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Landscape of RNA polyadenylation in E. coli

Alexandre Maes¹, Céline Gracia¹, Nicolas Innocenti²,³, Kaiyang Zhang⁴, Erik Aurell²,⁵ and Eliane Hajnsdorf¹,*

¹CNRS UMR8261 (previously FRE3630) associated with University Paris Diderot, Sorbonne Paris Cité, Institut de Biologie Physico-Chimique, 13 rue P. et M. Curie, 75005 Paris, France, ²Department of Computational Biology, KTH Royal Institute of Technology, AlbaNova University Center, Roslagstullsbacken 17, SE-10691 Stockholm, Sweden, ³Combient AB, Nettövägen 6, SE-175 41 Järfälla, Sweden, ⁴Systems Biology Laboratory, Research Programs Unit, Genome-Scale Biology, Faculty of Medicine, University of Helsinki, Helsinki, FIN-00014, Finland and ⁵Departments of Computer Science and Applied Physics, Aalto University, Konemiehentie 2, FI-02150 Espoo, Finland

ABSTRACT

Polyadenylation is thought to be involved in the degradation and quality control of bacterial RNAs but relatively few examples have been investigated. We used a combination of 5′-tagRACE and RNA-seq to analyze the total RNA content from a wild-type strain and from a poly(A)polymerase deleted mutant. A total of 178 transcripts were either up- or down-regulated in the mutant when compared to the wild-type strain. Poly(A)polymerase up-regulates the expression of all genes related to the FliA regulon and several previously unknown transcripts, including numerous transporters. Notable down-regulation of genes in the expression of antigen 43 and components of the type 1 fimbriae was detected. The major consequence of the absence of poly(A)polymerase was the accumulation of numerous sRNAs, antisense transcripts, REP sequences and RNA fragments resulting from the processing of entire transcripts. A new algorithm to analyze the position and composition of post-transcriptional modifications based on the sequence of unencoded 3′-ends, was developed to identify polyadenylated molecules. Overall our results shed new light on the broad spectrum of action of polyadenylation on gene expression and demonstrate the importance of poly(A) dependent degradation to remove structured RNA fragments.

INTRODUCTION

Polyadenylation is a ubiquitous post-transcriptional modification, which profoundly affects the activity and fate of RNAs (1). First discovered in prokaryotes, polyadenylation has been primarily studied in eukaryotes, where it contributes to the export of mRNAs to the cytoplasm and promotes mRNA stability and translation. Subsequently polyadenylation was also demonstrated to have an RNA destabilizing function that is conserved in bacteria, organelles and both the nuclei and cytoplasm of eukaryotes (2–5). In this paper we investigate for the first time the entire genome-wide polyadenylation landscape in Escherichia coli.

In E. coli the rate-limiting step in the degradation of mRNAs is an endoribonucleolytic cleavage, performed in the majority of cases, by RNase E, but also by RNase III for a small number of mRNAs. These cleavages generate small RNA fragments which are then degraded by the 3′-5′ exoribonucleases RNase II, PNPase and RNase R (4). In some cases the addition of poly(A) tails by poly(A)polymerase I (PAP I) to the 3′ end of RNA fragments has been shown to facilitate the degradation of short folded RNAs by providing a toe-hold for the exoribonucleases (4). Poly(A)-assisted decay is also implicated in RNA quality control, as demonstrated for a defective TRNA precursor (6). In contrast to eukaryotes, the pcnB gene, encoding poly(A)polymerase, is not essential in E. coli (7). This allows to compare polyadenylation in wild-type and a pcnB-deficient strain, as will be done here.

Polyadenylation is not only a biological waste-disposal system to recycle non-functional RNA molecules, it has also been shown to play an integral role in the regulation, either positive or negative, of the expression of certain bacterial genes in E. coli (8–12). The gene for PAP I (pcnB) was initially identified because it controls plasmid copy number. PAP I is required for the destabilization of RNA I, CopA, Sok and RNA-OUT antisense RNAs encoded by plasmids, phages and transposons and as a consequence controls their function (13). In addition, poly(A)polymerase I, with PNPase, participates in the end repair process of tRNA,
when tRNA nucleotidytransferase is absent, to maintain functional tRNA levels (14). PNPase also has a template-independent 3′-oligonucleotide polymerase activity, and it was reported to be responsible for the addition of short heteropolymeric tails detected in the absence of PAP I and of non-A residues inserted into poly(A)tails in the wild-type strain (15).

