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# Importance of a 5′ Stem-Loop for Longevity of *papA* mRNA in *Escherichia coli*

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**High-level expression of the major pilus subunit (PapA) of uropathogenic strains of *Escherichia coli* results in part from the unusually long lifetime of the mRNA that encodes this protein. Here we report that the longevity of *papA* mRNA derives in large measure from the protection afforded by its 5′ untranslated region. This *papA* RNA segment can prolong the lifetime of an otherwise short-lived mRNA to which it is fused. In vivo alkylation studies indicate that, in its natural milieu, the *papA* message begins with a stem-loop structure. This stem-loop is important for the stabilizing effect of the *papA* 5′ untranslated region, as evidenced by the significant acceleration in *papA* mRNA decay that results from its removal.**

The pyelonephritis-associated pili (*pap*) genes of uropathogenic strains of *Escherichia coli* encode the surface fimbrial structures by which *E. coli* cells attach themselves to the epithelium of the upper urinary tract, a critical step in pathogenesis (11). The protein products of the *pap* gene cluster are involved in pilus assembly and in the regulation of *pap* gene expression. Two of these genes, specifying the major pilus subunit PapA and the transcription factor PapB, are cotranscribed from an inducible promoter as a dicistronic *papBA* operon transcript. Despite their coordinate transcription, the PapA protein is produced in substantial molar excess over PapB due to a posttranscriptional regulatory mechanism involving the differential stability of the *papB* and *papA* segments of the 1.2-kb *papBA* transcript (3). The 5′-terminal *papB* segment of this transcript is rapidly degraded with a half-life of 2 to 3 min, whereas the 3′ *papA* segment decays slowly, with a half-life of 20 to 30 min, after being liberated as a discrete 0.7-kb mRNA processing product by RNase E cleavage at an intercistronic site (3, 19). The greater longevity of the *papA* message allows this mRNA to accumulate to a large molar excess over its *papBA* mRNA precursor, thereby accounting for much of the difference in expression of the *papB* and *papA* genes.

We set out to investigate the basis for the longevity of *papA* mRNA. Our studies indicate that the lifetime of this mRNA is determined primarily by its 5′ untranslated region (UTR) and that a stem-loop near the 5′ end of this UTR helps to protect the message from degradation.

**The *papA* 5′ UTR can stabilize a heterologous mRNA.** Earlier studies had shown that the unusual longevity of the *E. coli ompA* transcript is a consequence of its 5′ UTR, which functions as an mRNA stabilizer capable of prolonging the lifetime of a variety of heterologous messages to which it is fused (4, 10). To determine whether the 5′ UTR of *papA* can likewise function as an mRNA stabilizer, we decided to investigate whether the longevity of the short-lived *bla* message increases when its 5′ UTR is replaced with that of *papA*. To facilitate these studies, we first constructed a gene (*papAΔ3*) encoding a monocistronic *papA* message expressed from a constitutive promoter as a primary transcription product. Except for the absence of three nucleotides (AUU) from the 5′ end and the presence of a 5′-terminal triphosphate, this pseudo-wild-type transcript is identical to the *papA* message that arises by RNase E processing of the dicistronic *papBA* transcript in *E. coli* cells bearing the entire *pap* gene cluster. We then constructed a plasmid (pPBB1E) encoding a hybrid *papAΔ3-bla* transcript (*pbb1*) in which the 84-nucleotide *papAΔ3* 5′ UTR was joined precisely to the 286-codon protein-coding region and 3′ UTR of the short-lived *bla* message (Fig. 1A). As an internal control, this plasmid also bore a copy of the *papAΔ3* gene. For comparison, we constructed an additional plasmid (pBLAE) bearing both the wild-type *bla* gene and the *papAΔ3* gene.

