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Regulation and functions of bacterial PNPase

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ABSTRACT

Polynucleotide phosphorylase (PNPase) is an exoribonuclease that catalyzes the processive phosphorolytic degradation of RNA from the 3'-end and the reverse reaction of polymerization of nucleoside diphosphates that generates heteropolymeric tails at the RNA 3'-end. The enzyme is widely conserved and plays a major role in RNA decay in both Gram negative and Gram positive bacteria. Moreover, it participates in maturation and quality control of stable RNA. PNPase autoregulates its own expression at post-transcriptional level through a complex mechanism that involves the endoribonuclease RNase III and translation control. The activity of PNPase is modulated in an intricate and still unclear manner by interactions with small molecules and recruitment in different multiprotein complexes. Not surprisingly, given the wide spectrum of PNPase substrates, PNPase-defective mutations in different bacterial species have pleiotropic effects and perturb the execution of genetic programs involving drastic changes in global gene expression such as biofilm formation, growth at suboptimal temperatures, and virulence. Moreover, PNPase reversibly catalyzes phosphorolysis of ssDNA and is involved in DNA repair and recombination in distantly related bacteria such as *Escherichia coli* and *Bacillus subtilis*; it thus appears that this enzyme stands at the crossroad of RNA and DNA worlds.

In 1959 Severo Ochoa and Arthur Kornberg were awarded the Nobel Prize "*for their discovery of the mechanisms in the biological synthesis of ribonucleic acid and deoxyribonucleic acids*", respectively. As for Ochoa, the award referred to his studies on the *Azotobacter vinelandii* polynucleotide phosphorylase (PNPase, polyribonucleotide nucleotidyltransferase, EC 2.7.7.8), which reversibly catalyzes the 3-'to-5' phosphorolysis of polyribonucleotides, releasing nucleoside diphosphates (NDPs), and the reverse template

independent 5'-to-3' polymerization of ribonucleoside diphosphates, releasing inorganic phosphate (Pi),[1] as described by the equation

$$RNAn+Pi \leftrightharpoons RNAn-1+NDP$$

The reversibility of this reaction leads to the exchange between inorganic phosphate and the β-phosphate of NDPs, a reaction exploited as a sensitive PNPase assay.[2, 3] PNPase was the first enzyme to be found that was "*capable of catalyzing the synthesis of RNA… in the test tube from simple, naturally occurring precursors*".[4] Soon after, with the discovery of the DNA-dependent RNA polymerase, it became apparent that the template-independent RNA polymerizing activity of PNPase could not be implicated in transcription (Ref.[5] and references therein) and the reverse reaction, *i.e.* RNA degradation, was then assumed as the main *in vivo* activity of this enzyme.[6] More recently, however, it was shown that PNPase can add heteropolymeric tails to RNA 3'-ends (reviewed in Ref.[7]). The biological role of PNPase was far from obvious and six decades of biochemical, genetic and molecular studies delineated a complex scenario leaving several unsolved issues. PNPase is widely conserved in *Bacteria* and in eukaryotic organelles of bacterial origin (*i.e.* chloroplasts and mitochondria) with the exception of some unicellular organisms, whereas it is absent from *Archaea*.[8] This rather abundant enzyme (PNPase is in the top 5% of the most expressed proteins in *E. coli* and other bacteria[9]) is finely regulated at the level of its activity by diverse cellular factors and autogenously regulated at post-transcriptional level; PNPase may be associated in macromolecular complexes of diverse composition dedicated to RNA degradation, such as the RNA degradosomes, but also to ribosomes; although in most species analyzed PNPase is not an essential protein, it has been implicated in the control of a variety of complex phenotypes such as response to cold shock, oxidative and other environmental stresses, biofilm formation and virulence. Besides the key role of PNPase in modulating the abundance of a number of mRNAs and small RNAs (sRNAs), and thus the expression of

many genes, other features have emerged that link this enzyme to DNA metabolism. RNA phosphorolysis generates CDP, a precursors of both CTP and dCTP;[10] PNPase deficient *E. coli* mutants are more sensitive to UV[11] and exhibit a lower frequency of mutation;[12] remarkably, it was found that PNPase can catalyze template independent synthesis of DNA from dNDPs and DNA phosphorolysis.[13-19] While initially the latter observation had only practical consequences (PNPase was exploited in the early era of molecular biology for the synthesis of oligoribonucleotides and oligodeoxyribonucleotides), recent data implicate PNPase in DNA recombination, repair, and mutagenesis.[11, 12, 17, 18]

Finally, to further complicate this intricate framework, it is becoming clear that PNPase evolved biochemical peculiarities and different functional properties in different organisms. Thus this review will not aim a full coverage of this pleiotropic and still elusive enzyme; rather it will be focused on the regulation of bacterial PNPase expression and activity, attempting to trace common trends emerging from what, in many cases, are still scattered observations. The most studied *E. coli* PNPase (*Ec*PNPase) will be taken as the reference, whereas other bacterial PNPase will be considered to highlight either conserved features or peculiarities of different orthologues.

PNPase CATALYTIC ACTIVITY AND STRUCTURE

Characterization of enzymatic properties of PNPase from several organisms has been the subject of intense study in the first twenty years after its discovery. The complicated overall picture that emerged has been reviewed by Littauer and Grunberg-Manago.[1] For our purposes it will be sufficient to remind that PNPase catalysis with ribo-substrates requires Mg^{2+} and is inhibited by high Mn^{2+} concentration.[4, 20] On the contrary, polymerization of dNDPs and DNA phosphorolysis require Mn^{2+} (or other ions such as Fe^{2+}) and are inhibited by Mg^{2+}.[14-18]

Moreover, a large variety of nucleoside diphosphates may serve as substrates for the polymerizing reaction (see for instance Refs.[21,22]).