Previous reports on the degradation pathway of the rpsO mRNA have revealed that about 10% of these mRNA harbor short oligo(A) tails ranging from one to five A residues. Poly(A) tails were mapped to several positions within the rpsO mRNA, in addition to the 3′ end of the primary transcript, corresponding to positions expected to be both single stranded or within in a 3′ folded termini. This showed that any 3′ RNA extremity can be tagged by a poly(A) tail and that poly(A)polymerase has little or no sequence specificity for the addition of oligo(A) tails (16). This is in agreement with the idea that poly(A) tails are added indiscriminately and that repetitive rounds of polyadenylation and exonucleolytic digestion progressively can remove secondary structures that otherwise would impair 3′-5′ degradation of RNA (16–19).

Polyadenylation affects the decay of a significant number of mRNAs and has also been shown to affect a few non-coding RNA. However, these previous studies aiming to identify PAP I targets were performed in the absence of eoribonucleases or when PAP I was overproduced (6,20) but not under physiological conditions. In this paper, we make use of innovations in RNA-seq (21–26) to compare the tran-scriptomes of a wild-type strain and a strain deleted for the pcnB gene. Our proposal was to make a catalogue of RNAs, which are polyadenylated. By analyzing the 3′ extremities of these RNAs, we demonstrate the range of PAP I substrates and gain insight to the specificity of PAP I target selection. Inactivation of poly(A)polymerase induces the massive accumulation of RNA fragments containing stable secondary structures. We identify many transcripts which are up- or down-regulated in the pcnB mutant. This differential accumulation of functional transcripts brings further evidence of the complex role, either direct or indirect, of polyadenylation, in the control of gene expression in E. coli.

MATERIALS AND METHODS

Bacterial strains, growth conditions

Strains N3433 (HfrH lacZ43 ΔrelA spoT1 thi1 from D. Apirion) and its ΔpcnB KanR derivative, IBPC903 (8) were used in this work. Strains were grown in LB medium at 37°C.

RNA extraction, northern blot analysis, 5′ RNA tagging and RNA seq

Total RNA was prepared from bacteria grown to an A660 = 0.35–0.4 using the hot-phenol procedure (27). Ten micrograms of total RNA were electrophoresed either on 1% agarose formaldehyde gel or 6% polyacrylamide gels containing 7 M urea and analyzed by Northern blotting (28,29). Templates for the synthesis of the RNA probes were obtained by PCR amplification using the pair of m and T7 oligonucleotides (Supplementary Table S1). RNA probes were synthesized by T7 RNA polymerase with (α-32P)UTP yielding uniformly labeled RNAs (30). Membranes were also probed for 5S rRNA with 5′-labeled oligonucleotide (Supplementary Table S1). RNA levels were quantified by phosphorimager.

For RNA-seq analysis, after DNase RQ1 digestion (Promega), RNAs were re-extracted with phenol–chloroform and precipitated with ethanol. RNAs were prepared by using a modified version of the 5′-tagRACE approach, including TEX treatment to remove untagged 5′ mono-phosphates (26,31) (Figure 1A). Total RNA (40 μg) was ligated to the PSS RNA primer (Processing Start Site specific primer) (Supplementary Table S1) with T4 RNA ligase (Epicentre) in denaturing buffer (50 mM Hepes pH 7.5, 20 mM MgCl2, 3 mM DTT, 0.1 mM ATP, 10% DMSO, 10 μg/μl BSA) and precipitated with ethanol. Termination5′-Phosphate-Dependent Exonuclease (TEX) was added to digest remaining RNA having a 5′-monophosphate (Epibione Biotechnologies). RNA primers and nucleotides were removed by gel filtration (Illustra MicroSpin G-50 Columns). γ and β-phosphates were sequentially removed from 5′-triphosphorylated RNAs by RNA 5′-polyphosphatase (Epibione Biotechnologies). RNAs were re-extracted with phenol-chloroform and precipitated with ethanol. The TSS RNA primer (Transcription Start Site specific primer) (Supplementary Table S1) was then ligated and non-ligated primers removed by gel filtration. The PSS and TSS primers sequences were chosen as short sequences not found on E. coli chromosome similar enough to each other to avoid any sequence specific artifact during the ligations and without any secondary structure. RNAs were then fragmented according to the NEBnext Magnesium RNA fragmentation module and purified on an RNasea column (Qiagen). Purified fragmented molecules were incubated with antarctic alkaline phosphatase then T4 polynucleotide kinase (NEB). Those molecules were next prepared according to the TruSeq Small RNA Library Prep kit replacing the last gel purification step by magnetic beads purification for a larger size selection.