*E. coli* cells containing either pPBB1E or pBLAE were grown exponentially at 37°C. Rifampin was added to halt further initiation of transcription, and total cellular RNA was extracted from culture samples withdrawn at time intervals thereafter. The decay of *pbb1*, *bla*, and *papAΔ3* mRNA was then examined by S1 protection analysis of the extracted RNA samples (Fig. 1B) with a mixture of 5′-end-labeled DNA probes complementary to the first 0.3 kb of the *papAΔ3* transcript or the first 0.8 kb of *bla* mRNA. These studies revealed that the half-life of *bla* mRNA increases more than fourfold when its 5′ UTR is replaced with the 5′ UTR of *papAΔ3*, rising from 1.9 ± 0.4 min (*bla* mRNA) to 8.9 ± 1.1 min (*pbb1* mRNA). This half-life of the *papAΔ3-bla* mRNA hybrid is approximately half as long as that of the pseudo-wild-type *papAΔ3* transcript in the same cells (18 ± 2 min, a value similar to the half-life reported for wild-type *papA* mRNA in *E. coli* [3]). These findings show that the *papA* 5′ UTR can act in *cis* to prolong the lifetime of a heterologous message to which it is fused, which in turn suggests that this 5′ RNA segment plays a key role in protecting the *papA* message from degradation in *E. coli*.

**Secondary structure of the *papAΔ3* 5′ UTR in *E. coli*.** Previously, the secondary structure of the *papBA* intercistronic region was analyzed in vitro by cleavage with structure-specific ribonucleases (16). Because a variety of environmental perturbations in vitro (including the absence of bound ribosomes) can prevent mRNA from assuming its natural in vivo conformation, we decided to examine the secondary structure of the *papA* 5′ UTR in *E. coli* as a step toward understanding the

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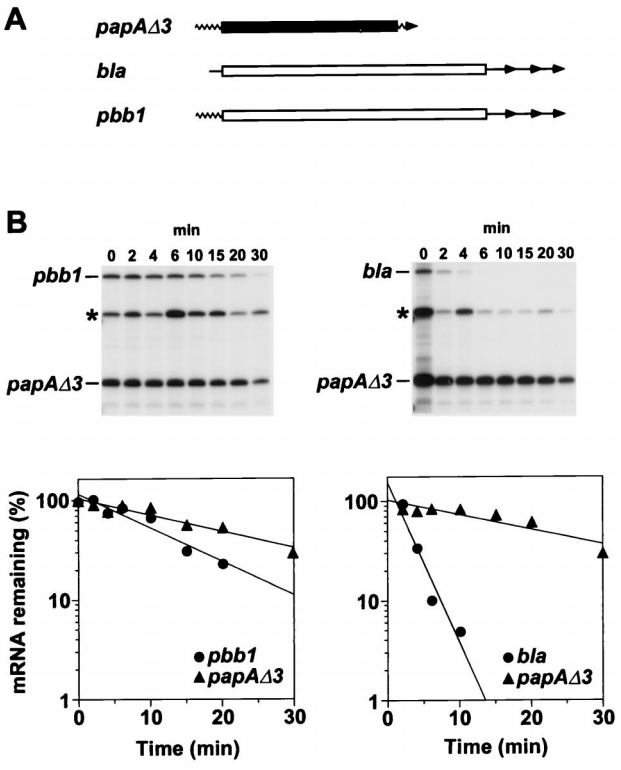