In vivo, the enzyme is an important component of the bacterial RNA decay machinery[23] and promotes the processive degradation of RNA molecules endowed with 10-12 nt long single stranded 3'-ends. PNPase is stopped by stable secondary structures;[24, 25] however, PNPase promotes also the reverse RNA polymerization reaction, thereby providing the RNA 3'-end with heteropolymeric tails.[7] In *Bacteria*, both heteropolymeric- and poly(A) tails, which in *E. coli* are added to the mRNA 3'-end by polyadenylpolymerase (PAPI) have an RNA destabilizing effect, as they function as toeholds for the recruitment of the exoribonucleases involved in RNA decay, which do not bind double stranded RNAs.[7, 26] It has been proposed that, while degrading a single RNA molecule, PNPase could go through cycles of polymerization and phosphorolysis triggered by changes in the local concentration of Pi and NDPs that could be instrumental in attacking structured regions.[27] Whereas in *E. coli* both PNPase and PAPI can elongate the RNA 3'-ends,[27-29] *Streptomyces* spp. lacks PAPI and thus RNA-tailing relies on PNPase.[30, 31]

PNPase is a homotrimer (Fig. 1A); the 711 aa-long protomer, encoded in *E. coli* by the *pnp* gene, is constituted by two RNase PH-like domains (PH1 and PH2; Fig. 1B), supposedly the result of an ancestral duplication event, connected by an all α-helical region (AAHD) and followed at the C-terminus by the KH and S1 RNA binding domains.[32-34] Although the molecular mechanism of PNPase catalysis is still unclear, mutagenesis and structural data locate the active site in the PH2 region; however, mutations in both PH1 and AAHD domains affect the enzymatic activity, suggesting that also these domains contribute to the overall architecture of the active site.[32, 35-37] The crystal structures of PNPase from different bacteria and of the *Ec*PNPase-RNA complex have been solved (Fig. 1A).[32, 34, 38, 39] In the doughnut-shaped enzyme, the RNA binding domains are superimposed on the ring made by the six

RNase PH-like domains. The RNA is fed in the central hole by the S1 and KH RNA binding domains, which contribute to the enzyme processivity.[34, 40, 41] Deletion of the S1 and KH domains severely impairs, but does not completely abolish RNA binding, which still occurs at the active site.[35, 41, 42] Moreover, although altered in the kinetic behaviour, the mutant PNPase is still enzymatically active, suggesting that the mutation does not compromise the enzyme catalytic site.[35, 41] Interestingly, deletion of the RNA binding domains of *Mycobacterium smegmatis* PNPase, while affecting RNA phosphorolysis and polymerization, enhances DNA synthesis and degradation by PNPase, suggesting that the S1 and KH domains may also contribute to increase PNPase specificity for RNA *vs*. DNA.[19]

Box 1. A common architecture for RNase PH, PNPase and the exosomes

The archaeal and eukaryotic exosomes are multiprotein complexes that processively degrade RNA from the 3'-end. They share with the bacterial phosphorolytic exoribonucleases-nucleotidyltransferases PNPase and RNase PH (a prokaryotic enzyme composed of three homodimers and mainly implicated in tRNA processing) a common architecture based on six RNase PH-like domains forming a pseudo-hexameric ring (Fig. 1A) (reviewed by Ref.[43]). The hexameric configuration of the archaeal exosomes is obtained by the assembly of three Rrp41-Rrp42 heterodimers. Since only Rrp41 is catalytically active, the complex has three active sites and, like PNPase, is capped by S1 and KH RNA binding domains that are carried by three copies of the Rrp4 or Csl4 subunits. The eukaryotic exosome is more complex. Its hexameric ring is formed by three catalytically inert RNase PH-like heterodimers (i.e. Rrp41-Rrp45, Rrp43-Rrp46 and Mtr3-Rrp42) assembled with three additional subunits on the top of the ring, which provide the RNA binding domains and stabilize the ring. The catalytic activity is conferred by a tenth subunit, either Rrp44 or Rrp6, evolutionarily related to RNase II/R and RNase D, respectively. Thus the eukaryotic exosome, despite its structural

relatedness with phosphorolytic exoribonucleases and the archaeal exosome, is a hydrolytic RNA-degrading machine. Evolutionarily, it is remarkable that the ancestral RNase PH imprinted the structure of several enzymes and complexes implicated in the RNA metabolism notwithstanding the loss of its enzymatic activity.

PNPase IS A COMPONENT OF HETEROGENOUS MULTIPROTEIN COMPLEXES

PNPase participates in the assembly of multiprotein complexes with variable composition. Early studies discovered that, besides the enzyme A form, which corresponds to the homotrimer, a B form of PNPase was present in crude extract. This heavier form derived from the association of the homotrimer with a dimer of a 48 kDa subunit that was lately identified as the RNA helicase RhlB, which confers to PNPase the ability of degrading structured RNAs.[44, 45] This strongly widens the spectrum of RNAs that can be degraded by PNPase to oligoribonucleotides and in fact the interaction with a helicase represents a conserved feature that bacterial PNPases share not only with their organelle counterparts, but even with the eukaryotic and archaeal exosomes.[8]

The propensity of factors involved in RNA decay to form composite machines, in which different enzymatic activities can be coordinated, has been further substantiated in bacteria by the discovery of the *E. coli* RNA degradosome, a high molecular weight complex assembled on the C-terminal region of the endonuclease RNase E that includes PNPase, RhlB and the metabolic enzyme enolase.[46] The definition of "RNA degradosome", however, has been subsequently stretched in order to include a variety of multiprotein complexes involved in RNA decay identified in other bacteria. For instance, in the Gram positive *Bacillus subtilis* and *Staphylococcus aureus* RNA degradosomes, RNase E, RhlB, and enolase are replaced by the endonuclease RNase Y, the helicase CshA, and the metabolic enzyme phosphofructokinase as partners of PNPase.[47] Even in a single bacterial specie as *E. coli,* the

composition of the RNA degradosome is highly dynamic and different components, only some of which with an established role in RNA metabolism, are present in substoichiometric amount in degradosome preparations. Moreover, RhlB is replaced by another helicase, CsdA, upon cold shock, a condition that leads to the stabilization of RNA secondary structures.[48] This suggests that the transient association of different factors may confer peculiar properties to the RNA degradosome that can be instrumental in responding to specific environmental stimuli (Ref.[47] and references therein).