Directional sequencing was performed by the high throughput sequencing platform of IMAGIF (Centre de Recherche de Gif–www.imagif.cnrs.fr) on an Illumina HiSeq 2000, in a 2 × 100 bp run on three technical replicates for each condition.

RNA sequencing alignment, differential expression analysis and RNA secondary structure prediction

After demultiplexing, tag sorting and quality trimming, reads were mapped on the E. coli K12 genome (Genbank accession U000966.3) using BWA (v0.7.5a-r405) with default option and allowing multiple mismatches. Paired-end reads were merged and counted in each annotated region and inter-genic region with bedtools multicov (v2.19.0). Read counts were normalized by total number of reads in each condition and by their length (rpkm). Looking at the mean-log distribution, regions with at least 40 rpkm in one of the two strains are enough covered to be above the background noise and were taken into account for the differential expression analysis.

Mapped paired-end reads:
Detection level threshold of the 3′ end extremity was determined as 2/3 of the value of the highest 5′-monophosphate site density in each penB PSS upregulated region. A nucleotide n bp downstream of the PSS was considered as part of the fragment as long as the derivative \( d_n = \frac{R_n - N_n}{N} \) (with \( N = 10 \) and \( R = \) pile-up value) is <0.5.

RNA extracted fragments were folded and minimum fold energy (mfe) determined using the RNAfold software v.2.1.9 (Vienna Package 2.0, [32]). Random sequences in the genome are picked up following the same normal length distribution as the extracted RNA fragments and were used as controls.

**Validation of the experimental approach**

An analysis of the RNA profiles in two regions, which have been previously studied in detail, were used to validate our method. Differential expression profiles from the RNAseq for the transcripts of the rpsO-pnp operon confirmed that the vast majority of the pnp transcripts harbored a PSS-tag at the downstream RNase III cleavage site (33), while the rpsO transcripts harbored either the TSS-tag, or the PSS-tag at the transcription start site (34) (Figure 1B and Supplementary Figure S1ABD). This indicates a conversion of the 5′ terminus of the rpsO transcript from a triphosphate to a monophosphate (Supplementary Figure S1D), as has been shown previously (35). PAP I facilitates the degradation of GlmY and GlmZ sRNAs, which are required for stabilization in the genome are picked up following the same normal length distribution as the extracted RNA fragments and were used as controls.

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<th>Int</th>
<th>PSS</th>
<th>TSS</th>
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<td>33 418 029</td>
<td>5 201 903</td>
<td>363 327</td>
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<tr>
<td>penB</td>
<td>33 374 213</td>
<td>4 666 615</td>
<td>346 133</td>
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Figure 1. Scheme of the RNA preparation prior to the RNA-seq. (A) A hypothetical transcript and its degradation intermediates are illustrated. A mRNA with 5′-monophosphate end (Supplementary Figure S1AC). Whilst being aware of this possible limitation to detect 5′-extremity of transcripts, we decided to continue with this approach because our goal was to compare the levels of 5′-tagged (and untagged, see below) RNAs in the wt and penB strains. After tagging the native 5′ ends in the RNA preparations, subsequent RNA fragmentation before the sequencing allowed to get an overview of the entire transcriptomes. These fragmented RNA did not harbor any tag and were labeled Int for internal fragments. Transcripts, harboring the PSS or the TSS tags, were labeled PSS and TSS transcripts, respectively. Gene counts representative of RNA levels in the tagged (PSS and TSS) and untagged (Int) fractions from the wt and penB mutant are presented in Supplementary Table S2. Inspection of the mean-log plots distribution in tagged and untagged fractions, two cut-offs based on fold changes (FC) were determined; one medium (log_{2} FC = 1.5) and one stringent (log_{2} FC = 2.0), the stringent one being of higher stringency (log_{2} FC = 2.0), the stringent one being of higher stringency.
confidence (Supplementary Figure S3). Transcripts which were either up- or down-regulated in the mutant when compared to the wild-type strain are presented in Table 1 and Supplementary Table S3.

Analysis of unmapped 3'-ends
Demultiplexed raw reads were processed using PEAR (Paired-End reAd mergeR) with default options (36) in order to simultaneously merge mates of paired-end reads into single but longer reads and remove sequencing adapters. The merging process succeeded for 84–85% of raw reads leading to ~52 million merged reads for each sample. Tags were removed using Flexbar v2.5 (37) and command line arguments “-be LEFT_TAIL -bt 2.5”.