FIG. 1. Decay of a hybrid *papA-bla* mRNA. (A) The *papA-bla* hybrid transcript *pbb1* is represented diagrammatically along with its mRNA progenitors *papAΔ3* and *bla*. These mRNAs were expressed from plasmid pPBB1E or pBLAE, each a derivative of plasmid pBLA200 (8), by transcription from a *bla* promoter. Wavy lines, *papA* UTRs; straight lines, *bla* UTRs; solid rectangle, *papA* coding region: open rectangles, *bla* coding region; arrowheads, mRNA 3′ ends. There are three alternative sites of transcription termination downstream of the *bla* gene. (B) Cultures of a *recA* mutant derivative of *E. coli* MG1693 (2) harboring either pPBB1E (left) or pBLAE (right) were grown exponentially for several generations at 37°C in Luria-Bertani medium supplemented with glucose (0.4%) and Casamino Acids (0.5%). At time intervals after transcription inhibition with rifampin (200 μg/ml), total cellular RNA was isolated. Equal amounts of each RNA sample (2 μg) were then analyzed by S1 protection with a mixture of two 5′-end-labeled probes complementary to a 5′-terminal segment of *papAΔ3* mRNA or *bla* mRNA. The radioactivity in bands that correspond to *papAΔ3*, *pbb1*, and *bla* mRNA was quantitated with a Molecular Dynamics PhosphorImager. Also marked is a band corresponding to the reannealed *papA* probe DNA (*). Beneath each autoradiogram is a semilogarithmic plot of mRNA concentration versus time. Half-lives were calculated from the slope of each plot, and half-life errors were estimated from the standard deviation of the slopes. The measured half-lives were 8.9 ± 1.1 min for *pbb1* mRNA and 18 ± 2 min for *papAΔ3* mRNA (left) and 1.9 ± 0.4 min for *bla* mRNA and 20 ± 4 min for *papAΔ3* mRNA (right). For procedural details concerning bacterial cell growth, RNA isolation, and S1 analysis, see reference 8.

basis for mRNA stabilization by this RNA segment. This was accomplished by chemical modification with dimethylsulfate (DMS), an alkylating agent whose reactivity is sensitive to base pairing (14). DMS alkylates unpaired adenosine residues at N1 and unpaired cytidine residues at N3. In contrast, 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) alkylates unpaired uridine residues at N3 and, to

