

Review



Cite this article: Hajnsdorf E, Kaberdin VR.

2018 RNA polyadenylation and its consequences in prokaryotes. *Phil. Trans. R. Soc. B* **373**: 20180166.

<http://dx.doi.org/10.1098/rstb.2018.0166>

Accepted: 24 July 2018

One contribution of 11 to a theme issue ‘5’ and 3’ modifications controlling RNA degradation’.

Subject Areas:

microbiology, molecular biology, biochemistry

Keywords:

polyadenylation, mRNA stability, tRNA surveillance, small RNA turnover, gene expression, bacteria

Authors for correspondence:

Eliane Hajnsdorf

e-mail: eliane.hajnsdorf@ibpc.fr

Vladimir R. Kaberdin

e-mail: vkaberdin@gmail.com

RNA polyadenylation and its consequences in prokaryotes

Eliane Hajnsdorf¹ and Vladimir R. Kaberdin^{2,3,4}

¹CNRS UMR8261 associated with University Paris Diderot, Institut de Biologie Physico-Chimique, 13 rue P. et M. Curie, 75005 Paris, France

²Department of Immunology, Microbiology and Parasitology, University of the Basque Country UPV/EHU, 48940 Leioa, Spain

³IKERBASQUE, Basque Foundation for Science, Maria Diaz de Haro 3, 48013 Bilbao, Spain

⁴Research Centre for Experimental Marine Biology and Biotechnology (PIE-UPV/EHU), 48620 Plentzia, Spain

EH, 0000-0002-8249-2007; VRK, 0000-0002-7166-5136

Post-transcriptional addition of poly(A) tails to the 3’ end of RNA is one of the fundamental events controlling the functionality and fate of RNA in all kingdoms of life. Although an enzyme with poly(A)-adding activity was discovered in *Escherichia coli* more than 50 years ago, its existence and role in prokaryotic RNA metabolism were neglected for many years. As a result, it was not until 1992 that *E. coli* poly(A) polymerase I was purified to homogeneity and its gene was finally identified. Further work revealed that, similar to its role in surveillance of aberrant nuclear RNAs of eukaryotes, the addition of poly(A) tails often destabilizes prokaryotic RNAs and their decay intermediates, thus facilitating RNA turnover. Moreover, numerous studies carried out over the last three decades have shown that polyadenylation greatly contributes to the control of prokaryotic gene expression by affecting the steady-state level of diverse protein-coding and non-coding transcripts including antisense RNAs involved in plasmid copy number control, expression of toxin–antitoxin systems and bacteriophage development. Here, we review the main findings related to the discovery of polyadenylation in prokaryotes, isolation, and characterization and regulation of bacterial poly(A)-adding activities, and discuss the impact of polyadenylation on prokaryotic mRNA metabolism and gene expression.

This article is part of the theme issue ‘5’ and 3’ modifications controlling RNA degradation’.

1. Introduction

Polyadenylation refers to an enzymatic process carried out by poly(A) polymerase to add adenine residues to the 3’ extremity of RNAs. Following its discovery, various poly(A) polymerases and their functional homologues were found in a variety of pro- and eukaryotic cells and were partially purified and characterized (reviewed in [1]). These findings and the detection of poly(A) sequences in phylogenetically distant organisms [2] suggested the widespread presence of polyadenylation in pro- and eukaryotic organisms. The poly(A) tails at the 3’ end of mRNA were considered for a long time a characteristic feature of eukaryotic mRNAs (except those encoding metazoan replication-dependent histone mRNAs) and were known to stabilize eukaryotic mRNAs and promote their translation. By contrast, polyadenylation in prokaryotes gained little attention and was neglected until the pioneering work of Nilima Sarkar published in 1992 [3,4].

