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Growth-rate dependent RNA polyadenylation in *Escherichia coli*

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RNA polyadenylation occurs not only in eukaryotes but also in bacteria. In prokaryotes, polyadenylated RNA molecules are usually degraded more efficiently than non-modified transcripts. Here we demonstrate that two transcripts, which were shown previously to be substrates for poly(A) polymerase I (PAP I), *Escherichia coli lpp* messenger RNA and bacteriophage λ *oop* RNA, are polyadenylated more efficiently in slowly growing bacteria than in rapidly growing bacteria. Intracellular levels of PAP I varied in inverse proportion to bacterial growth rate. Moreover, transcription from a promoter for the *pcnB* gene (encoding PAP I) was shown to be more efficient under conditions of low bacterial growth rates. We conclude that efficiency of RNA polyadenylation in *E. coli* is higher in slowly growing bacteria because of more efficient expression of the *pcnB* gene. This may allow regulation of the stability of certain transcripts (those subjected to PAP I-dependent polyadenylation) in response to various growth conditions.

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INTRODUCTION

Despite the fact that bacterial poly(A) polymerase was described 40 years ago (August *et al*., 1962), specific polyadenylation at the 3′ end of RNA was for a long time believed to be unique to eukaryotic messenger RNAs. Studies of the last ten years, initiated by the discovery of the structural gene for poly(A) polymerase I (PAP I) in *Escherichia coli* (Cao & Sarkar, 1992a), clearly demonstrated that prokaryotic RNAs are also polyadenylated.

Two poly(A) polymerases, PAP I and PAP II, were discovered in *E. coli*. PAP I, which is responsible for over 90% of poly(A) polymerase activity in *E. coli* cells (O'Hara *et al*., 1995; Mohanty & Kushner, 1999a), is encoded by the *pcnB* gene (Cao & Sarkar, 1992a), but the gene encoding PAP II is still unknown (Mohanty & Kushner, 1999b). One could speculate that polynucleotide phosphorylase (PNPase) functions as an exonuclease and also as a poly(A) polymerase, which could account for the activity of PAP II. Various RNA molecules are polyadenylated with different efficiency, and it appears that the susceptibility of certain RNAs to polyadenylation depends on the presence of single-stranded segments at either the 5′ or the 3′ end and monophosphorylation at an unpaired 5′ terminus (Feng & Cohen, 2000; Yehudai-Resheff & Schuster, 2000).

There are many reports indicating that bacterial RNA polyadenylation leads to decreased stability of transcripts (O'Hara *et al*., 1995; Xu & Cohen, 1995; Szalewska-Pałasz *et al*., 1998; Blum *et al*., 1999). Results of other experiments showed either stabilization of specific transcripts, or a lack of effect on the half-lives of other mRNAs after enhanced polyadenylation (Mohanty & Kushner, 1999a). However, transcripts stabilized or unaffected by polyadenylation are very rare, and it is therefore generally accepted that this process may regulate gene expression by promoting RNA degradation (for reviews see Sarkar, 1996; Carpousis *et al*., 1999; Rauhut & Klug, 1999; Regnier & Arraiano, 2000; Steege, 2000).

If RNA polyadenylation plays a regulatory role in gene expression, it follows that the activity or level of poly(A) polymerase in cells should be modulated under certain physiological conditions, such that stability of various RNAs could be regulated in response to environmental factors. However, although there has been remarkable progress in understanding the biochemical properties and functions of PAP I, the biological significance of bacterial RNA polyadenylation, and that of the resultant enhanced transcript degradation, remains unclear. We therefore investigated the efficiency of RNA polyadenylation in *E. coli* under different growth conditions. We employed *lpp* mRNA and bacteriophage λ *oop* RNA as model transcripts. Both transcripts were previously demonstrated to be specifically polyadenylated by PAP I (Cao & Sarkar, 1992b; O'Hara *et al*., 1995; Wróbel *et al*., 1998; Mohanty & Kushner, 1999a), and polyadenylation of *lpp* and *oop* RNAs leads to a significant decrease in their stability in *E. coli* cells (O'Hara *et al*., 1995; Szalewska-Pałasz *et al*., 1998; Mohanty & Kushner, 1999a).