The assembly of PNPase in multiprotein complexes impacts not only on its physiological role, but also on its spatial localization within the cell. In fact, there is evidence that PNPase, as other components of the RNA degradation apparatus, is not evenly distributed in the cell cytoplasm and that its localization may depend on the association to other factors, in particular RNase E (reviewed by Ref.[49]). Indeed, the assembly in the RNA degradosome, which has been reported to interact with translating ribosomes, accounts for the co-sedimentation of part of PNPase with the ribosomal fraction, as the co-sedimentation is lost in an RNase E mutant lacking the degradosome scaffold.[50-52] Moreover, a recent survey of *E. coli* peripherome has detected PNPase and other components of the degradosome among proteins located at the inner face of the cell membrane, in agreement with the documented association of RNase E with the cytoplasmic membrane.[47, 53]

Interestingly, the uneven distribution of PNPase-containing RNA degradation machineries is maintained also in other bacteria, such as *Caulobacter crescentus*, where the RNA degradosome is located in proximity of the nucleoid, and *B. subtilis*, whose RNase Y-based degradosome is membrane-associated.[47] The functional consequences of the spatial organization of the RNA decay and the mechanisms of its compartmentalization in the bacterial cells still need to be ascertained.

REGULATION OF PNPase EXPRESSION AND ACTIVITY

The *E. coli pnp* gene control: an example of post-transcriptional regulation

The *pnp* gene is located immediately downstream of the *rpsO* gene,[54] which encodes the ribosomal protein S15. Regulation of the *rpsO-pnp* locus mainly occurs at post-transcriptional level, through modulation of mRNA stability and translatability and the study of regulatory mechanisms acting at this locus has greatly contributed to the development of current models of mRNA stability control in Gram-negative bacteria. Control of *rpsO* expression[55] (Ref. [56] and references therein) is largely independent from the autogenous regulation of *pnp* and will not be hereby reviewed.

Autogenous regulation of pnp in E. coli

The *pnp* gene can be transcribed from two promoters, namely P1, located upstream of *rpsO*, and P2, mapping in the intergenic *rpsO-pnp* region.[57] The post-transcriptional regulation steps of PNPase expression are outlined in Fig. 2. Transcripts from both P1 and P2 are quickly and efficiently processed by the endonuclease RNase III, which makes a staggered cut in a long stem-loop located at the 5'-end of the *pnp* 5'-untranslated region (5'-UTR) (step a), thus generating a new 5'-end of the *pnp* mRNA (step b) 80 nt upstream of the *pnp* UUG start codon. (Ref.[58]; our unpublished data). In RNase III-deficient (*rnc*) mutants, the non-processed *pnp* mRNA is more stable than in *rnc*$^+$ strains and PNPase expression increases.[59, 60] However, PNPase acts as a translational repressor on the primary (RNase III-unprocessed) *pnp* mRNAs,[61] thus exerting a first level of autoregulation. Interestingly, this RNase III-independent (RTI) regulatory pathway requires PNPase RNA binding, but not its catalytic activity.[61] It has been proposed that PNPase represses translation by binding the primary transcript at an unmapped site downstream of the 5'-stem-loop, in proximity of the translation initiation region (steps a, b).

Once cleaved by RNase III, the *pnp* mRNA enters an RNase E-dependent degradative pathway promoted by PNPase (steps b-e). This second autoregulatory mechanism requires both catalytic and RNA binding activities of PNPase.[35, 37, 41, 62] According to the elegant model proposed by Claude Portier,[63] after RNase III cleavage, the 5'-monophosphate end of the processed mRNA remains annealed with a small RNA (sRNA) corresponding to the first 37 nt of the transcripts from P2 (RNA37; step b). This double-stranded structure would protect the mRNA from RNase E.[64] Then PNPase, with the dispensable assistance of PAPI,[63, 65] digests the short RNA, thus releasing the processed *pnp* mRNA, which is terminally degraded through a PNPase-independent pathway triggered by a first cut by RNase E within the 8th codon of the *pnp* open reading frame (ORF; step c).[66] Thus PNPase targets its own messenger to a decay pathway by degrading a sRNA, generated by RNase III cleavage, that protects it from RNase E.

A former model for *pnp* autoregulation proposed by the same author postulated that PNPase could act as a translational repressor of the processed *pnp* mRNA by binding to a specific region in the 5'-UTR and targeting the untranslated mRNA to degradation by other nucleases.[67] Experimental evidence suggests that translation efficiency may indeed play a role in *pnp* autoregulation mechanism. In fact, while the primary transcript and the processed mRNA paired with the RNA37 are translatable, the unpaired *pnp* mRNA seems to be poorly, if at all, translated both in *vivo* and in *vitro*.[61, 63, 65] The molecular basis of the untranslatability of this mRNA is not known. RNA secondary structure prediction suggests that, in the absence of the interacting RNA37, the mRNA 5'-end could pair with codons 3-7 of the ORF (step d). A hypothesis that awaits experimental testing is that this structure may inhibit translation. This implies that by degrading RNA37, PNPase would promote the transition of the 5'-UTR from a translation-competent to an inhibitory secondary structure that, in turn, would destabilize the *pnp* untranslated transcript. This could explain the derepressed phenotype

exhibited by a mutant with a base substitution in the *pnp* 4th codon (GFX5311 mutant in Ref.[67]) and would reconcile the current view of PNPase autoregulation[63] with the former model[67] by postulating that PNPase could indirectly act as a translational repressor also on the processed *pnp* mRNA by degrading a translation activator (the RNA37 sRNA). Thus PNPase autoregulates its own expression by several mechanisms. Whereas after the RNase III cleavage PNPase irreversibly targets the *pnp* mRNA to degradation, the upstream RNase III-independent pathway would represent a reversible step in PNPase autoregulation. What is the relative impact of the two autoregulation steps on PNPase expression and whether it may change in different physiological conditions are open questions. However, as in *rnc*$^{+}$ strains the primary *pnp* transcript is very quickly processed by RNase III, it is plausible that the RNase III-dependent pathway plays the major role in PNPase expression regulation.