Reads which included an RNA sequence, with a 3' tail that does not match the sequence on the coding strand of the DNA sequence, so called ‘unmapped’ 3' ends, were detected by an iterative aligning-and-trim procedure as follows: reads are aligned with Bowtie v1.0.0 (38), allowing only for perfect matches (‘-v 0’ command line option). For unaligned reads that are longer than 25 nt, one nucleotide is trimmed from the 3'-end and memorized as the 'tail' of the read before a new mapping attempt is performed. At the end of this procedure, reads that still do not align are reconstructed and, for those longer than 25 nt, trimmed of one nucleotide at the 3'-end and sent through a new series of iterative align-and-trim attempts. In total, 98% of merged reads were mapped this way in each sample.

For all reads that align after the removal of one or more nucleotides from their 3' end, we constructed four signals similar to ‘pile-ups’ but counting, instead of aligned reads, reads were mapped this way in each sample. Tags were either up- or down-regulated in the wt strain and the mutant (15,39), we searched in Supplementary Figure S5A shows that there are many more A-rich unmapped tails in the wild-type strain. On the other hand RNAs with unmapped tails in the pcnB strain correspond to nucleotides added by PN-Pase (15,39) or could be the result of experimental artifacts. By choosing a cut-off, defining the most asymmetrical points in the wt strain (depicted by the dashed line in Supplementary Figure S5), we retrieved 878 unmapped 3'-ends, which cluster into 138 regions (Supplementary Table S4).

Newly identified polyadenylated molecules
mRNAs. A striking new example of a polyadenylated RNA is the rplT mRNA, encoding the L20 ribosomal protein (Supplementary Table S4). The rplT RNA levels were considerably higher in the pcnB mutant in both the PSS and Int fractions (Supplementary Table S2).

Analogous results were obtained for

rep sequences represent a major class of polyadenylated transcripts (5/110) which are terminated by Rho-dependent (SibD) and Rho-independent terminators (IstR and GlmY) (41,42), indicating that Rho-dependent terminators are also poly(A) targets (Supplementary Table S4). No poly(A) tails were detected at the internal polyadenylation site of GlmY (9,10) but rather at the extremity of its Rho-independent terminator, which correlates with the down-regulation of the full length sRNA.

These examples illustrate another proposed function of poly(A)polymerase, which is to participate in the matura-
Table 1. Differential expression analysis

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<th>(Int)</th>
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<td>Up-high</td>
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<td>Up-medium</td>
<td>41</td>
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<tr>
<td>unchanged</td>
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<td>Down-medium</td>
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The values indicate the number of coding and non-coding regions up- and down-regulated in the pcnB mutant in the untagged (Int) and PSS fractions. Cut-off values for gene expression fold changes higher than log₂FC = 2.0 determined up-high and up-down transcripts, while up-medium and down-medium transcripts present a FC determined as (1.5 < log₂FC < 2).

Figure 2. Polyadenylation sites at the 3’ end of rplT. (A) Signals from unmapped tails detected in the 3’-UTR of the rplT transcript in the wt and the pcnB strain are presented with A residues in red, C in blue, G in green, U in orange and total in grey. Sequence and predicted RNA structure obtained with RNAfold (ViennaRNA package 2) of the 3’end of rplT containing the Rho independent terminator are indicated. (B) Tail pile-up signals obtained with the estimator, representing the number of residues and their A content in the unmapped sequences in the wt (red) and pcnB (blue) strains (see Materials and Methods). (C) Expression profile of 3’ end of rplT in the Int fraction in both strains. Post-transcriptionally A-added nucleotides detected as unmapped 3’ additions are indicated in red dots in the wt strain.

Figure 3. Small structured RNA molecules accumulate in the pcnB mutant

In order to further investigate the involvement of PAP I in the degradation of RNAs, we next analyzed PSS tagged molecules, which are more abundant in the pcnB strain. Their sequences were extracted, the resulting fragments were folded in silico and the minimum free energy of folding (mfe) of these molecules was calculated (Materials and Methods). This pool of fragments includes 102 short RNA fragments from full-length transcripts (including ptsG, pheS, malT and fur fragments), 21 REP sequences and 6 sRNAs, whose lengths range from 75 to 342 nt. They have stronger secondary structures than random sequences, and their level of accumulation is significantly higher than folding energy of random sequences (Figure 3). This is particularly true for REP sequences which, as shown above, are major targets of polyadenylation, thus confirming the direct role of PAP I in the destabilization of these stable RNA fragments. Most of these fragments had a mfe below 0.35 kcal/mol/nt (Figure 3). This value could represent the limiting energy above which an RNA structure requires addition of poly(A) for degradation. These small structured RNAs are thus likely to be direct targets for PAP I, even though poly(A) tails were not found on all of them using the estimator.