RESULTS

Increased RNA polyadenylation in slowly growing cells

To investigate the efficiency of RNA polyadenylation in *E. coli* cells cultured at different growth rates, we used various culture media. This method was previously proved to be adequate for achieving different generation times of the same strain with

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Table 1 | Generation times of *E. coli* strains growing in different media at 37 °C and copy numbers of plasmid pBR322 in the MG1655 strain under these conditions

Medium	Generation time (min)		Plasmid copy number[a]
	MG1655/pKB2	VH1000:: ppcnB-lacZ	MG1655/pKB2
LB	34	28	15 ± 3
MMGlu	76	50	29 ± 5
MMGly	119	77	42 ± 5
MMSuc	140	86	60 ± 6
MMAce	216	134	76 ± 8

[a]Results from three experiments are shown, as means ± s.d.

relatively minor effects on other physiological parameters (Nilsson *et al.*, 1984; Hadas *et al.*, 1977; Gabig *et al.*, 1998; Węgrzyn *et al.*, 2000). Growth rates of the *E. coli* strains in different media were determined (Table 1).

The percentage of polyadenylated *lpp* and *oop* transcripts was determined using the kinetic polymerase chain reaction with reverse transcription (RT–PCR) method described previously (Ferre *et al.*, 1996; Mohanty & Kushner, 1999a). Using this method, we were able to estimate amounts of polyadenylated RNA and total RNA (which included both polyadenylated and non-polyadenylated RNAs) for each transcript. We demonstrated that putative annealing of the oligo-dT primer to internal stretches of A residues does not impair the analysis, and that RT–PCR amplification is proportional to the amount of polyadenylated RNA (Fig. 1). When the reactions were repeated using RNA isolated from a *pcnB* mutant, very little polyadenylation of *lpp* and *oop* transcripts was observed (data not shown). When different amounts of RNA isolated from *pcnB*$^+$ cells were adjusted to identical amounts of total RNA with RNA isolated from *pcnB* mutant cells, the expected percentage of polyadenylated transcripts was always very similar (less than 5% difference assuming the expected value to be 100%) to the value estimated on the basis of the RT–PCR procedure (data not shown).

We found that efficiency of polyadenylation of the *lpp* transcript depends on bacterial growth rate: the slower the cell growth, the higher the percentage of polyadenylated *lpp* mRNA observed (Fig. 2A). A very similar result was observed for *oop* RNA, transcribed from a bacteriophage λ-derived plasmid (Fig. 2B), despite considerable differences between these two transcripts in the general efficiency of their polyadenylation.

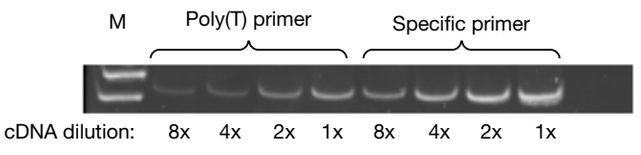

Fig. 1 | An example of RT–PCR experiments. The *lpp* transcript was detected by RT–PCR in samples of RNA isolated from bacteria growing in the MMGly medium. Different dilutions of cDNA were used, as indicated. The specific primer (REVLPP) was diluted to a concentration of one-half that of the poly(T) primer. Lane M contains molecular weight markers.

Level of PAP at various bacterial growth rates

It has been demonstrated that overexpression of the *pcnB* gene, encoding PAP I, results in a significant increase in the percentage of polyadenylated transcripts (Mohanty & Kushner, 1999a). We therefore investigated whether increased efficiency of polyadenylation observed in slowly growing bacteria arises from higher PAP I levels under these conditions.