Interestingly, autoregulation of *pnp* expression involving RNase III-dependent processing of the *pnp* transcript is a conserved mechanism not only in other Gram negative bacteria such as *Photorabdus luminescens*, *Pseudomonas putida* and *Yersinia enterocolitica*,[68-70] but also in the soil Gram positive bacterium *Streptomyces coelicolor*.[71, 72] RNase III controls the expression of both stable RNAs and a large number of mRNAs[73, 74] and its activity is affected by a variety of stimuli, including environmental stresses (recently reviewed in Ref. [75]). We speculate that the inverse relationship between RNase III activity and PNPase expression might partially compensate RNase III activity variations under certain conditions and provide alternative PNPase-dependent decay pathways to a subset of its target RNAs. The evolutionarily conserved RNase III-dependent regulation of PNPase highlights the need for a coordinate expression of the players of seemingly redundant degradation pathways.

Cold shock relieves pnp autoregulation

Cold (i.e. a temperature below 20°C cell for mesophilic bacteria) is a physical stress that influences enzyme kinetics, membrane fluidity and affects conformation, topology and reciprocal interactions of macromolecules. In *E. coli*, during the cold acclimation phase the rate of synthesis of several proteins (cold shock proteins) transiently increases, whereas the synthesis of most other gene products appears to be repressed. Post-transcriptional mechanisms, notably control of mRNA stability and translatability, play a major role in the adaptive response to cold temperature. In particular, mRNA stabilization has been identified as the major factor responsible for the dramatic increase of several cold shock transcripts.[76] PNPase belongs to the cold shock proteins and its expression transiently increases about twofold in the acclimation phase. However, contrarily to mRNAs of other cold shock genes, *pnp* transcripts do not seem to be efficiently translated. In fact, in contrast with the two-fold increase of PNPase levels, the cumulative expression of the monocistronic and polycistronic *pnp* mRNAs, which trespass *pnp* terminator t_{pnp}, rises about 10-fold in the first two hours after the shift to low temperature.[77] This enhancement is due to transient suppression of both autoregulation and transcription termination at intrinsic terminators.[77-81] Whereas abrogation of transcription termination is not specific for the *pnp* locus,[81] reversal of autoregulation should occur through a specific mechanism as PNPase appears to be active upon cold shock.[79, 82, 83] Although the enzyme is still able to degrade in *vitro* the RNA37 annealed to the *pnp* mRNA at 20 °C,[63] it is possible that in *vivo* the augmented stability of the RNA duplex may protect the paired RNA37. In agreement with this hypothesis, we observed increased abundance of RNA37 in the acclimation phase (unpublished data). However, differential stability of the RNA duplex at different temperatures cannot be the only operating mechanism, as autogenous regulation occurs at low temperature in acclimated cells. It is possible that upon cold shock, the general impairment of translation may transiently increase

the pool of ribosomal subunits available for binding, and thus stabilizing, the relatively small number of translation-competent mRNAs, among which the *pnp* mRNA. Accordingly, at the end of acclimation phase, when general translation resumes, also *pnp* autoregulation is restored.[77]

PNPase induction in the cold has been reported also for *S. coelicolor* and the psychrotrophic bacteria *P. luminescens* and *Y. enterocolitica.* However, in such systems, a different mechanism based on the activation of specific promoters at low temperature is involved.[68, 69, 72] Conversely, in *P. putida,* the *pnp* mRNA level strongly decreases at 9 °C, but the messenger appears to be translatable since the amount of PNPase remains constant in cells growing in the cold.[70]

PNPase activity is modulated by cellular effector molecules

Another layer of PNPase regulation is modulation of its catalytic activity by several low molecular weight effectors, which connect PNPase enzymatic activity to the cell metabolic state. However, a caveat is needed because for some effectors, the actual *in vivo* physiological relevance of the *in vitro* interaction is unsettled.

This is not the case of the Krebs cycle metabolite citrate, whose effect on PNPase has been examined *in vitro* and *in vivo*. In particular, magnesium-chelated citrate inhibits PNPase by binding, and conceivably occluding, its catalytic site, whereas the metal-free citrate acts as an allosteric activator of PNPase by interacting with a vestigial active site of the enzyme. *In vivo*, a PNPase-dependent strain, defective for both RNase II and RNase R, grows slowly on magnesium-citrate; on the other hand, PNPase activity impacts on the cell metabolome.[84] While this interaction establishes a link between PNPase and the central metabolism, the finding that *in vitro Ec*PNPase is allosterically inhibited by ATP suggests that PNPase activity responds to the cell energy state.[85, 86] In particular, PNPase should be maximally

active *in vivo* when the energy charge is low, such as upon transition from exponential to stationary phase and in late stationary phase.[87, 88] Albeit still awaiting an experimental demonstration, this seems a sensible assumption considering that RNA phosphorolysis and tailing by PNPase, by generating and consuming NDPs, respectively, are more energy-saving processes than RNA hydrolysis by ribonucleases, which produce NMPs, and polyadenylation by PAPI, which consumes ATP.[85, 89] Accordingly, the alarmone ppGpp, whose concentration peaks at the entry in stationary phase,[87] stimulates in *vitro* the catalytic activity of *Ec*PNPase.[86] Conversely, the same effector acts as an inhibitor for the PNPase orthologues of the Gram-positive *Nonomuraea,* which is not affected by ATP,[86] and *Streptomyces*. This observation has been rationalized in the light of the rich secondary metabolism of such actinomycetes, which lack RNase II and R and for which shutting off PNPase in stationary phase may represent a strategy to counterbalance the decrease in transcription efficiency by preserving RNA from degradation.[86, 90]

All the above effectors have been found to interact with purified PNPase, whereas another modified nucleotide, namely cyclic di-GMP (c-di-GMP), may modulate *Ec*PNPase when the enzyme is part of a specialized RNA degradosome containing, besides RNase E and enolase, the termination factor Rho, the diguanylate cyclase DosC and the phospohodiesterase DosP. Thanks to the interaction with c-di-GMP synthesized by DosC in the presence of oxygen, the activity of PNPase in the complex would be inhibited in aerobiosis.[91] However, further analyses are required to strengthen this model. In fact, the actual concentration of c-di-GMP associated with the complex at different [O_2] was not determined, an aspect that appears to be relevant since the c-di-GMP degradative activity of DosP is also activated by oxygen. Moreover, c-di-GMP neither activates nor inhibits purified PNPase.[91, 92]