Poly(A)polymerase deficiency increases the level of specific regions of transcripts

Next we wanted to determine whether poly(A)polymerase also impacts the level of functional/full size transcripts (both mRNAs or non-coding RNA). We compared the FC values of RNA in the wt and the pcnB mutant in the Int fraction versus the FC values in the PSS fraction (Supplementary Figure S7). The majority of RNAs upregulated in the pcnB mutant shows a higher fold change in the PSS
fraction indicating that it was predominately endonucleolytically generated RNA fragments which were stabilized in the absence of PAP I, and they may also represent di- 

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frac, which is regulated by the sRNA GlmY, itself a direct poly(A) target (9).

We found 65 RNAs containing REP sequences which were upregulated in the PSS fraction in the penB mutant, which have similar FC in the Int and the PSS fractions could correspond to indirect targets, such as glmS, which is regulated by the sRNA GlmY, itself a direct poly(A) target (9).

Full size functional RNAs upregulated in the PAP I mutant

In addition to the direct effects on small RNA fragments, Poly(A) Polymerase also affects the abundance of functional, full length transcripts, of both sRNAs and mRNA.

Thirteen sRNAs, were present in higher amounts in the penB strain, and were detected in the PSS and Int fractions (Supplementary Table S2). In the case of SroH and SraF both full-length transcripts and processed forms accumulated in the penB mutant (Supplementary Figure S9 and Table S2) and poly(A) sequences were detected at the 3’ extremity of the SroH primary transcript (Supplementary Table S3). In the case of RyjA (SraL) (Supplementary Figure S9) which is also polyadenylated (Supplementary Table S4) (44,45) only the full-length transcript accumulated in the mutant. This indicates that the degradation of the primary transcript was initiated by polyadenylation.

The flu transcript (encoding the surface antigen, Ag43) is an example demonstrating the stabilization of whole mRNA in the penB mutant (Supplementary Tables S2, S3 and Figure S9GH). Its expression is controlled by antitermination of the upstream IsrC RNA (46). However, the stabilized flu mRNA did not contain IssC, indicating that issC-flu transcript had been processed and the leader region degraded. Although Ag43 expression is controlled by OxyR (repression) and Dam (activation) (47), there was no substantial variation in the levels of the oxyR or dam transcripts upon penB inactivation (Supplementary Table S2). At the moment we cannot distinguish between a direct or indirect effect of penB on flu levels.

Poly(A)polymerase affects the levels of some toxin–antitoxin pairs

The dimQ-agr locus shows the classical features of a Type I toxin/antitoxin (TA) system. It encodes AgrA and AgrB sRNAs transcribed in the opposite direction to dimQ with their own promoters and terminators (48). Both were up-regulated in the mutant and AgrB was polyadenylated in the wt (Supplementary Table S2 and Figure S6). Shorter AgrA/B forms were also detected in the penB strain, revealing that these RNAs were processed (Figure 4AB). Formation of the dimQ RNA/AgrB complex inhibits the endonucleolytic cleavage that normally produces the translatable dimQ mRNA (48). The dimQ transcript was less abundant in the penB mutant, probably as a consequence of the higher levels of AgrA and AgrB in the same strain (Figure 4AB). SibD and IstR, two other polyadenylated Type I antitoxin sRNAs, were more abundant in the mutant strain as well as their corresponding 1bsD and 1tsB toxin RNA (Supplementary Tables S2 and S4). The chromosome of E. coli encodes for five Hok-like toxins with an associated Sok antisense RNA, among which only SokB displayed a 3-fold higher level in the penB mutant (Supplementary Table S2). Two other antisense sRNAs, 1bsA and 1tsB were abundant and downregulated in the mutant by 2- and 2.5-fold, respectively. They are transcribed antisense to the 1bsD and 1tsB genes, encoding short toxic proteins (49) whose transcripts were nearly undetectable (Supplementary Table S2). These examples demonstrate that polyadenylation, by modulating the levels of some RNA antitoxins, participates in the control of Type I TA systems.
Antisense transcripts are only minimally affected by \textit{pcnB} inactivation