A hexahistidine (His_6)-tagged truncated form of PAP I was overexpressed, purified and used for the production of a rabbit anti-PAP I serum. The specificity of this serum was proven, as no signal was detected when cell lysates from Δ*pcnB*::*kan* mutants were analysed by performing western blots, whereas PAP I bands were easily detectable in similar samples from wild-type bacteria (Fig. 3). The intensity of the signal was found to be proportional to the amount of PAP I (data not shown).

We found that levels of PAP I are significantly higher in slowly growing cells than in bacteria cultivated in media supporting high growth rates (Fig. 3). These results suggest a correlation between levels of PAP I in *E. coli* cells and the percentage of polyadenylated *lpp* and *oop* transcripts.

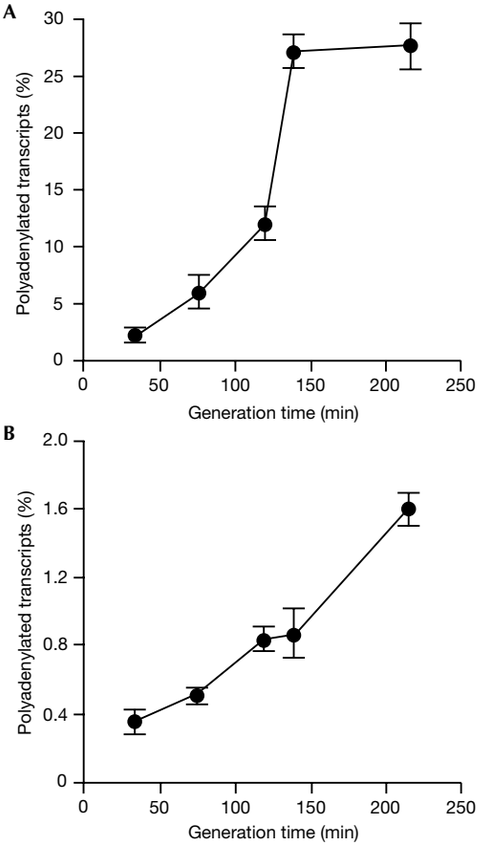

Fig. 2 | Efficiency of polyadenylation of *lpp* and *oop* transcripts in *E. coli* MG1655/pKB2 strain at different bacterial growth rates, as estimated by the kinetic RT–PCR method. The percentage of polyadenylated *lpp* (**A**) and *oop* (**B**) transcripts are shown. Average values from three experiments are shown, with error bars ± s.d.

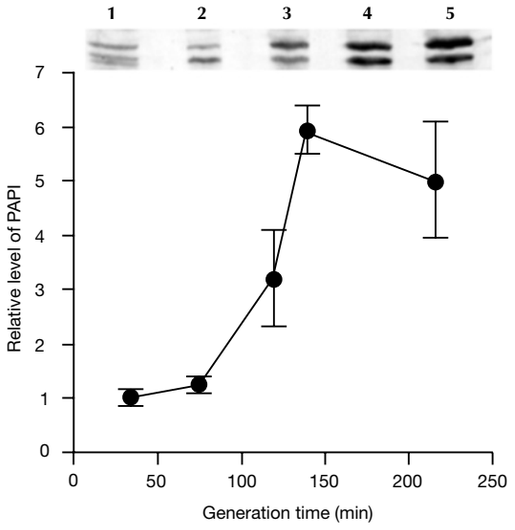

Fig. 3 | Relative amounts of PAP I in *E. coli* MG1655/pKB2 strain at different bacterial growth rates, as estimated by western blotting. A western blot is shown above the graph (lanes, labelled 1 to 5, correspond to consecutive points on the graph). PAP I migrates on denaturing PAGE as a doublet band (see Cao & Sarkar, 1992a). Average values from three experiments are shown, with error bars ± s.d.