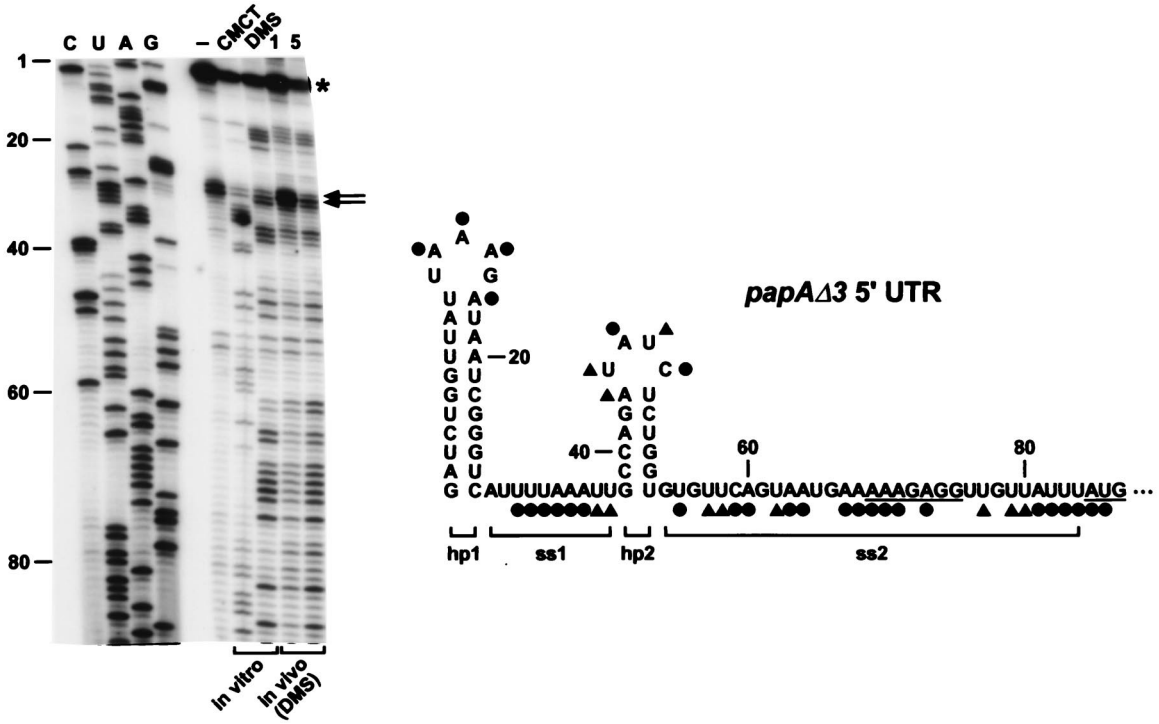


FIG. 2. Alkylation of the 5′ UTR of *papAΔ3* mRNA. (Left) Primer extension analysis of alkylated *papAΔ3* mRNA. Total cellular RNA was isolated from an exponential-phase culture of *E. coli* C600S (17) containing pPAPAΔ3 after treatment of aliquots of the culture with DMS (in vivo, 1 or 5 μl per ml of culture). In addition, samples of RNA extracted from an untreated culture were alkylated in vitro with DMS or CMCT. Sites of alkylation were mapped by primer extension with avian myeloblastosis virus reverse transcriptase and a 5′-end-labeled DNA primer (5′-AAGACACCACTGCCATAGCT-3′) complementary to the coding region of *papA* mRNA. The resulting extension products were then analyzed by gel electrophoresis beside sequencing ladders that were generated by extension of the same 5′-end-labeled primer on a *papAΔ3* DNA template. Unalkylated RNA (lane −) served as a negative control to identify primer extension products unrelated to alkylation. Blockage of primer extension by an alkylated RNA base results in the production of a complementary DNA fragment one nucleotide shorter than that arising from incorporation of a dideoxynucleotide opposite the same base. In the experiment shown, CMCT did not react detectably with guanosine nucleotides, precluding a direct assessment of base pairing by those residues. The sequencing lanes (C, U, A, G) are labeled to indicate the sequence of the RNA, not the complementary DNA. Calibration is in nucleotides from the *papAΔ3* 5′ end. An asterisk marks the site of transcription initiation. The degree of chemical modification can be difficult to assess at sites where it is no greater than the basal level of termination by reverse transcriptase on an unalkylated RNA template. With the avian myeloblastosis virus enzyme, such sites often correspond to the 3′ boundary of secondary structure elements of significant thermodynamic stability (6), and in this case, the two major sites of premature termination on unalkylated RNA (marked by arrows) map to the foot of the 5′-terminal *papAΔ3* stem-loop. For procedural details, see reference 9. (Right) Summary of the alkylation data for the *papAΔ3* 5′ UTR. ●, heavy alkylation; ▲, moderate alkylation. Brackets delineate the boundaries of the four structural domains (hp1, ss1, hp2, and ss2) within the *papAΔ3* 5′ UTR. The Shine-Dalgarno element and initiation codon are underlined.

a lesser extent, unpaired guanosine residues at N1 (14). Nucleotides engaged in Watson-Crick base pairing are protected from alkylation by these two reagents. Sites of alkylation can be mapped readily, as these base modifications block primer extension with reverse transcriptase.

Being a small, uncharged molecule able to pass through cell membranes, DMS can be used to probe the secondary structure of mRNAs in their native conformation within the cytoplasm of living cells (6). It is also possible to treat extracted RNA with DMS to allow a comparison of RNA conformations in vivo and in vitro. In contrast, CMCT only can be used to alkylate RNA in vitro because this reagent does not penetrate cell membranes.

The secondary structure of the *papAΔ3* 5′ UTR was analyzed by alkylation with DMS and CMCT, followed by primer extension (Fig. 2, left). As a negative control, primer extension was also performed with an unalkylated RNA sample to identify sites where reverse transcriptase naturally pauses or terminates. The pattern of alkylation by DMS was similar in *E. coli* and in vitro, indicating that the conformation of this RNA segment does not change perceptibly upon extraction from cells. This finding validated the in vitro alkylation data that we obtained by using CMCT. Overall, the in vivo alkylation data were consistent with data from earlier studies based on RNase cleavage in vitro (16). Together, these data indicate that the *papAΔ3* 5′ UTR contains two stem-loops, one of which (hp1) is situated at the 5′ terminus (Fig. 2, right). These two stem-loops are separated by a short single-stranded RNA segment (ss1) and followed by a second single-stranded segment (ss2) that contains the signals for translation initiation.