The polyadenylation story in prokaryotes began in 1962 [5,6], with the partial purification and characterization of *Escherichia coli* poly(A) polymerase I (PAP I). In the following years, polyadenylated RNAs were detected in *Caulobacter crescentus* and *E. coli* and later in *Bacillus brevis*, *Bacillus subtilis*, *Rhodospseudomonas capsulata* and *Rhodospirillum rubrum* (reviewed in [7,8]). By analysing the length of poly(A) tracts in prokaryotic RNAs bound to oligo(dT) cellulose, it was found that the median length of poly(A) stretches was between

14 and 60 nucleotides and that only a small fraction of total RNA was polyadenylated. Further progress in the study of polyadenylation was achieved after the systematic analysis of poly(A) tracts of *E. coli trpA* and *lpp* transcripts [3,9,10]. Then the *E. coli pcnB* gene, a non-essential gene previously reported to control ColE1 plasmid copy number [11–14], was identified as the gene encoding poly(A) polymerase I [3,15], providing the first evidence of the biological function of polyadenylation in *E. coli*. Namely, it was shown that polyadenylation promotes the rapid turnover of RNAI, a small antisense RNA complementary to RNAII, which, in turn, serves as a primer for DNA polymerase during replication of ColE1-type plasmids [15–17]. Later, the elucidation of the degradation pathways of both non-coding and protein-coding RNAs demonstrated the involvement of *E. coli* poly(A) polymerase I in the turnover of several RNAs, thus reinforcing the idea that polyadenylation often destabilizes bacterial transcripts and their decay intermediates. The destabilizing role of polyadenylation is not restricted to bacteria. It was later demonstrated in Archaea and chloroplasts, and in mitochondria and nuclei of diverse eukaryotes including yeasts, plants and humans [18–25].

2. *Escherichia coli* poly(A) polymerase I and functionally related enzymes able to carry out poly(A) addition in bacteria

A few years prior to the identification of the biological function of the *E. coli pcnB* gene, it was noted that the product of this gene shares a high sequence similarity with tRNA nucleotidyltransferase [14], an enzyme that catalyses the addition of A and C residues to incomplete CCA termini of tRNAs [26]. Both enzymes are members of the class II polymerase beta-superfamily of nucleotidyltransferases [27] and poly(A) polymerase I, upon its overexpression, can partially compensate for the absence of tRNA nucleotidyltransferase by adding (although with a lower efficiency) a 3'-terminal CCA sequence to defective tRNAs in *E. coli* [28]. Moreover, it was demonstrated that the high sequence similarity of these enzymes opens up the possibility to swap amino acids within the evolutionarily conserved N-terminal catalytic domain and to generate recombinant polypeptides with altered specificities [29].

The difficulty in distinguishing poly(A) polymerases from other closely related enzymes makes it challenging to identify poly(A) polymerase genes in other bacteria, including Gram-positive species. However, additional genetic tools and functional assays have been used to assure the enzyme's identity. For instance, among three candidates tested for the role of poly(A) polymerase in *Streptomyces coelicolor*, only polynucleotide phosphorylase (PNPase) was found to be responsible for RNA 3'-tail synthesis *in vivo* [30]. PNPase, known for its 3'-5' degradative activity of RNA, also possesses a 3'-terminal oligonucleotide polymerase activity at low inorganic phosphate concentrations. Namely, the enzyme is able to add A, C and U residues to an RNA chain in *E. coli* in the absence of PAP I [31]. Attempts to identify a poly(A)-adding enzyme in another Gram-positive bacterium, *B. subtilis* [32], showed that PNPase was not able to carry out polyadenylation of RNA *in vivo* and also did not identify any other enzyme(s) possessing a poly(A)-adding activity.

Therefore, it was proposed that polyadenylation in *B. subtilis* was likely performed by a still unknown enzyme [30,32].