ROLE OF PNPase IN RNA DECAY AND QUALITY CONTROL

RNA degradation relies on the concerted actions of endoribonucleases, which cut RNA at internal sites and in most cases catalyze the first step in RNA decay, and exoribonucleases, which processively degrade from one end the RNA fragments generated by the endoribonucleases. Accessory functions, mainly RNA helicases and enzymes that modify the RNA ends to either facilitate or prevent the access of exoribonucleases to the substrate, are also involved (recently reviewed by ref.[93)]. Besides oligoribonuclease, which degrades RNA fragments 2-5 nt long, the principal *E. coli* RNA exonucleolytic activities responsible of RNA decay are PNPase and the hydrolytic ribonucleases RNase II and RNase R (encoded by *rnb* and *rnr* genes, respectively), which, like PNPase, degrade the RNA from the 3'- to the 5'-end without sequence specificity.[94] Only oligoribonuclease is essential[95]; however, while the cells can tolerate the concomitant lack of RNase II and R, deletion of *pnp* gene is lethal in the absence of either RNases.[24, 96] This suggests that PNPase can substitute for RNase II and R specific functions that are not shared between the two hydrolytic RNases. Indeed, like RNase R and unlike RNase II, in complex with RhlB or the RNA degradosome PNPase can degrade structured RNA.[97, 98] On the other hand, RNase R, which in the exponential phase is mostly associated with ribosomes, could be targeted to specific decay pathways[99] thus being not suitable to sustain the entire burden of bulk RNA decay.

Control of mRNA decay by PNPase

Decay pathways have been analyzed only for a handful of model mRNAs, which have been taken as paradigmatic examples, and mainly in *E. coli.*[100] Whereas this has allowed to reconstruct some decay routes that specifically involve different exoribonucleases, the sample of model RNAs is too small to draw general conclusions on the relative impact on gene expression regulation exerted *in vivo* by each exoribonuclease. Early estimates suggested that degradation of unstable RNA mainly occurs through hydrolysis in *E. coli*[101] and that RNase II

plays quantitatively the most relevant role in RNA degradation in crude extracts.[89] While it seems sound that this abundant enzyme may have a relevant role in bulk RNA degradation, this does not necessarily implies a major role in mRNA decay. Indeed, spacer regions left by maturation of the rRNA and tRNA primary transcripts or the rRNA itself represent in fast and slowly growing cells, respectively, the most abundant substrates for the degradative machinery.[102] As a matter of fact, microarray analysis of the transcriptome of *E. coli* mutants lacking either PNPase or RNase II showed that much more mRNAs (17.3% *vs* 7.4%) were overexpressed in the absence of PNPase than RNase II. Conversely, in the RNase II mutant, one third of cellular mRNAs resulted more unstable, suggesting that RNase II may indeed have a protective role on many mRNAs as already shown for the *rpsO* mRNA.[103, 104] In agreement with these results, a recent RNA-Seq global mRNA profiling of *E. coli* mutants lacking either PNPase, RNase II or RNase R has shown that the three enzymes control the expression of a comparable number of transcripts (about 200 transcripts each). However, most RNAs were actually destabilized by the absence of RNase II or RNase R (66.9 and 54.5 %, respectively), whereas the lack of PNPase caused an increase in the level of the majority (the 59 %) of its target RNAs.[105] On the whole, these data confer a prominent role to PNPase in mRNA degradation.

rRNA and tRNA as PNPase targets

In *E. coli* PNPase has been implicated in the processing of 16S rRNA 3'-end[106] and in the maturation and 3'-end repair of tRNAs[107-110], two processes characterized by high redundancy. For instance, besides PNPase, the RNases T, PH, D, II and BN are also implicated in tRNA 3'-end processing; however, in single mutants lacking any of these enzymes, tRNA precursors do not accumulate.[111] This suggests that several alternative pathways recruiting any of these enzymes can indifferently perform this process. Moreover,

*Ec*PNPase is involved in quality control pathways that eliminate RNA molecules deriving from the incorrect maturation of 16S and 23S rRNAs. Also in this case, another exoribonuclease, RNase R, can fully replace PNPase.[112] The accumulation of RNA fragments interfering with correct ribosome maturation has been causally related to the loss of viability of *rnr pnp* double mutants, which lacks both enzymes.[112] PNPase is also implicated in quality control systems that eliminate defective tRNA precursors and in tRNA degradation.[105, 113]

Less clear is whether in *E. coli* PNPase participates in rRNA degradation that occurs upon carbon starvation[114, 115] or at the entry in stationary phase.[116] On the contrary, in *Deinococcus radiodurans*, PNPase is converted into a degradation machine specific for structured RNA, named RYPER, by its recruitment in a ribonucleic complex that comprises the Ro orthologue Rsr and the small noncoding Y RNA. Ro protein and Y RNAs, together with La protein, form the core of Ro ribonucleoprotein (RNP) complexes, originally identified in humans as major targets of autoantibodies, whose still controversial functions in animal cells include stabilization of newly synthesized RNA polymerase III transcripts.[117] In *D. radiodurans*, RYPER is responsible for extensive rRNA decay in stationary phase.[118, 119] A RYPER-like complex has been purified also from *Salmonella enterica* and putative Y RNAs have been identified in > 250 bacteria and phages encoding a Ro orthologue, which have thus the potential to assemble RYPER-like complexes.[119, 120] These findings open the interesting possibility that PNPase, by the assembly in ribonucleic machines with different composition, may acquire specificity towards peculiar classes of RNAs.