**A 5′-terminal stem-loop contributes to the stability of *papA* mRNA.** Like the *papAΔ3* 5′ UTR, the 5′ UTR of the long-lived *E. coli ompA* transcript functions as an mRNA stabilizer. Previous studies have shown that a stem-loop present at the 5′ terminus of the *ompA* 5′ UTR plays an important role in protecting the *ompA* message from degradation (1, 9). To determine whether the 5′-terminal stem-loop of *papAΔ3* is similarly important for message longevity, we constructed a plasmid (pPAPAΔhp1E) encoding both *papAΔ3* mRNA and a variant *papA* transcript (*papAΔhp1*) from which the 5′ stem-loop had been deleted. The rates of decay of these two plasmid-encoded mRNAs were monitored simultaneously in *E. coli* by primer extension analysis of RNA samples extracted at time intervals after rifampin addition (Fig. 3A).

Consistent with our previous experiments (Fig. 1), the pseudo-wild-type *papAΔ3* transcript decayed slowly in these cells, with a half-life of 18 ± 2 min (Fig. 3C). The *papAΔhp1* transcript, on the other hand, decayed more rapidly, with a half-life of 6.6 ± 0.9 min, corresponding to a degradation rate nearly three times faster than that of *papAΔ3* mRNA (Fig. 3C). Structural analysis of the *papAΔhp1* 5′ UTR by chemical alkylation confirmed that deletion of the 5′ stem-loop had not disrupted the secondary structure of the remainder of the UTR (Fig. 3D). We conclude that this 5′-terminal stem-loop makes an important contribution to the stabilizing effect of the *papAΔ3* 5′ UTR. This finding raises the possibility that hp1 may help to shield *papAΔ3* mRNA from degradation via an RNase E-dependent pathway (the principal pathway for mRNA decay in *E. coli* [2, 13, 15, 20]), as internal cleavage by this endonuclease in *E. coli* can be hindered by the presence of a stem-loop near the RNA 5′ end (5).

The protective effect of hp1 suggests a similar role for the nearly identical 5′ stem-loop of the wild-type *papA* message, an mRNA that arises by cleavage of the *papBA* precursor transcript at an intercistronic site two nucleotides upstream of this stem-loop (19). In this regard, it is noteworthy that the protective stem-loop at the 5′ end of *ompA* mRNA retains its stabilizing influence even when it is preceded by two unpaired nucleotides (9). The 5′ *papA* stem-loop might be particularly important for the longevity of the wild-type *papA* message if this mRNA is targeted for subsequent digestion by RNase E, as this 3′ RNA processing product is thought to begin with a 5′ monophosphate. Recent in vitro studies have shown that

