reason for this behavior but do not expect this small difference to complicate the assay. As mentioned above, PNPase in cell

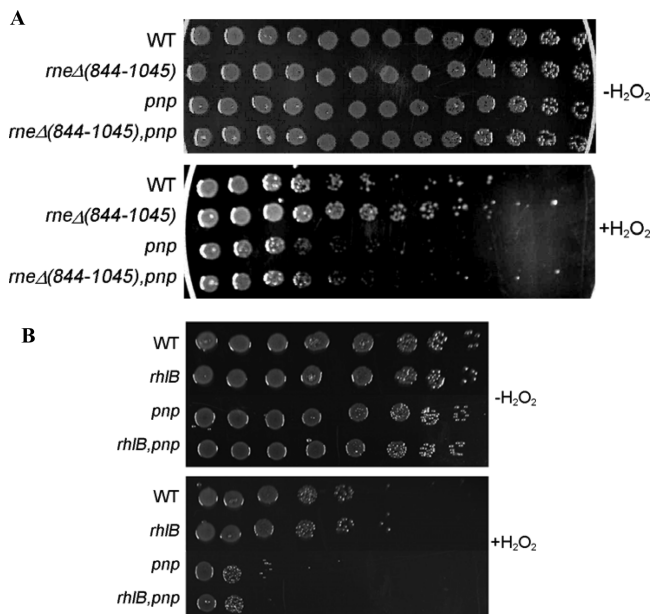


FIGURE 7: Responses of strains defective in PNPase-associated proteins to H_2O_2 treatment. (A) Sensitivity of wild-type, *rneΔ(844–1045)*, and their respective *pnp* derivative cells to H_2O_2 . Exponentially grown cultures were serially diluted 1:3 and inoculated to YT plates with or without 0.5 mM H_2O_2 . (B) Sensitivity of wild-type, *rhlB*, and their respective *pnp* derivative cells to H_2O_2 . Exponentially grown cultures were serially diluted by 1:5 and inoculated to YT plates with or without 0.5 mM H_2O_2 .

extracts and purified PNPase bind oxidized RNA, indicating that PNPase is also sufficient for recognizing damaged RNA. Taken together, these data suggest that PNPase acts directly to protect cells from oxidative insult.

Association with the Degradosome or with RhlB Is Not Required for PNPase To Protect Cells under Oxidative Stress. Because a large portion of PNPase exists in the RNA degradosome, we tested whether association of PNPase with the degradosome is important for its protective role under oxidative stress. We made use of a mutant form of RNase E, *RneΔ(844–1045)*, which lacks the PNPase binding domain, amino acids 844–1045 (28), but interacts with other members of the degradosome, RhlB, and enolase. In the absence of oxidative challenge, cells substituting wild-type RNase E with *RneΔ(844–1045)* grow relatively normally (28) (Figure 7A).

If association with degradosomes is required for PNPase to protect cells against oxidative stress, then cells expressing *RneΔ(844–1045)* should also be hypersensitive to H_2O_2 . This was not the case (Figure 7A). In fact, the *rneΔ(844–1045)* strain grew significantly better than wild type in the presence of H_2O_2 . We do not understand the increased resistance of these cells to oxidative challenge; however, cell lysate made from the *rneΔ(844–1045)* culture displayed PNPase activity nearly 40% higher than the wild-type extract. The *rneΔ(844–1045),pnp* double mutant shows H_2O_2 sensitivity similar to that of the *pnp* single mutant. These results suggest that PNPase is ultimately responsible for protecting cells from oxidative stress in our assay and that it does so independently of degradosomes.

It has also been suggested that PNPase may interact with RNA helicase B (RhlB) independent of the degradosome (16). We therefore examined the response of *E. coli* mutants

lacking RhlB to H_2O_2 insult. The results parallel those with the degradosome assembly mutant. In the presence of H_2O_2 , *rhlB* cells grow similarly to wild type, and *rhlB,pnp* double mutant cells grow similarly to *pnp* single mutant cell (Figure 7B). Therefore, RhlB and its potential interaction with PNPase do not affect cell viability under oxidative stress, revealing that the mechanism of PNPase protection does not require RhlB, either alone or associated with degradosomes.