3. Biochemical properties of *Escherichia coli* poly(A) polymerase I

PAP I is a monomer of approximately 53 kDa. The enzyme requires divalent metal ions (i.e. Mg²⁺ or Mn²⁺) for activity and it uses ATP as a substrate to add adenosine residues at the 3' end of RNA molecules. Moreover, *in vitro* PAP I prefers to carry out polyadenylation of non-structured RNAs and its activity is inhibited by 3'-terminal stem-loop structures [33]. Although CTP can also be used by PAP I, the efficiency of its incorporation is only 5% of that observed for ATP [33,34]. These properties may explain why Cs are occasionally incorporated in poly(A) tails [35]. *In vivo* poly(A) tails were detected at the 3' ends of many (but not all) RNA, including primary transcripts, processed RNAs and intermediate products of exonucleolytic degradation [36–39]. However, folding predictions and searches for conserved motifs potentially present in polyadenylated RNA could not reveal any consensus sequence or structure that could serve as a signal for PAP I to begin poly(A) addition [37]. Instead, *in vitro* studies suggested that PAP I activity is controlled by more general features of RNA. Namely, it was found that unpaired 5' and 3' termini, as well as the presence of 5'-monophosphate in substrate RNAs, could greatly increase the rate of poly(A) addition [34,40]. These findings are consistent with the well-documented role of polyadenylation in accelerating the decay of intermediate products of RNA degradation that are produced in the initial steps of RNA decay, i.e. after the removal of 5'-diphosphate by the combined action of RppH and an unknown enzyme [41] followed by the initial RNase E cleavage (figure 1a) or after 'direct entry cleavages' made by RNase E or RNase III (figure 1b) [43]. As the resulting decay intermediates carry 5'-monophosphate groups and likely possess unstructured 5' and 3' termini, they are further targeted for polyadenylation and hence rapid turnover (figure 1c).

4. Regulation of poly(A) polymerase I level and activity

The intracellular level of *E. coli* PAP I is usually low, as its overproduction is detrimental to cell growth [13,44]. Previous work has shown that an increase in the abundance of this enzyme beyond its normal level (e.g. by plasmid-dependent overexpression) leads to polyadenylation of mature tRNA. This results in a dramatic decrease in the level of functional tRNAs and a concomitant inhibition of translation, ultimately leading to cell death [45].

The tight control of PAP I production is exerted at the transcriptional and post-transcriptional levels. Transcription of *pcnB* mRNA can be initiated from five distinct promoters (figure 2a; [47]). The activities of at least two of them are inversely dependent on the growth rate, which is manifested by an increase in the abundance of PAP I and higher levels of RNA polyadenylation observed in slowly growing bacteria [48,49]. The intricate regulation of *pcnB* transcription in *E. coli* was shown to be dependent on two sigma factors, σ^{70} and σ^S , responsible for promoter recognition by RNA