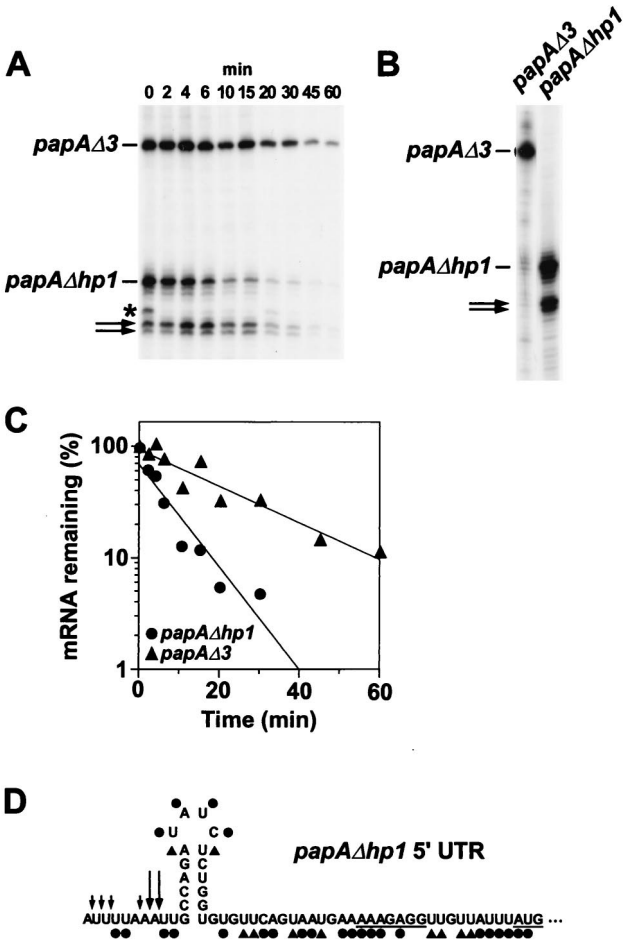


FIG. 3. Accelerated decay of a truncated *papA* transcript lacking the 5′ stem-loop. (A) A culture of *E. coli* JC10287 (7) harboring pPAPAΔhp1E was grown exponentially for several generations in supplemented Luria-Bertani medium at 37°C. At time intervals after transcription inhibition with rifampin (200 μg/ml), total cellular RNA was isolated. Equal amounts of each RNA sample (2 μg) were then analyzed by primer extension with avian myeloblastosis virus reverse transcriptase and a 5′-end-labeled DNA primer (5′-AAGACACCACT GCCATAGCT-3′) complementary to the coding region of *papA* mRNA. Bands that correspond to *papAΔ3* and *papAΔhp1* mRNA are indicated, as are two bands corresponding to a pair of apparent *papA* mRNA cleavage products (arrows). The origin of an additional band (*) seen only at 0 min is not known. (B) That the two cleavage products are derived principally from *papAΔhp1* mRNA and not significantly from *papA* mRNA is demonstrated by an additional primer extension experiment performed with RNA samples isolated from a pair of isogenic *E. coli* strains that expressed either *papAΔ3* mRNA or *papAΔhp1* mRNA. (C) Semilogarithmic plot of mRNA concentration versus time after rifampin addition. The measured half-lives were 6.6 ± 0.9 min for *papAΔhp1* mRNA and 18 ± 2 min for *papAΔ3* mRNA. (D) Summary of the DMS and CMCT alkylation data obtained in vivo and in vitro for the 5′ UTR of *papAΔhp1* mRNA by the procedure described in Fig. 2. ●, heavy alkylation; ▲, moderate alkylation. Sites of premature termination by reverse transcriptase on an unalkylated RNA template are indicated by arrows: large arrows, major termination sites thought to represent the 5′ ends of RNase E cleavage products generated in vivo; small arrows, minor termination sites that preclude a direct assessment of base pairing by the preceding residue. The Shine-Dalgarno element and initiation codon are underlined.

RNase E is especially aggressive at cleaving RNAs that begin with a 5′ monophosphate rather than a 5′ triphosphate, but that this accelerated cleavage can be significantly impeded by base pairing at or near the RNA 5′ terminus (12). Therefore, the contribution of *papA* hp1 to mRNA stability may be even greater for the natural *papA* message than it is for *papAΔ3*, which presumably begins with a 5′ triphosphate. We note that the presence of hp1 not only enhances the longevity of *papAΔ3* mRNA, but also reduces the relative abundance of a pair of apparent degradation intermediates arising from cleavage in the vicinity of a known RNase E site (16) within the unpaired ss1 segment (see bands marked by arrows in Fig. 3A and B). Thus, by deterring RNase E from degrading the *papA* message following its liberation from the *papBA* operon transcript, hp1 may be critically important for the differential stability of the *papB* and *papA* segments of the *papBA* transcript and hence for the differential expression of these two cotranscribed genes. Consistent with this conclusion, a large deletion within the *papBA* intercistronic region that removes hp1 together with 85 flanking nucleotides (81 upstream and 4 downstream) reduces PapA protein production substantially, resulting in truncated cell surface fimbriae (18). The stem-loop structures present just downstream of endonucleolytic processing sites in various other mRNAs may have a similar protective function.