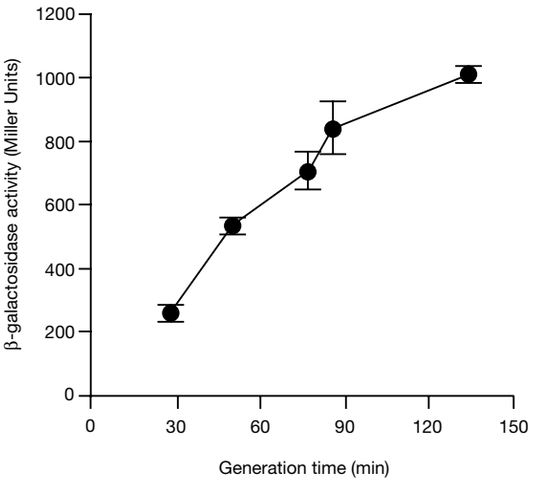

Fig. 4 | Activity of β-galactosidase in an *E. coli* VH1000 host bearing a ppcnB-lacZ fusion at different cell growth rates. Average values from three experiments are shown, with error bars ± s.d.

Growth-rate-dependent activity of the *pcnB* gene promoter

The region of the *pcnB* promoter was cloned into a specific vector to construct a multicopy fusion of this promoter with the *lacZ* reporter gene. A strain bearing a single copy of this fusion was then constructed by transferring the fusion into a bacteriophage λ-derived vector and subsequent lysogenization of the *lacZ* mutant host. The activity of this fusion construct was measured in bacteria growing in various media, and it was found that the *pcnB* promoter is more active in slowly growing cells than in media supporting higher growth rates of *E. coli* (Fig. 4).

Degradation of RNA at different bacterial growth rates

In previous studies, no significant effect of bacterial growth rate on degradation of *lpp* mRNA ws observed (Nillson *et al*., 1984). This is in contrast to our demonstration of enhanced RNA polyadenylation in slowly growing cells. We therefore measured the stability of *oop* RNA and another transcript polyadenylated by PAP I, RNA I of a ColE1-like plasmid, pBR322, in bacteria growing in different media. We found that degradation of both *oop* RNA and RNA I is more efficient at low bacterial growth rates (Fig. 5).

pBR322 plasmid copy number at different host growth rates

RNA I is an antisense transcript which negatively controls replication of ColE1-like plasmids. We therefore measured the copy number of pBR322 in bacteria growing in different media and found that the plasmid is present at higher copy numbers in slowly growing cells (Table 1). These results are compatible with the finding that the degradation of RNA I is dependent on growth rate (Fig. 5), although recent studies indicate that polyadenylation of RNA I may also decrease the interaction of this antisense transcript with the RNA II pre-primer (Xu *et al*., 2002).

DISCUSSION

Polyadenylation of transcripts in bacteria is believed to be involved in the regulation of gene expression by influencing the efficiency of RNA degradation. However, any biological process can have a regulatory significance only if its efficiency depends on physiological conditions. Despite remarkable progress in understanding functions of the main *E. coli* poly(A) polymerase, PAP I, physiological factors controlling the efficiency of RNA polyadenylation have previously been unknown. However, it has been demonstrated that low levels of polyadenylation (at most a small percentage of polyadenylated RNA molecules), observed in *E. coli* strains growing in a rich medium, can be increased significantly by overexpression of the *pcnB* gene (Mohanty & Kushner, 1999a). It therefore appears that the level of PAP I in bacterial cells may be a limiting factor for RNA polyadenylation efficiency. If this is true, physiological conditions that cause enhanced expression of the *pcnB* gene should result in more efficient polyadenylation of certain transcripts, possibly influencing their degradation. Such regulation might be an important element of cellular responses to changing environmental conditions.