Our RNAseq analysis detected transcripts on the opposite strand of open reading frames in the Int fraction (Supplementary Table S2). Some of these antisense transcripts have already been described; e.g. antisense to sRNAs MicA or ArcZ, in a RNase III deficient strain (23,50) and to mRNA, e.g. \textit{asutB} and \textit{ashole} (24). We also detected two new antisense transcripts; \textit{aslpp} and \textit{asdeoA} transcripts (Supplementary Table S2). However, \textit{pcnB} inactivation had no impact on their levels or sizes (data not shown). Our analysis also detected antisense transcripts in the \textit{ykfBK} operon from the CP4-6 prophage (Supplementary Table S2). It did not reveal any \textit{ykfF} transcripts, while an \textit{asykF} RNA about 500 nt in length was detected in the wild-type strain which was slightly, more abundant in the \textit{pcnB} mutant. In contrast, \textit{ykfB} transcript was more abundant in the \textit{wt} than in the mutant and an \textit{asykF} about 200 nt long was only detected in the mutant (Figure 4CD). It was recently shown that the \textit{yafY} gene located upstream of \textit{ykfF} contains a binding site for the FliA sigma factor that drives significant expression of \textit{ykfF} (51). The increased expression of \textit{fliA} in the \textit{wt} strain compared to the \textit{pcnB} mutant could be responsible for the higher level of \textit{ykfB} mRNA in the \textit{wt} strain.

RNAs downregulated in the PAP I mutant

Transcripts downregulated in the \textit{pcnB} strain are expected to be indirect targets of the destabilizing function of PAP I, since no transcripts are known to be stabilized by poly(A) addition in \textit{E. coli}. We previously showed that polyadenylation positively controls the expression of FliC, FliA and FlhD (11). The RNAseq analysis showed that this down-regulation in the \textit{pcnB} strain extends to the whole FliA regulon, controlling flagella biosynthesis (Supplementary Table S2, Figure S10).

\textit{TnaC} is a regulatory peptide encoded by the leader of the \textit{tnaAB} operon, which codes tryptophanase (52,53). \textit{tnaC}, \textit{tnaCA} and \textit{tnaCAB} transcripts were all down-regulated in the \textit{pcnB} mutant (Supplementary Figure S11A and B, Supplementary Tables S2 and S3). Global investigation in enterohemorrhagic \textit{E. coli} (EHEC) showed that expression of the analogous \textit{tnaABL} operon was upregulated when the GlmY and GlmZ sRNAs were inactivated (54). Thus, the higher levels of these two sRNAs in the \textit{pcnB} mutant could explain the lower level of \textit{tnaCAB} transcripts in this strain (9).

A transcript the size of \textit{srlAEBD} was down-regulated in the \textit{pcnB} mutant (Supplementary Figure S11C and D, Supplementary Tables S2 and S3). \textit{pcnB} inactivation also decreased by 3-fold the level of the downstream \textit{srlM} (\textit{gutM}) activator, with a smaller effect on the downstream \textit{srlR} repressor (Supplementary Tables S2 and S3). This modifica-
tion of the balance between the activator and the repressor may explain the down-regulation of transcripts from this operon in the mutant.

The *fluACDB* operon, which encodes components of the ferrichrome transport system, was down-regulated in the mutant but with *fluC* and *fluD* more strongly affected (Supplementary Tables S2 and S3 and Figure S11E).Northern analysis, using a probe specific for the 5′ part of the *fluA* gene, revealed a smear of degraded RNA both in the wt and the *pcnB* mutant (Supplementary Figure S11F). These RNA fragments were produced by a cleavage in the 3′-extremity of *fluA* mRNA as revealed by probing the 3′-part of the messenger (Supplementary Figure S11G). This cleavage initiated the 3′-5′ degradation of the 5′ part of the *fluA* message and generated the smaller 3′ RNA fragment of about 700 nt, detected only in the mutant. This cleavage is independent of RNase E or RNase III in spite of the stabilization of the *fluA* transcript when RNase E is inactivated (Supplementary Figure S12). Surprisingly, the PAP I-dependent down-regulation of the full-length transcript remained dominant in the absence of both RNase III and RNase E activity. Hfq inactivation had no impact on the level or the cleavage of the transcript in the wt or the *pcnB*, which would rule out a role for a Hfq-dependent sRNA (Supplementary Figure S12). Another hypothesis, that PAP I may modulate the level of a transcription regulator, also seems unlikely, since the 3′-part of *fluA* mRNA was still detectable at a high level in the *pcnB* mutant. Fur is an inhibitor of *fluA* transcription (55), which may contribute to the *pcnB*′ mediated accumulation of *fluA* since fur mRNA increased in the *pcnB* strain (Supplementary Figure S8H band c). Altogether, this shows that poly(A) polymerase can exert complex and indirect effects that have major consequences on gene expression in *E. coli*.