DISCUSSION

In this work, we have shown that polynucleotide phosphorylase plays an important role in protecting *E. coli* cells under oxidative stress. Cells lacking PNPase are hypersensitive to H_2O_2 and PMS and contain more 8-oxoG in RNA under oxidative challenge. The viability and RNA oxidation of the *pnp* mutant cells are restored by introduction of a plasmid-borne *pnp* gene. Interestingly, this protective role of PNPase is independent of its association with the RNA degradosome or with RhlB RNA helicase.

Our results seem to contradict the previous observation that *pnp* mutant cells are much more resistant to oxidation by paraquat than wild-type cells. Paraquat has been widely used as an oxidant to treat eukaryotic cells or tissues, and when used in *E. coli* cultures, paraquat generates ROS exclusively outside the cell, presumably causing primary oxidative damage to periplasmic components (36). The apparent discrepancy prompted us to ask if paraquat causes oxidative damage to *E. coli* cytoplasmic components, particularly RNA. We have shown that the level of the oxidative lesions in RNA and DNA do not increase upon treatment with paraquat (Figure 2B,C). In contrast, treatment with H_2O_2 induces a substantial increase in RNA 8-oxoG (Figures 4A and 5B). Therefore, unlike H_2O_2 , paraquat may kill *E. coli* cells by a mechanism unrelated to RNA oxidation. The hyperresistance of *pnp* mutant cells to paraquat is likely caused by an indirect effect on some other process, such as altered expression of outer membrane proteins (21).

E. coli PNPase was previously shown to bind poly(8-oxoG·A) RNA with higher affinity than poly(U·A) (2). We have shown here that PNPase also preferentially binds to an RNA of natural sequence that is oxidized by H_2O_2 treatment. Such oxidation introduces various types of damage to RNA including the formation of 8-oxoG (9). It will be interesting to know in the future if the high affinity of PNPase to damaged RNA is solely due to its interaction with the 8-oxoG residues or if it also involves other damaged nucleotide residues. There are two conserved RNA-binding domains (RBDs), the KH and S1 motives, located in the C-terminal region of PNPase protein. Removal of one or both RBDs greatly reduces the binding and moderately affects the 3' → 5' degradation of an RNA substrate *in vitro* (37). Intriguingly, the oxidized RNA substrate used in our experiment is covalently cross-linked to agarose beads at the 3' end. Consequently, the preferential binding of PNPase to the oxidized RNA must occur independent of a free 3' end. It remains to be elucidated if the binding involves the conserved RBDs or other unidentified domains of PNPase.

The association of PNPase with the RNA degradosome or RhlB helicase is not required for the protective effect of PNPase under oxidative stress. This is not surprising since the degradosome or RhlB may only play a limited role in the degradation

of certain mRNA species or structures (18, 38). In fact, the strain carrying the truncated RNase E lacking the PNPase binding site is hyperresistant to H₂O₂ treatment (Figure 7A) and has slightly higher PNPase activity *in vitro*. Consistent with this observation, an *E. coli* mutant containing a transposon insertion blocking expression of the C-terminal half of RNase E was also shown to be hyperresistant to oxidative stress generated by introduction of the human Bax protein (39). The truncated RNase E proteins likely confer antioxidant activities through modulating the activity of PNPase. Consistent with this explanation, the hyperresistance of the *rne*Δ(844–1045) strain to H₂O₂ is entirely lost upon deletion of *pnp* (Figure 7A).

PNPase specifically binds oxidized RNA *in vitro* and is required for the reduction of oxidized RNA *in vivo* and cell survival under oxidative stress. The most straightforward explanation of these data is the specific removal of oxidized RNA by PNPase. However, it is difficult to establish such a role because PNPase has been reported to bind, but not degrade, oligoribonucleotides made of 8-oxoG *in vitro* (2). It is not yet known whether this is a peculiar feature of this unnatural RNA. It remains to be elucidated whether PNPase preferentially binds and degrades naturally occurring RNA molecules with oxidative damages *in vivo*, either alone or facilitated by other factors. Until these data become available, we cannot exclude an alternative model suggested by our data, that PNPase acts as a sentinel protein, monitoring RNA molecules for oxidative damage and preventing them from functioning, and recruiting additional ribonucleases to destroy damaged transcripts. In this case, PNPase would need to interact with novel partners apart from RhlB or the degradosome. These interesting points warrant further studies in the future.

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