Here we demonstrate that polyadenylation of two transcripts, *lpp* and *oop*, is significantly increased in slowly growing cells. Both the activity of the *pcnB* gene promoter and the level of PAP I varied in inverse proportion to bacterial growth rate. Results of our preliminary experiments, in which we tried to detect modified transcripts directly on electrophoretic gels rather than after RT–PCR (see Szalewska-Pałasz *et al*. (1998) for technical details), suggest that the proportion of polyadenylated RNAs, but not lengths of poly(A) tails, vary in bacterial cells growing with different generation times (data not shown). One might argue that this is in contrast to previous reports, which concluded that both the proportion of polyadenylated RNA molecules and the length of poly(A) tails are increased in cells overexpressing PAP I (Mohanty & Kushner, 1999a, 2000). However, these studies used strains highly overexpressing a modified *pcnB* gene from a plasmid, whereas in our experiments the wild-type *pcnB* gene was expressed from a bacterial chromosome. Moreover, the plasmid-mediated *pcnB* overexpression was toxic to bacteria and caused cell

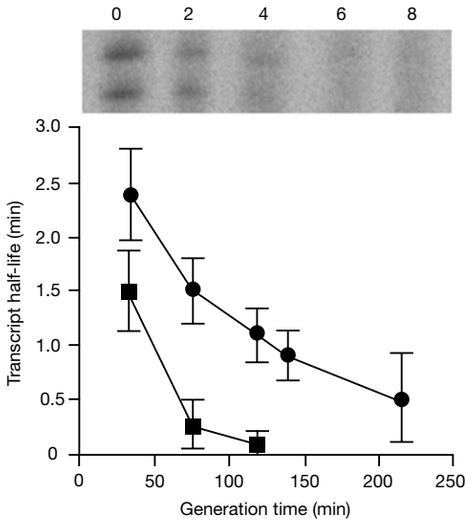

Fig. 5 | Degradation of RNA I and *oop* RNAs in *E. coli* MG1655/pBR322 and MG1655/pKB2 strains at different bacterial growth rates. Half-lives for RNA I (circles) and *oop* (squares) transcripts are indicated. Average values from three experiments are shown, with error bars ± s.d. A representative autoradiograph (samples isolated from bacteria growing in the MMGlu medium and tested for RNA I) is shown above the graph (numbers represent minutes after addition of rifampicin).

death (Mohanty & Kushner 1999a, 2000), whereas bacteria were still growing in all the media used in the experiments described in this report. This indicates that the physiological conditions in both these sets of experiments could be significantly different.

Recent studies have suggested that expression of the *pcnB* gene is regulated at the level of translation (Binns and Masters, 2002). These studies have shown that translation of *pcnB* mRNA is initiated from an unusual and inefficient start codon (AUU), which is subject to a negative regulation such that most initiation events are aborted. In this report we demonstrate that expression from the *pcnB* promoter is dependent on growth rate. It therefore appears that expression of the *pcnB* gene is precisely regulated at various levels. Such precise regulation usually corresponds to a physiological importance of achieving optimal levels of a gene product under particular environmental conditions. Our results indicate that bacteria may adapt the expression levels of certain genes to various growth conditions by changing the levels of polyadenylation of transcripts, and thus by controlling the rates of their degradation. Because poly(A) polymerases act more readily on some transcripts than on others, (Mohanty & Kushner, 1999a; Feng & Cohen, 2000), polyadenylation may significantly influence transcriptomes of bacteria growing under conditions supporting different growth rates.

Growth-rate-dependent regulation of mRNA stability in *E. coli* was demonstrated almost two decades ago by Nilsson *et al*. (1984). In fact, in those studies, some transcripts were found to be less stable in slowly growing cells. One might speculate that this is at least partially due to changes in efficiency of RNA polyadenylation. Although the role of polyadenylation in the decay of full-length transcripts was considered controversial (Coburn & Mackie, 1999) and despite the fact that in a previous study no significant effect of bacterial growth rate on degradation of *lpp* mRNA was observed (Nilsson *et al*., 1984), we have demonstrated that degradation of RNA I and *oop* RNAs depends on bacterial growth rate. The reason for this discrepancy remains to be discovered. One possible explanation is that expression of genes encoding particular RNases may be dependent on growth rate. Moreover, the correlation between levels of polyadenylation and the rate of RNA degradation is not necessarily as straightforward under different cellular growth conditions as in the case of studies performed without changing culture media.