**DISCUSSION**

The destabilizing activity of 3′ poly(A) addition is involved in RNA degradation and quality control found in almost all organisms. While the predominant function of poly(A) tails is to facilitate degradation of short structured RNA decay intermediates, Poly(A)polymerase also impacts gene expression (2,4). In this study, we identify new RNAs whose expression level changes in the *pcnB* strain (Supplementary Tables S2 and S3) and RNAs that harbor a poly(A)tail (Supplementary Table S4), revealing the landscape of RNA polyadenylation at the transcriptome level. This work provides extensive new evidence that PAP I controls gene expression both positively and negatively in *E. coli*.

The major targets of poly(A) addition are short structured RNAs and it is therefore not surprising that structured REP containing RNA fragments are abundant poly(A) targets. Interestingly REP RNA fragments are poly(A) targets but not REP containing transcripts, indeed when part of long functional mRNAs, REP have little effect on poly(A) sensitivity of the mRNA. We show that both Rho-dependent and Rho-independent terminated RNAs are PAP I targets. Several new sRNAs as exemplified by regulatory RNAs, Type I antitoxin RNAs of TA systems and tRNA precursors were also modulated by poly(A)polymerase.

Our experiments also give invaluable information on 3′-5′ degradation mechanisms of individual RNAs. In the wt strain, using a rather stringent criteria, we identified only 110 poly(A) sites out of a total of 16,867 non-encoded 3′ ends. Their detection means that at these sites oligo(A) removal is slower than its addition. The repartition of poly(A) tails at the 3′ end of Rho-independent terminators together with a reduction in transcript abundance in the wt strain reveals the difficulty of 3′-5′ exoribonucleases to overcome these secondary structures and the need to add A residues to degrade these molecules (Figure 2, Supplementary Figure S6). In contrast in the *pcnB* mutant the vast majority of 3′ non-encoded ends are spread over this secondary structure and most, if not all, transcripts have their 3′ end at the terminator. This led to the conclusion that the unmapped tails detected in the *pcnB* mutant have no role in RNA decay. The nucleotide content of these 3′ end tails does not present any preference for A, C, G or U residues and does not support a previous finding of residual polyadenylation in the mutant (39). Moreover there is no evidence for any uridylation of *E. coli* RNAs, that has been reported to participate in the control of RNA stability in various eukaryotes (56,57). Whether the heterologous tails detected in our RNAseq analysis are synthesized by PNPase (15,58) or correspond to experimental noise is unknown.

Three classes of RNA molecules were individually quantified in our analysis; 5′ tri- (TSS), 5′ mono-phosphorylated (PSS) fragments and the bulk RNA after fragmentation (Int). Very few transcripts with a 5′-triphasphate with the exception of sRNAs, were affected by inactivation of PAP I (Supplementary Table S2). This may be due to the conversion of the 5′-terminus of primary transcript from a triphosphate to a monophosphate by RppH (35) (Figure 1B and Supplementary Figure S1D) or to the presence of a stable hairpin at the 5′ end, making them inaccessible to RNA polyphosphatase and/or to 5′-ligation (Supplementary Figure S1C). Either scenario would lead to an underestimation of the number of TSS tagged RNA molecules. As a consequence we cannot draw any conclusion on a difference in the polyadenylation between primary and processed targets. The recovery of all kinds of transcripts after fragmentation as untagged RNAs reveals additional full-length transcripts misregulated in the *pcnB* strain, as exemplified by *srlAEBD* or *tnaA(C)* mRNAs. Numerous RNAs harboring a PSS tag were also affected. They should correspond to indirect PAP I targets, via an effect of polyadenylation on a regulator or a sigma factor (e.g. FliA, see below). When upregulated in the *pcnB* mutant, they mostly corresponded to degradation products generated by an endoribonuclease and are direct PAP I targets. This is exemplified by REP-containing transcripts and transcripts with Rho-independent terminators. One possible explanation is that degradation of translated (and unstructured) transcripts mainly depends on endoribonucleolytic cleavages that generate fragments, which are further degraded by exonucleases without the help of poly(A) tails, while the structured 3′ terminators and REP sequences require PAP I to facilitate their exonucleolytic decay as previously proposed (17,59). However, when the initial cleavage is inhibited or inefficient, these transcripts are
The first number represents the number of regulated transcripts with a log2FC polyadenylated at their 3' ends and degraded through the poly(A) dependent exonucleolytic pathway (18).