Regulation of gene expression by PNPase *via* degradation and processing of sRNAs

Recent research by different groups has revealed a central role exerted by PNPase in the stability control of small noncoding RNAs (sRNAs).[105, 121] It has been suggested that *Ec*PNPase protects several sRNAs from degradation by other nucleases in exponential

cultures, whereas it would be responsible of their decay upon entry in stationary phase, an activity counteracted by the RNA chaperone Hfq.[122-124] However, this seems not generally applicable, as some sRNAs appear to be more abundant in exponentially growing cultures of a *pnp* mutant, suggesting that they could be degraded by PNPase in this growth phase.[105] Similarly to what occurs with rRNAs and tRNAs, besides its role as a degradative enzyme, PNPase is also responsible for the maturation of some sRNAs from longer RNA precursors, thus implying that depending on the substrate, PNPase can switch from processive RNA digestion to the precise removal of few nucleotides. This is for instance the case of the CI RNA, the immunity factor of bacteriophage P4. PNPase plays a major role, with PAPI, in the correct CI RNA 3'-end formation, which in turn stimulates the 5'-end processing by RNase P.[125, 126] Interestingly, ca. 600 CI-like encoding loci, not necessarily associated with integrated phages, are scattered in sequenced proteobacterial genomes (see c4 antisense RNA motif, Table 1 in ref.[127]).

Recently, PNPase has been found to be responsible for the maturation of the CRISPR RNA RliB in *Listeria monocytogenes*; interestingly, PNPase seems to contribute also to the DNA interference activity of RliB. It remains to be established whether PNPase role in this process is restricted to modulation of RliB stability or entails a direct action on DNA.[128]

PNPase CONTROLS COMPLEX CELL ADAPTIVE RESPONSES

PNPase is essential for growth at low temperature

The *pnp* gene is essential for growth at low temperature in different Gram positive and negative bacteria such as *B. subtilis, Campylobacter jejuni, E. coli* and *Yersinia enterocolitica*.[69, 77, 129, 130] On the other hand, *Salmonella enterica* serovar Typhimurium *pnp* mutants show limited cold sensitivity and PNPase is not required for *Pseudomonas putida* growth at temperature as low as 5 °C.[70, 96, 131] The hypothesis that PNPase may be involved in

a cold-essential process conserved in the first group of bacteria and absent in the second one seems unlikely in the light of the lack of correlation between the cold sensitivity of *pnp* mutants of different bacterial species and their evolutionary distance or adaptation to specific ecological niches. Most likely, in some bacteria the lack of PNPase at low temperature could be at least partially compensated by other factors, and in particular other nucleases. The idea that nuclease-dependent pathways may have partially redundant functions in the cold is supported by the observation that in *E. coli*, the overexpression of RNase II can suppress the growth defect of a *pnp* mutant at low temperature. However, *pnp* strains overexpressing RNase R remain cold sensitive, indicating once again that RNases are not completely interchangeable.[132]

The molecular bases of PNPase essentiality in the cold have been mainly investigated in *E. coli*. In such system, incubation at temperatures ≤ 20 °C has a bacteriostatic, reversible effect on the growth of *pnp* mutants, which seem to be stalled in the cold acclimation phase.[82] Moreover, both PNPase RNA degradation and binding activities are essential in the cold.[35, 41, 132] On the whole, these observations suggest that PNPase may be required to degrade RNAs that prevent the cells from exiting the cold acclimation phase and resuming growth. Indeed, both in *E. coli* and in *Y. enterocolitica*, PNPase is required to promote the degradation of the mRNAs of cold inducible members of CspA family proteins, which are transiently induced in the acclimation phase and have been proposed to sequester the ribosomes.[82, 83, 133-135] In fact, ribosomal subunits and monosomes accumulate in a Δ*pnp E. coli* strain at 15 °C, suggesting that translation is impaired.[132]

PNPase may be also required for ribosome assembly in the cold. Indeed, in a double *pnp rph* mutant, which lacks also the second phosphorolytic exoribonuclease RNase PH, the number of 50S ribosomal subunits is reduced at 31 °C, but not at 42 °C, and the 23S rRNA is

degraded.[133] This suggests that ribosome maturation at sub-optimal temperature may require at least one of the phosphorolytic ribonucleases.

PNPase controls the expression of aggregation factors that promote biofilm formation

Biofilms are multicellular communities of microorganisms adhered to a surface. Biofilm formation is a complex developmental process that involves cell morphological changes, such as the expression of pili at the cell surface and the secretion of an extracellular polymer matrix mainly constituted by exopolysaccharides (EPS). A complex network of regulators including quorum sensing molecules and modified nucleotides controls the transition from planktonic to sessile life style and biofilm maturation.[136] Evidence from different groups suggest that PNPase contributes to biofilm regulation in *E. coli* and *Salmonella.* [105, 137, 138] In particular, in *E. coli* C, PNPase inhibits the synthesis of the EPS poly-N-acetylglucosamine (PNAG) by negatively regulating the PNAG biosynthetic operon *pgaABCD* at post-transcriptional level through a still unclear mechanism that involves the 5'-UTR of the operon. As a consequence of PNAG overexpression, cellular aggregation and biofilm production are stimulated in a Δ*pnp* mutant.[137] In *E coli* K12, mRNAs related to the production of several adhesion factors (including *pgaC* and *pgaD* PNAG biosynthetic genes) are specifically overexpressed in Δ*pnp*, but not in strains lacking either RNase R or II.[105] However, in such genetic background, the role of PNPase in biofilm formation is less clear as deletion of *pnp* has been reported to either stimulate[137] or, as in *Salmonella,*[138] prevent[105] biofilm formation. These differences, whose molecular bases are unknown, could be imputed to strain-specific mechanisms that may have evolved to respond to the variety of environmental signals impacting on biofilm development.

PNPase as a virulence regulator in pathogenic bacteria

Virulence regulation by PNPase in various bacteria has been recently reviewed[139] and we will limit here to some general comments. Disparate virulence-related functions respond to PNPase in pathogenic bacteria and the molecular mechanisms by which PNPase controls virulence functions are also variable. Only in some systems PNPase contribution to pathogenesis is clearly imputable to its activity as an exoribonuclease.[140] In fact, in other cases, PNPase RNA binding rather than enzymatic activity is required for virulence regulation. For instance, PNPase confers increased resistance to killing by murine macrophages to *Yersinia pseudotuberculosis* and *Yersinia pestis,*[139] an effect that has been linked to secretion of Yop effectors by type 3 secretion system (T3SS) specifically promoted by PNPase. Interestingly, the ability to modulate T3SS function is retained by a *pnp* mutant defective in the catalytic activity, whereas it is lost when the PNPase S1 binding domain is deleted. Moreover, the overexpression of the isolated S1 domain (from PNPase or other proteins) suppresses T3SS downregulation in a *pnp* deletion mutant.[141] Overall, these data suggest that PNPase may act in this system by binding (and maybe stabilizing) an unidentified RNA.