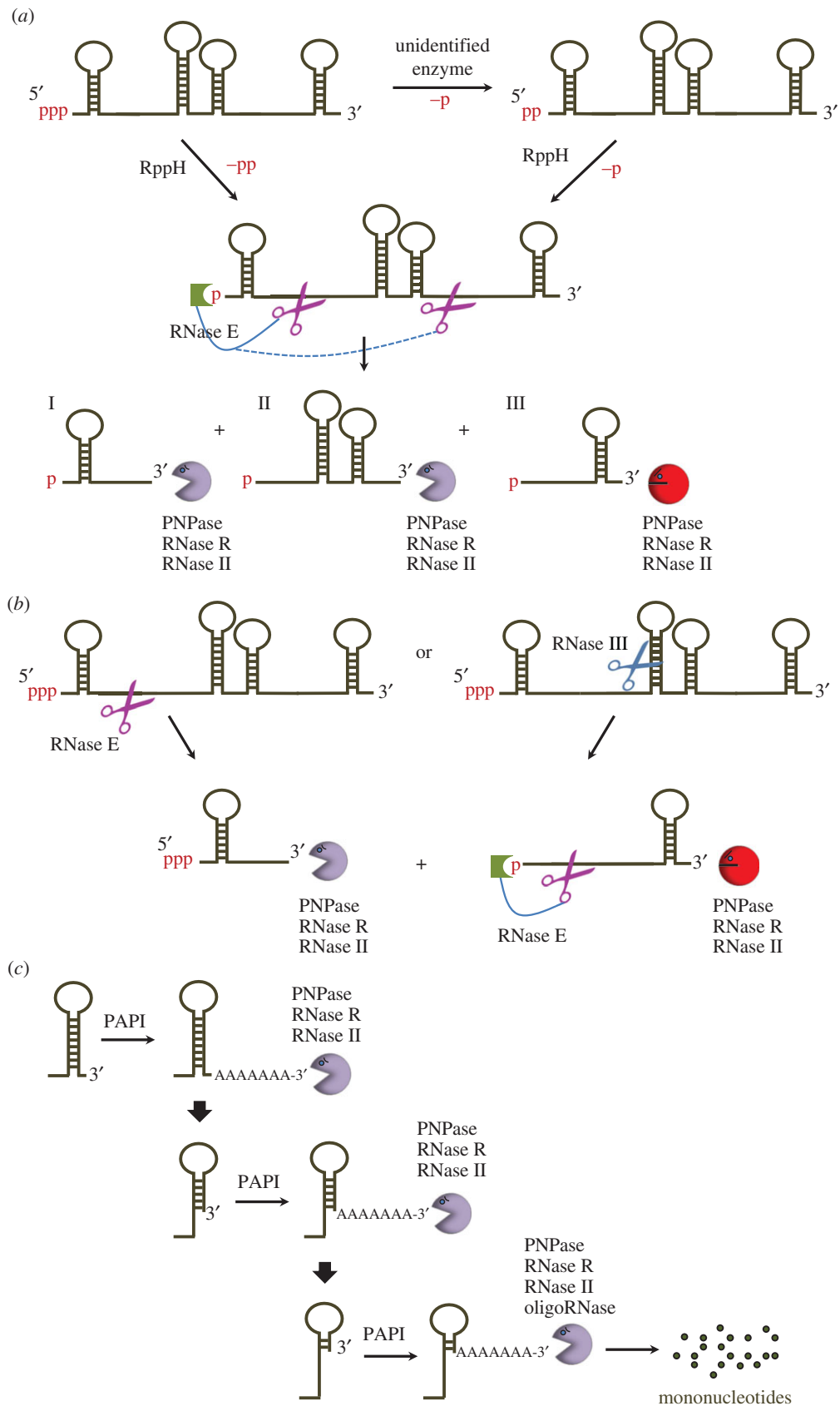


Figure 1. RNA turnover in *E. coli* and its facilitation by polyadenylation. (a) The principal pathway for RNA turnover in *E. coli*. RNA decay begins with pyrophosphate removal by the combined action of RppH and an unknown enzyme [41], followed by endonucleolytic cleavage(s) made by the 5'-monophosphate-dependent endoribonuclease, RNase E. The resulting intermediate products such as I, II and III (schematically shown above) are further degraded by the combined action of 3'–5' exonucleases, primarily including polynucleotide phosphorylase (PNPase), ribonuclease R (RNase R) and ribonuclease II (RNase II). (b) Degradation of some transcripts can involve 'direct entry' cleavages made by RNase E or RNase III. The resulting decay intermediates are further degraded by exo- and endonucleases. (c) The PAP I-dependent degradation of structured RNAs in *E. coli*. Although stable stem–loop structures at the 3' end of *E. coli* transcripts and their decay intermediates (e.g. fragment III shown in (a)) *per se* are resistant to the action of major *E. coli* exoribonucleases, their decay can be facilitated by polyadenylation. Moreover, repeated cycles of polyadenylation and subsequent 3'–5' degradation of the polyadenylated species by exoribonucleases ensure their progressive degradation *in vivo* [42], in turn rendering short oligonucleotides that are further digested by oligoribonuclease (oligoRNase) to yield mononucleotides. (Online version in colour.)