METHODS

Bacterial strains. All experiments were performed in the *E. coli* MG1655 (*pyrE*) strain (Jensen, 1993) and its *pyrE*$^+$ *lacI lacZ* derivative, VH1000 (provided by M.S. Thomas), as hosts.

Plasmids, bacteriophages and gene fusions. A ColE1-like plasmid, pBR322 (Bolivar *et al*., 1977), was used. A standard, wild-type bacteriophage λ plasmid, pKB2 (Kur *et al*., 1987), was used to express *oop* RNA. For production of truncated and His-tagged poly(A) polymerase I (His_6-PAPI-L), plasmid pQPAPL was constructed by PCR amplification of an *E. coli* MG1655 chromosome fragment using *Pfu* DNA polymerase (Fermentas) and primers PCNLATG (5′-CAGAATTCATTAAAGAGGAGCTGAAGGTAATGTACAGGCTC-3′) and PCNLKON (5′-CATAGATCTTGCGGTACCCTCACGACGTGG-3′), digestion of the PCR product with *Bgl*II and *Eco*RI, and its insertion into corresponding restriction sites of the pQE60 vector (Qiagen). Plasmid pHG-ppcnB, bearing a multicopy ppcnB-lacZ fusion, was constructed by cloning a PCR-amplified DNA fragment containing a region of the putative *pcnB* promoter into the *Bam*HI and *Eco*RI sites of the pHG86 plasmid (Giladi *et al*., 1992). The PCR reaction was performed using the following primers: PCNE (5′-ATGGATCCCACCGT CACCTGTGGAC) and PCNECO (5′-TAGAATTCATGCTGAGCT ATGATTAGCCGC). To construct a single-copy ppcnB–lacZ fusion plasmid, *in vivo* recombination between phage vector λB299 (Giladi *et al*., 1995) and plasmid pHG-ppcnB was performed according to a previously described procedure (Giladi *et al*., 1995). The phage strain carrying the ppcnB–lacZ fusion was then used for lysogenization of strain VH1000. The presence of a single copy fusion in the host (VH1000-ppcnB–lacZ) chromosome was verified using a PCR-based method (described in Powell *et al*., 1994).

Culture media and growth conditions. Bacterial strains were cultured at 37 °C in media supporting different growth rates (Table 1). Luria–Bertani (LB) medium (Sambrook *et al*., 1989) and different minimal media were used. The minimal media contained salts (per litre of the medium: 6.0 g Tris, 5.8 g maleic acid, 2.5 g NaCl, 2.0 g KCl, 1.0 g NH_4Cl, 1.0 g $MgCl_2{\cdot}6H_2O$, 0.132 g Na_2SO_4, 0.322 g $Na_2HPO_4{\cdot}12H_2O$; pH 7.2), 0.01 g l^{-1} thiamine, and different carbon sources (one of the following: 2 g l^{-1} glucose (MMGlu), 2 g l^{-1} glycerol (MMGly), 6 g l^{-1} sodium succinate (MMSuc) or 8 g l^{-1} sodium acetate (MMAce)).When appropriate, kanamycin (up to 25 μg ml^{-1}) and/or ampicillin (up to 50 μg ml^{-1}) were added.

Isolation of total RNA and reverse transcription. A sample of bacterial culture was centrifuged and the pellet was frozen in liquid nitrogen. Total RNA was isolated using Total RNA Prep Plus kit (A&A Biotechnology) in accordance with the manufacturer's instructions. Total RNA (1 μg) was used, along with one of the primers described in the next section, for the first-strand complementary DNA synthesis, which was catalysed by Moloney Murine Leukaemia virus reverse transcriptase (Fermentas) in accordance with the manufacturer's instructions.