Interestingly, while RNase R, like PNPase, has an established function in bacterial pathogenesis[142] and in growth at low temperature[143-145] of various bacteria, to our knowledge RNase II has not been found to be essential in the cold or implicated in virulence in any bacteria. It is unclear whether this may reflect an actual specificity in the roles of different bacterial ribonucleases, with overlapping functions between RNase R and PNPase, or only the paucity of studies specifically addressing RNase II role in these phenomena.

PNPase and nucleic acids stress: removal of damaged RNA and DNA repair

In the last years several apparently unrelated observations implicated PNPase in the response to nucleic acid damage and/or DNA recombination and repair. It should be mentioned that

although a direct role of PNPase in these mechanisms has been proposed, in most cases an indirect effect of PNPase, which could control the expression of factors operating in the above pathways, has not been ruled out.

Concerning RNA damage, it has been shown that *Ec*PNPase binds RNA containing 8-hydroxyguanine (8-oxoG) residues with higher affinity than normal RNA[146, 147] and that PNPase defective *E. coli* mutants are more sensitive to H_2O_2 treatment[147], which increases the levels of oxidized ribo- and deoxyriboguanosines. Although the effects of RNA modifications on the physiology of the bacterial cell are still largely unexplored, it is believed that, by altering base-pairing interactions, RNA modifications may interfere with the functions of both mRNA and stable RNAs, thus impairing translation and regulation of gene expression.[148] Thus PNPase, by acting as a specific scavenger of oxidized RNA, may protect the cell from the many harmful consequences caused by oxidized bases.

Recently, PNPase has been implicated in both spontaneous and induced mutagenesis in *E. coli.*[12] It has been shown that the increased frequency of spontaneous mutations caused by the lack of either the mismatch repair (MMR) system, which corrects spontaneous mutations resulting from replication errors, or the MutT protein, which destroys the pool of oxidized dGTP, is suppressed in *pnp* deletion mutants. Moreover, such mutants exhibit lower levels of mutagenesis induced by some base analogues such as 5-bromodeoxyuridine.[12] An explanation of these facts has been related to phosphorolysis of RNA, which generates NDPs that can be converted to dNDPs by ribonucleotide reductase. It was hypothesized that the pool of dNDPs derived from PNPase activity could *per se* be responsible for the spontaneous mutations observed in MMR- and MutT-deficient backgrounds upon treatment with 5-bromodeoxyuridine.[12] Such hypothesis takes in account only the ability of PNPase to bind and degrade RNA. However, other observations have more directly implicated PNPase in DNA repair and recombination in evolutionary distant bacteria, possibly through its ability to

bind, degrade and/or polymerize DNA. In *B. subtilis* PNPase copurifies with RecN, a key protein for the repair of DNA double strand breaks (DSB),[149] and it is required for the formation of RecN-promoted discrete repair centers upon DNA DSBs induction, where it provides the RecN-associated 3'→5' ssDNA exonucleolytic activity.[17, 18] Notably, unlike *E. coli*, which is endowed with a panoply of 3'→5' exodeoxyribonucleases, *B. subtilis* is lacking other genes encoding such activity. Moreover, PNPase activity on ssDNA degradation and polymerization is modulated *in vitro* by RecN, RecA, and SSB.[17, 18] In *E. coli* PNPase was found to co-purify with RecA.[150] More recently, it was observed that PNPase deficient mutants are sensitive to UV and that in the absence of nucleotide excision repair system, *pnp* mutants show hypersensitivity to UV radiation. This *pnp* phenotype is epistatic to *uvrD*, *recB* and *ruvA*, thus implicating PNPase in the recombinational repair process.[11]

The ability of PNPase to resect and synthesize ssDNA 3'-ends and its interaction with recombination and repair proteins leave open the possibility that PNPase directly participates in recombination, repair and mutagenesis pathways.

CONCLUSION

After 60 years of research, we are still far from having a complete picture of the multifaceted role played by PNPase in bacterial cells. It is interesting that a "non-essential" protein has been so widely conserved through evolution in *Bacteria* and *Eukarya*, notwithstanding the functional redundancy of RNA decay pathways. On the other hand, PNPase exhibits a great evolutionary plasticity as it appears involved in a variety of processes and supramolecular structures in which this protein may play diversified structural and/or enzymatic roles in different bacterial species and eukaryotic organelles. Moreover, the ability to operate on either RNA or DNA, modulated by divalent cations, further expands the repertoire of functions in which PNPase is implicated and opens new research fields that may elucidate

new interconnections between RNA and DNA metabolism. A deeper knowledge of PNPase assembly in (nucleo)protein complexes like the various RNA degradosomes, RYPERs, and DNA recombination/repair centers, interactions with small molecules, and modulation of its activity in response to growth phase and environmental stimuli is needed to fully understand PNPase physiological role. Lastly, the spatial localization of PNPase and other factors of the RNA decay and DNA transactions apparatus in specific cell districts is a fascinating aspect emerged in the last years. Relating such localization to the functioning of the RNA and DNA transactions machineries is a major challenge for future research in the field.

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FIGURE CAPTIONS

Fig. 1. Structural similarities between PNPase, RNase PH and the exosomes. (A) The homotrimeric structure of the *Ec*PNPase (PDB ID: 3CDI[34]) and the hexameric rings of RNase PH, archaeal and eukaryotic exosomes (PDB ID: 1UDN,[151] 2C37[152] and 2NN6,[153] respectively) are reported. In the exosomes, only the subunits constituting the rings are shown in colour and named. (B) Domain structure of bacterial PNPase.