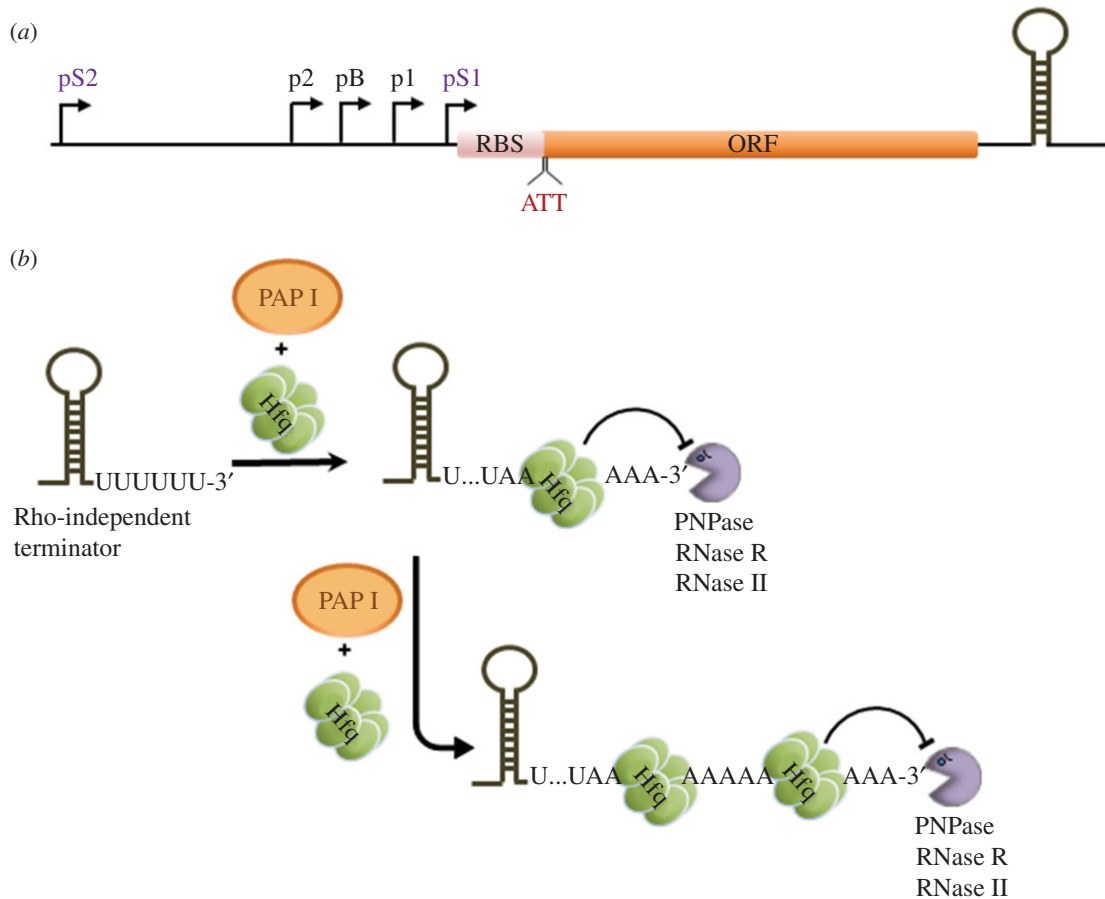


Figure 2. Control of poly(A) polymerase I level and activity in *E. coli*. (a) Control of *E. coli pcnB* expression. Transcription of the *pcnB* gene coding for PAP I is driven by three σ^{70} -dependent (i.e. p1, pB and p2) and two σ^S -dependent (pS1 and pS2) promoters [46]. Moreover, ppGpp and DksA were found to directly inhibit transcription from pB, pS1 and pS2 and the overall low level of PAP I is maintained owing to lower efficiency of translation caused by the presence of a non-canonical start codon (AUU) within the ribosome-binding site (RBS). (b) Effect of Hfq on poly(A) addition. The RNA chaperone Hfq can bind to poly(A) tails, thereby impeding their exonucleolytic degradation and concomitantly increasing the processivity of the poly(A) addition by PAP I. ORF, open reading frame. (Online version in colour.)

polymerase, and on ppGpp and DksA, two effectors of the stringent response produced in response to amino acid starvation [46]. Moreover, transcriptional control of *pcnB* expression in other bacteria seems to also involve additional transcription factors [50]. The use of the non-canonical initiation codon AUU to initiate the translation of *pcnB* may be another way to limit the availability of PAP I. Moreover, there are indications that PAP I activity could potentially be regulated post-translationally. It was reported that a PAP I variant, with a C-terminal His tag, can be phosphorylated both *in vivo* and *in vitro* and that this modification impairs its activity [48]. However, it remains unknown whether phosphorylation of the untagged enzyme occurs *in vivo* or *in vitro* and can likewise impact PAP I activity.