Quantification of polyadenylated RNA by RT–PCR. The percentages of polyadenylated *lpp* and *oop* transcripts were determined using the kinetic RT–PCR method (Ferre *et al*., 1996). Total RNA (1 μg) was used in reverse transcription reactions using the poly(T) primer, $(T)_{18}$, which results in the production only of cDNAs reverse-transcribed from polyadenylated RNAs, and 3′ end gene-specific primers (REVLPP: 5′-TGTTGTCCAGACGCTGGTTAG-CACGAG-3′ for *lpp*, and REVOOP: 5′-CGGCGGCAACCGAG-3′ for *oop*), which amplify the gene-specific total cDNA, including both polyadenylated and non-polyadenylated transcripts. These cDNAs were amplified in separate RT–PCR reactions using a 5′-specific primer for either *lpp* (LPP1: 5′-TGGTACTGGGCGCG-GTAATCCTG-3′) or *oop* (OOP2: 5′-GTTGATAGATCCAGTAAT-GACCTCAG-3′) and the 3′-specific primer (REVLPP for *lpp* or REVOOP for *oop*). During the exponential phase of the PCR, amplified products were run on a 6% polyacrylamide gel in TBE buffer (Sambrook *et al*., 1989), and the gel was stained with ethidium bromide. Quantification of band densities was performed in the Kodak Digital Science 1D system. The percentage of polyadenylated RNA was calculated based on the densitometrically determined values obtained from poly(A)-dependent cDNA versus total cDNA.

Overproduction and purification of the truncated and His-tagged poly(A) polymerase I (His_6-PAP I-L). The *E. coli* JM109 strain (Yanisch-Perron *et al*., 1985) bearing pQPAPL was grown at 37 °C in LB medium supplemented with ampicillin (50 μg ml^{-1}) until the culture reached an A_{578} of 0.3. The culture was then induced with 1 mM isopropyl-β-D-thiogalactoside for 2 h. The truncated and His-tagged poly(A) polymerase I (His_6-PAPI-L) was purified under denaturing conditions using Ni^{2+}-nitrilotriacetate affinity chromatography (Qiagen), in accordance with the manufacturer's instructions.

Production and testing of anti-PAP I serum. Purified His_6-PAPI-L protein was dialysed against 0.9% NaCl. This protein (0.5 mg) was used for immunization of a rabbit as described by Harlow & Lane (1988). The anti-PAP I serum obtained was tested by western blotting with protein lysates of an *E. coli* wild-type (MG1655) strain and its Δ*pcnB*::*kan* derivative (Wróbel *et al*., 1998).

Western blotting analysis. Samples of equal bacterial mass (corresponding to an A_{578} of 0.4) were collected by centrifugation (4,000*g* for 10 min) and frozen in liquid nitrogen. Cell lysates, prepared by boiling of cells in loading buffer (Sambrook *et al*., 1989), were separated electrophoretically in SDS–polyacrylamide gels and proteins were transferred to polyvinylidene difluoride membranes. The proteins were detected using ECL Western-blot detection reagents (Amersham Life Science) and a PhosphorImager (Bio-Rad). Bands were quantified using the Kodak Digital Science 1D system.

Estimation of β-galactosidase activity. Activity of β-galactosidase in *E. coli* cells was estimated according to Miller (1972).

Estimation of RNA degradation rates. Bacteria carrying either pBR322 or pKB2 plasmids were grown in various media to mid-log phase. Rifampicin was added up to 200 μg ml^{-1} to inhibit transcription. Samples were withdrawn at different times, total RNA was isolated as described above, and amounts of specific transcripts were determined by primer extension as described previously (Hajnsdorf *et al*., 1995), using the gene-specific primers REVOOP for *oop* and COLREV (5′-TACCAACGGTG GTTTGTTTGCCGG) for RNA I.

Estimation of plasmid copy number. Plasmid copy number in bacteria growing in different media was estimated as described previously (Węgrzyn *et al*., 1996).

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