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## REFERENCES

1. **Arnold, T. E., and J. G. Belasco.** 1998. mRNA stabilization by the *ompA* 5′ untranslated region: two protective elements hinder distinct pathways for mRNA degradation. RNA **4:**319–330.
2. **Babitzke, P., and S. R. Kushner.** 1991. The Ams (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **88:**1–5.
3. **Båga, M., M. Göransson, S. Normark, and B. E. Uhlin.** 1988. Processed mRNA with differential stability in the regulation of *E. coli* pilin gene expression. Cell **52:**197–206.
4. **Belasco, J. G., G. Nilsson, A. von Gabain, and S. N. Cohen.** 1986. The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. Cell **46:**245–251.
5. **Bouvet, P., and J. G. Belasco.** 1992. Control of RNase E-mediated RNA degradation by 5′-terminal base pairing in *E. coli*. Nature **360:**488–491.
6. **Chen, L.-H., S. A. Emory, A. L. Bricker, P. Bouvet, and J. G. Belasco.** 1991. Structure and function of a bacterial mRNA stabilizer: analysis of the 5′ untranslated region of *ompA* mRNA. J. Bacteriol. **173:**4578–4586.
7. **Csonka, L. N., and A. J. Clark.** 1979. Deletions generated by the transposon Tn10 in the *srl recA* region of the *Escherichia coli* K-12 chromosome. Genetics **93:**321–343.
8. **Emory, S. A., and J. G. Belasco.** 1990. The *ompA* 5′ untranslated RNA segment functions in *Escherichia coli* as a growth-rate-regulated mRNA stabilizer whose activity is unrelated to translational efficiency. J. Bacteriol. **172:**4472–4481.
9. **Emory, S. A., P. Bouvet, and J. G. Belasco.** 1992. A 5′-terminal stem-loop structure can stabilize mRNA in *Escherichia coli*. Genes Dev. **6:**135–148.
10. **Hansen, M. J., L.-H. Chen, M. L. S. Fejzo, and J. G. Belasco.** 1994. The *ompA* 5′ untranslated region impedes a major pathway for mRNA degradation in *Escherichia coli*. Mol. Microbiol. **12:**707–716.
11. **Hultgren, S. J., S. Normark, and S. N. Abraham.** 1991. Chaperone-assisted assembly and molecular architecture of adhesive pili. Annu. Rev. Microbiol. **45:**383–415.
12. **Mackie, G. A.** 1998. Ribonuclease E is a 5′-end-dependent endonuclease. Nature **395:**720–723.
13. **Melefors, Ö., and A. von Gabain.** 1991. Genetic studies of cleavage-initiated mRNA decay and processing of ribosomal 9S RNA show that the *Escherichia coli ams* and *rne* loci are the same. Mol. Microbiol. **5:**857–864.
14. **Moazed, D., S. Stern, and H. F. Noller.** 1986. Rapid chemical probing of conformation in 16S ribosomal RNA and 30S ribosomal subunits using primer extension. J. Mol. Biol. **187:**399–416.
15. **Mudd, E. A., H. M. Krisch, and C. F. Higgins.** 1990. RNase E, an endoribonuclease, has a general role in the chemical decay of *E. coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. Mol. Microbiol. **4:** 2127–2135.
16. **Naureckiene, S., and B. E. Uhlin.** 1996. In vitro analysis of mRNA processing by RNase E in the *pap* operon of *Escherichia coli*. Mol. Microbiol. **21:**55–68.
17. **Nilsson, G., J. G. Belasco, S. N. Cohen, and A. von Gabain.** 1987. Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc. Natl. Acad. Sci. USA **84:**4890–4894.
18. **Nilsson, P., S. Naureckiene, and B. E. Uhlin.** 1996. Mutations affecting mRNA processing and fimbrial biogenesis in the *Escherichia coli pap* operon. J. Bacteriol. **178:**683–690.
19. **Nilsson, P., and B. E. Uhlin.** 1991. Differential decay of a polycistronic *Escherichia coli* transcript is initiated by RNase E-dependent endonucleolytic processing. Mol. Microbiol. **5:**1791–1799.
20. **Taraseviciene, L., A. Miczak, and D. Apirion.** 1991. The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. Mol. Microbiol. **5:**851–855.