Apart from the regulation of *pcnB* expression, polyadenylation can be controlled by the RNA chaperone Hfq. The latter was found to bind to poly(A) tails [34] and stimulate their elongation *in vitro* by increasing the processivity of PAP I (figure 2b; [51]). Moreover, inactivation of Hfq affects recognition and polyadenylation of transcripts terminated by Rho-independent terminators. For instance, the average length, occurrence of polyadenylated transcripts and percentage of poly(A) tails located at the 3' end of the *rpsO* Rho-independent transcription terminator were considerably higher in the wild-type strain than in the *hfq* mutants [51,52]. Therefore, it is possible that oligo(A) tails less than 10 nt in length may be too short to form stable complexes with Hfq, but seem to be long enough to be used by 3'-5'

exoribonucleases which carry out the degradation of structured RNAs [53]. When the tails are longer, Hfq binds tightly to these oligo(A) sequences and may protect polyadenylated RNA from RNase II and PNPase-mediated decay as shown *in vitro* (figure 2b [54]).

5. Poly(A)-dependent mechanisms and their role in RNA metabolism and gene expression

An increasing number of studies indicate that polyadenylation is broadly used by bacteria to control RNA metabolism (mRNA turnover, stable RNA surveillance and recycling of small RNAs), thereby regulating gene expression at the post-transcriptional level.

(a) Effects of polyadenylation on mRNA and stable RNA metabolism

Polyadenylation of prokaryotic transcripts is best known for its ancillary role in RNA turnover. Although the rate-limiting step in the degradation of prokaryotic mRNA usually involves an endoribonucleolytic cleavage at the 5' end of the transcript subsequently resulting in its functional inactivation (figure 1a), the remaining steps that are responsible for further fragmentation of the primary cleavage products and their ultimate conversion to nucleotides often involve the combined action of endo- (RNase E/G, RNase III,

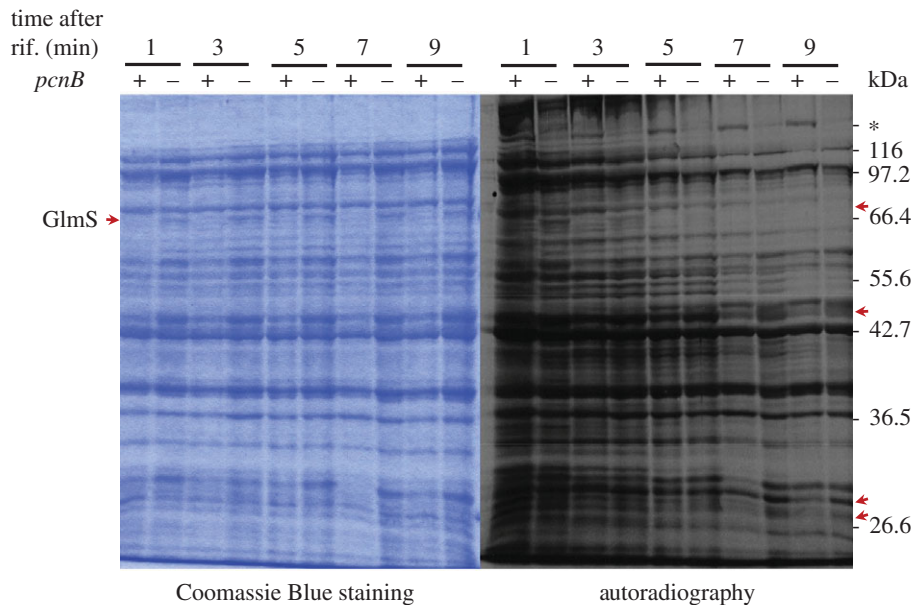


Figure 3. Effects of PAP I inactivation on functional mRNA stability and protein synthesis in *E. coli*. The residual capacity of *E. coli* mRNAs to direct protein synthesis after inhibition of RNA synthesis by rifampicin (rif.) was assessed in the wild-type ($pcnB^+$) and $pcnB$ mutant ($pcnB^-$) strains. Soluble proteins extracted from cells that were treated with rifampicin for 1, 3, 5, 7 and 9 min and pulse-labelled with radioactive methionine as described in [74] were separated on SDS-polyacrylamide gels. The steady-state level of polypeptides and those synthesized *de novo* were revealed by Coomassie Blue staining and autoradiography, respectively. The position of GlmS and unknown polypeptides overproduced in the mutant strain are indicated by arrowheads, while the position of an unknown protein overproduced in the wild-type strain is indicated by an asterisk.