FIG. 2. Regulation of PNPase expression. Detailed explanation of the model and supporting references are provided in the text. The *pnp* primary and processed transcripts have been drawn according to the most stable secondary structure predicted by mfold.[154] Different regions of *pnp* mRNA are represented by lines of different colours: turquoise, *pnp* coding region; black, 5'-UTR of the processed *pnp* mRNA; blue, stem-loop removed by the RNase III staggered cuts; green, sRNA37. The 5'-tri- and monophosphate ends of primary and processed RNAs, respectively, are indicated by the number of arrow nocks, and the 3'-OH ends by arrowheads. Other symbols are explained in the box at the bottom of the figure. a) The *pnp* primary transcript is translatable and stable in the absence of RNase III. PNPase acts as a translation repressor (⊥), probably by competing with S1 ribosomal protein for mRNA binding. However, the native transcript is quickly processed by RNase III, which

makes two staggered cuts in the long hairpin within the 5'-UTR; b) RNase III cleavage removes about half of the stem loop; the processed *pnp* mRNA with the small RNA37 annealed at its 5'-end is still translatable and stable; c) PNPase degrades the small RNA37; d) the processed *pnp* mRNA with a single stranded 5'-end is not translation proficient and is functionally inactivated by a first RNase E cut within the *pnp* ORF; e) upon RNase E cleavage, rapid degradation of the *pnp* mRNA ensues.

Related Articles

Article ID	Article title
	Focus: Connections between 3' end processing and DNA repair
	Influence of the 5' end on bacterial expression/decay

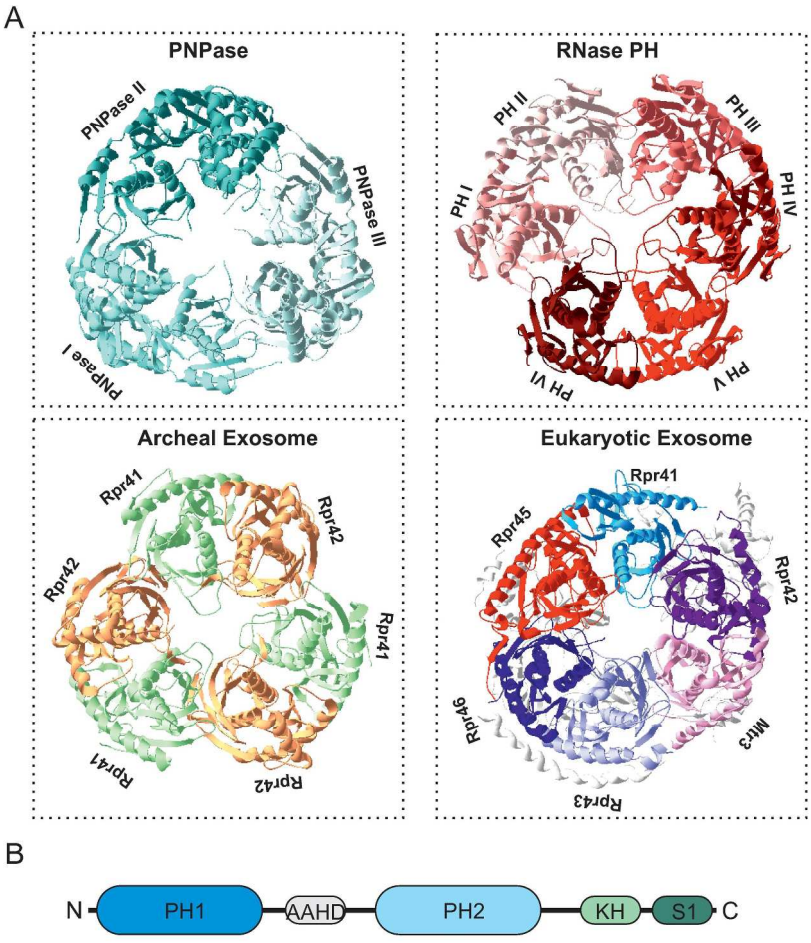

Fig. 1

Fig. 1. Structural similarities between PNPase, RNase PH and the exosomes. (A) The homotrimeric structure of the EcPNPase (PDB ID: 3CDI[34]) and the hexameric rings of RNase PH, archaeal and eukaryotic exosomes (PDB ID: 1UDN,[151] 2C37[152] and 2NN6,[153] respectively) are reported. In the exosomes, only the subunits constituting the rings are shown in colour and named. (B) Domain structure of bacterial PNPase.
196x291mm (300 x 300 DPI)

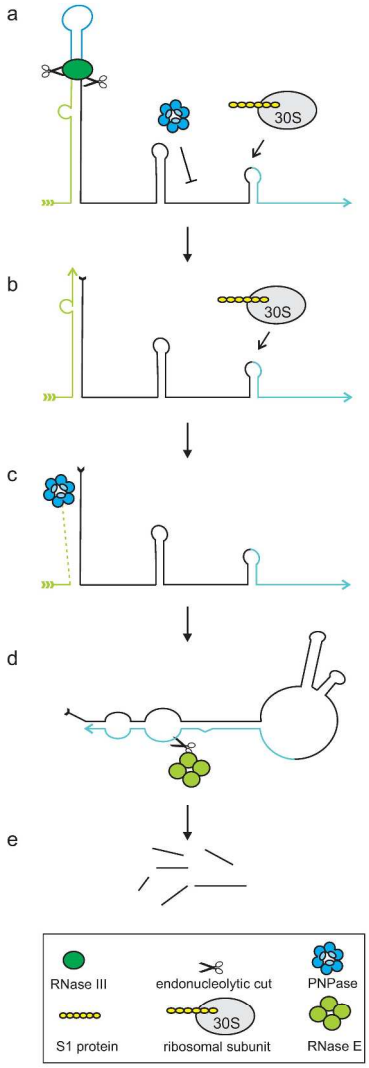

Fig. 2

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RNase E cut within the *pnp* ORF; e) upon RNase E cleavage, rapid degradation of the *pnp* mRNA ensues.
248x590mm (300 x 300 DPI)