A recent paper proposes a new function for a REP containing RNA in the organization of the E. coli chromosome by connecting chromosomal domains through the histone like protein HU (60). The determinant role of PAP I in the degradation of REP containing RNAs may impact such regulatory network. Finally, the many RNA fragments, which are mostly small and highly structured and sometimes harbor a Rho-independent terminator as exemplified by the pscG 3' extremity pose questions on their possible biological functions. We hypothesize that some of them may interact with other RNA molecules and therefore regulate gene expression as reported for the 3' part of the gltL transcript (61).

One other notable outcome of this work is the generalization of our previous observation, that FliC is down-regulated in the absence of polyadenylation (11), to an effect on the whole FliA regulon (Supplementary Figure S10 and Table 2). The levels of the vast majority of the transcripts of the Class 2 flagella operon flhLMNOPQR, and Class 3 operons that encode products required in late flagellar assembly, chemotaxis and cyclic-di-GMP regulation of motility (51) were decreased in the penB mutant. In agreement, the FliA promoters located inside flhc, yjdA and yajF genes also drive increased transcription of the downstream motA, yjeZ and ykJB genes in the wild-type strain (51). The flhDC operon that encodes the master regulator of the flagellar gene cascade acting upstream of FliA is targeted by numerous regulators. For example, the 5' untranslated region of the flhDC mRNA is targeted for repression by ArcZ, OmrA, OmrB and OxyS sRNAs and for activation by McaS (62,63). Our observation that McaS sRNA is three times more abundant in the wild-type strain than in the penB mutant could be one clue to the mechanism of how PAP I enhances expression of FlhDC (11). However we cannot exclude the involvement of another regulator in the poly(A) dependent control of fliA transcription.

tar, tap, trg encode methyl-accepting chemotaxis proteins that together with cheA and cheY products, are the signaling intermediates, which allow the bacteria to sense the environmental stimuli leading to chemotaxis (64). This permits the bacteria to change either the rotational direction or speed of flagella rotation and causes tumbling of the cell. Our observation that all these transcripts are less abundant in the penB mutant explains the decreased motility of the mutant on soft agar (11). Another finding of this work concerns the misregulation of numerous membrane proteins in the absence of PAP I (Table 2). Membrane proteins make up 22% and 27% of up- and down-regulated transcripts, respectively, which is in agreement with the increased sensitivity of the penB mutant to cell envelope stress (11). The fact that many membrane proteins are regulated by sRNAs, together with the major effects of poly(A) polymerase on the degradation of numerous regulatory RNAs (Table 3), provides an important clue on how polyadenylation may impact on membrane protein expression. This is exemplified by SroH sRNA whose function is still unknown, but the sroH-deleted mutant is sensitive to cell envelope stress (65).

Finally we also show that poly(A) polymerase is part of the fine-tuning of Type I TA systems, by modulating the level of antitoxin RNAs (AgrB, SibA, SibB, SokB RNAs).

We suspect that this function is underestimated due to the low level of expression of these RNAs in our experimental conditions.

This study has enlarged the vision we had on the role of polyadenylation, which is not only a way to eliminate molecular waste but which contributes to more complex regulatory network acting post-transcriptionally to control gene expression in bacteria.

<table>
<thead>
<tr>
<th>Table 2. Functional categories of genes differentially expressed in the penB mutant</th>
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<tbody>
<tr>
<td>geneclass</td>
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<tr>
<td>membrane protein</td>
</tr>
<tr>
<td>flagella</td>
</tr>
<tr>
<td>miscellaneous</td>
</tr>
<tr>
<td>RNA metabolism</td>
</tr>
<tr>
<td>ammonium metabolism</td>
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<tr>
<td>prophase</td>
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<tr>
<td>fimbriae</td>
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</tbody>
</table>

Up- and down-regulated transcripts in the penB strain in the Int fraction classified by functional categories according to the Gene Ontology database. The first number represents the number of regulated transcripts with a log2FC > 2 and the second, regulated transcripts with 1.5 < log2FC < 2.

<table>
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<th>Table 3. Summary of differential expression analysis</th>
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<tr>
<td>Expression detected only in penB</td>
</tr>
<tr>
<td>sdsR</td>
</tr>
<tr>
<td>micF</td>
</tr>
<tr>
<td>ohsC</td>
</tr>
<tr>
<td>arrS</td>
</tr>
<tr>
<td>sokA</td>
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Genes the expression of which was detected in only one of the strain together with the five most up-regulated and down-regulated genes in penB deficient mutant (positive log2 fold-change indicates a higher expression in the penB strain).

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SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

REFERENCES