RNase Y or RNase J1/J2) and exonucleases (e.g. PNPase, RNase II, RNase R and oligoribonuclease) [55]. Moreover, the three major exonucleases (i.e. PNPase, RNase II and RNase R) were shown to play different roles in the PAP I-dependent pathway in *E. coli* (figure 1c). In contrast to PNPase and RNase R, which initiate and efficiently degrade polyadenylated structured RNAs [56–60], RNase II appears to play a protective role. RNase II is unable to degrade structured RNAs, but because it can eliminate poly(A) tails from polyadenylated decay intermediates, it thereby inhibits their exonucleolytic degradation by PNPase and RNase R [34,61]. In addition to the above mechanism, polyadenylated primary transcripts and their decay intermediates can also be stabilized by Hfq (see above), which binds to their poly(A) tails and thus protects them from exonucleolytic decay [54].

The efficiency of mRNA turnover is often regulated by additional *cis*- and *trans*-acting factors differentially affecting the susceptibility of transcripts to the components of the translation apparatus or the RNA decay machine (reviewed in [62]). *In vivo* studies have shown that stable RNA structures (e.g. transcriptional terminators or repetitive extragenic palindromic (REP) sequences [63,64]) at the 3' end of decay intermediates or processed transcripts [35,36,65] can confer resistance to degradation by 3'–5' exonucleases and therefore can protect the adjacent upstream regions from exonucleolytic decay *in vivo*. Analysis of this resistance mechanism *in vitro* has revealed that *E. coli* can employ at least two different strategies to overcome the stabilizing effect of secondary structures.

The first one is associated with the formation of the *E. coli* degradosome, a multienzyme ribonucleolytic complex, whose major components include RNase E, PNPase, the RNA helicase B (RhlB) and enolase [66,67]. It has been shown that the interaction of RhlB and PNPase in the context of the degradosome enables their functional cooperation in the degradation of structured RNAs [67,68]. Namely, the

RNA-unwinding activity of RhlB partly unfolds structured RNAs, yielding single-stranded 3' ends, thereby facilitating 3'–5' degradation by exonucleases.

An alternative mechanism involves the action of poly(A) polymerase I (figure 1b). PAP I adds poly(A) tails, thus providing a toe-hold for 3'–5' exonucleases unable to bind in the absence of such single-stranded stretches of nucleotides downstream of stable stem–loop structures. However, once exonucleases become attached to free 3' ends, they can progressively digest structured RNAs [42,68,69]. As the processivity of exonucleases such as PNPase on highly structured RNA is low, they cannot finish digestion of RNA during a single round owing to their dissociation from substrates [42]. Nevertheless, consecutive cycles of polyadenylation and exonucleolytic degradation are believed to ensure the complete degradation to mononucleotides (figure 1c [42]). In principle, the physical interaction of PAP I and the degradosome detected *in vivo* [70] suggests that *E. coli* can likely orchestrate mRNA turnover by coordinating the action of the degradosome- and poly(A)-dependent pathways.

Although polyadenylation of stable RNA precursors *in vivo* was discovered a long time ago, its biological significance remains largely unknown, in particular for 5S and 23S RNAs [71,72]. As to the contribution of polyadenylation to tRNA metabolism, it is proposed to be linked to the role of PAP I in a quality-control mechanism. The latter helps to eliminate defective tRNAs by targeting them for 3'–5' exonucleolytic decay [37,73], apparently independent of an interaction between PAP I and the degradosome.

(b) Polyadenylation and its role in the regulation of gene expression

Apart from its contribution to recycling of structured RNA decay intermediates and surveillance of tRNA, polyadenylation also plays an important role in the regulation of gene

expression. The regulatory function of polyadenylation can affect the steady-state levels of mRNAs available for translation either positively or negatively (figure 3 and table 1) [37,39,74–77].

Besides its apparently direct role in the regulation of mRNA abundance, polyadenylation can also indirectly influence gene expression controlled by numerous *cis*- and *trans*-encoded small RNAs. Previous studies have shown that the addition of poly(A) tails has a destabilizing effect on several *cis*-encoded antisense RNAs known for their role in the control of plasmid replication (e.g. RNAI [17,56] and CopA [78] controlling the copy number of ColE1-type and R1 plasmids, respectively), bacteriophage lambda development (Oop RNA [79]), bacteriophage P4 immunity (CI RNA [80]) and expression of Type I toxin–antitoxin systems (Sok, [81], and others [39]).

Likewise, polyadenylation is known to lead to destabilization of *trans*-encoded sRNAs. Recent studies have shown that polyadenylation destabilizes the small RNA GlmY involved in the GlmZ-dependent control of *glmS* expression [82,83]. Besides controlling the level of GlmY, polyadenylation also affects the steady-state levels of RyjA, RybB, SroH [84] and many other sRNAs [39] that become more abundant in *pcnB* mutant strains. Nevertheless, the actual impact of poly(A) tails on the biological functions of most of these riboregulators remains unknown and merits further analysis.

(c) Perspectives

Despite the significant progress recently achieved in revealing the multifaceted role of polyadenylation in the regulation of prokaryotic RNA metabolism and gene expression, there are still many open questions that have to be addressed in future.

In particular, more work needs to be done to characterize the positive effect of polyadenylation on prokaryotic gene expression. Namely, in contrast to the well-known examples, in which the addition of poly(A) tails to the 3' end of prokaryotic RNAs or their decay intermediates promotes RNA turnover and reduces the level of functional RNA, the presence of an active poly(A) polymerase in certain cases can enhance the steady-state transcript level (e.g. those expressed from the *E. coli* flagellar operon [76] and others [39]). The mechanisms, direct or indirect, by which polyadenylation can increase RNA levels and whether this also enhances gene expression are poorly understood and warrant further analysis.

In addition, future studies should reveal more details regarding the complex regulatory networks used by *E. coli* and other bacteria to coordinate and modulate the action of poly(A) polymerase I and its interaction with known (e.g. the degradosome and Hfq) or still unknown interacting partners to exert post-transcriptional control of gene expression.

Moreover, although the *pcnB* gene *per se* is not essential for sustaining *E. coli* survival and growth under laboratory

Table 1. Role of polyadenylation in global RNA turnover [39]. Indicated are the numbers of genes that were up- or downregulated in the *pcnB* mutant when compared with the wild-type (wt) strain. Their functional classification is provided according to the Gene Ontology database. The first number represents the number of regulated transcripts with \log_2 fold change (\log_2FC) greater than 2, whereas the second one corresponds to the transcripts with \log_2FC between 1.5 and 2.

functional categories	<i>pcnB</i> versus wt	
	up	down
membrane proteins	7/7	9/12
prophage proteins	1/4	2/0
RNA metabolism	2/2	1
ammonium metabolism	3/1	1/4
motility (flagella proteins)	0	11/15
cell adhesion (fimbriae)	1/5	0
catabolism	0	2/5
miscellaneous	10/22	9/10

conditions, the gene might play a more critical role in cell survival in adverse environments. Therefore, it is of particular interest to compare the regulation of PAP I expression and activity along with its impact on gene expression under normal and various stress conditions. This might yield particularly interesting results by testing the effect of major stress factors (such as limitation of iron and nutrients, oxidative stress, etc.) faced by bacterial pathogens in their hosts.

Finally, as the main findings regarding the role of polyadenylation in gene expression and cell physiology were obtained by using *E. coli* as a model organism, there is little known about the actual mechanisms controlled by polyadenylation in other bacteria. In this regard, the recently reported function of the *Pantoea agglomerans* YS19 *pcnB* gene in the indole regulatory pathway [85] found to be involved in host–pathogen interactions is intriguing and clearly demonstrates the merits of using phylogenetically distant organisms to discover new biological functions.

Data accessibility. This article has no additional data.

Authors' contributions. E.H. and V.R.K. wrote the paper.

Competing interests. We have no competing interests.

Funding. This work was supported by the Centre National de la Recherche Scientifique (UMR8261), University Paris-Diderot, 'Initiative d'Excellence' programme from the French State (grant 'DYNAMO', ANR-11-LABX-0011) (to E.H.), Spanish Ministry of Economy and Competitiveness grant CGL2015-70929-R and IKER-BASQUE (Basque Foundation for Science) (to V.R.K.).

Acknowledgements. The authors are indebted to Jackie Plumbridge for critical reading of the manuscript.

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