Maturation and degradation of RNA in bacteria

Ciarán Condon

RNA decay plays an important role, not only in recycling nucleotides but also in determining the rapidity with which cells can react to changing growth conditions. The degradation process can be regulated, thus providing an often-underestimated means of controlling gene expression. Recent developments in the field of RNA maturation and decay in two key model organisms, *Escherichia coli* and *Bacillus subtilis*, include the resolution of the structures of many of the participants in these processes in *E. coli* and the identification of an enzyme in *B. subtilis* that appears to fit the bill as a major player in RNA decay in this organism.

Addresses
CNRS UPR 9073 (affiliated with Université de Paris 7 — Denis Diderot), Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

Corresponding author: Condon, Ciarán (condon@ibpc.fr)

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Introduction

The degradation of RNA was long considered a constitutive process that only came into play at the end of the usefulness of an mRNA for translation. That perception has changed over time, however, and it is now well established that degradation of RNA can play an important role in the control of gene expression. Bacterial RNA degradation has been best studied in *Escherichia coli* and significant headway has been made recently in *Bacillus subtilis*, where the enzymes and pathways appear to be radically different. In this review, the techniques routinely employed in studying RNA maturation and degradation in bacteria are briefly discussed. The principles of RNA maturation and decay in *E. coli* and *B. subtilis* are developed in more detail, placing particular emphasis on discoveries made in the past two years and, where interesting, highlighting the methods used.

Techniques used in the study of RNA maturation and decay

Detection and quantification of RNA

The techniques used to study the phenomena of RNA maturation and degradation have not changed much in 30 years. Northern blot analysis still remains the method of choice to detect RNAs *in vivo*. Northern blots have the advantage of providing a visual confirmation of RNA quality and size. Many researchers have switched to real-time RT-PCR analysis to study the fate of a single RNA under many different experimental conditions. Once the effort has been made to optimize the method for a favorite RNA, many samples can be processed rapidly and variations in low-abundance RNAs can be detected more readily than by Northern blot. In bacteria, RNA half-lives are usually measured by quantifying the amount of RNA remaining at different times after addition of rifampicin to cultures to inhibit new initiation events by RNA polymerase. Although this method does have secondary effects on cellular metabolism and may not always give a precise absolute measurement of half-life, it is still a valid method to compare half-lives in two experimental conditions, wild type versus mutant, for example. Global effects of RNase mutations on mRNA half-lives have been successfully analyzed using gene arrays performed on RNAs isolated at different times after rifampicin addition (e.g. [1]).

Mapping of 5′ and 3′ extremities of RNA

Whereas mapping the 5′ extremity of primary transcripts or processing/degradation intermediates is a straight-forward exercise using a primer-extension assay, mapping the 3′ end provides a little more of a challenge. RNase protection assays have given way to both 5′ and 3′ RACE (rapid amplification of cDNA ends) experiments, in which an oligonucleotide is ligated to either the 5′ or 3′ end, and the ligated species is amplified by RT-PCR. The PCR fragment is then cloned and sequenced to determine the 5′ or 3′ extremities. A variation of this technique (5′/3′ RACE), in which RNAs are circularized by ligation, reverse transcribed across the ligated junction and amplified by PCR, permits simultaneous mapping of both 5′ and 3′ ends (e.g. [2]).

Assay of ribonuclease activity *in vitro*

To be able to associate an activity with a particular enzyme, particularly when identifying new RNases, requires the setting up of processing assays *in vitro*. With the development of protein-tagging techniques, purification of enzymes has become much simpler. In general, assays of purified proteins are performed with radiolabelled substrate RNAs, synthesized by T7 RNA polymerase *in vitro*. The products are then analyzed on polyacrylamide sequencing gels, which give a better overview than, say, filter-binding assays. Fluorescence-based assays have recently become popular for labor-intensive kinetic studies. Some groups have used such an assay for *E. coli* RNase

E endonucleolytic activity, where the substrate has a fluorescence tag and quencher on either side of the cleavage site. Cleavage of this substrate by RNase E causes an easily detectable increase in fluorescence that can be measured in real time (e.g. [3]).

Recent developments in the field of RNA maturation and decay

Messenger RNA decay under 'normal' growth conditions in *E. coli*

E. coli mRNAs are generally degraded by endonucleolytic cleavage, followed by 3′-to-5′ exonucleolytic RNA decay (Figure 1). The principal instigator of this process under equilibrium growth conditions is the essential ribonuclease RNase E, with minor roles attributed to other more specialized endonucleases, such as its paralog RNase G, RNase III, RNase P and, more recently, RNase Z [4]. RNase E does not generally act alone; it has a long unstructured C-terminal tail that serves as a scaffold for the 3′-to-5′ exoribonuclease polynucleotide phosphorylase (PNPase), an ATP-dependent helicase (RhlB) and enolase. This complex, which can also have other proteins [such as poly(A) polymerase] attached in sub-stoichiometric amounts, is called the RNA degradosome and has been found under different guises in many bacteria (below). Although it cleaves mRNAs internally, RNase E is a 5′-end-dependent nuclease. It requires at least four nucleotides of single-stranded RNA at the 5′ end to be able to bind efficiently and it has a significant preference for 5′-monophosphorylated extremities over the 5′-triphosphate ends of primary transcripts. Using an interesting approach, based on the ability to ligate monophosphorylated but not triphosphorylated ends of primary transcripts, Belasco and colleagues (H Celesnik, A Deana, JG Belasco, personal communication) have proposed that a pyrophosphatase acts on mRNAs to enable RNase E to launch its initial attack, perhaps analogous to the decapping step of eukaryotic mRNAs.

The 3′ end of bacterial mRNAs is protected from attack by 3′-to-5′ exonucleases by secondary structure, in most cases a Rho-independent transcription terminator. Endonucleolytic cleavages generate new unprotected 3′ ends that are rapidly degraded by exonucleases. The main exoribonucleases acting on *E. coli* mRNAs are RNase II, PNPase and RNase R. RNase II can only degrade single-stranded RNA and is completely inhibited by secondary structure. Both PNPase and RNase R can get through secondary structure, however, provided a 3′ toehold is available. This toehold often comes in the form of a poly(A) tail, synthesized by poly(A) polymerase (PAP1) with the help of the small RNA-binding protein Hfq. Although a toehold of 7 nt is sufficient for RNase R [5] because of its intrinsic ability to advance processively through secondary structure, PNPase might also require assistance from a helicase for particularly stable structures. Degradation of highly structured repeated extragenic palindromic (REP) elements, for example, can be accomplished by a combination either of RNase R and PAP1 [6•], or of degradosome-bound PNPase and RhlB [7•]. Multiple rounds of polyadenylation and exonucleolytic degradation might be required before secondary structures are completely degraded.

Several lines of evidence suggest that endonucleolytic cleavage by RNase E provides the signal for polyadenylation of fragments. This could be due to the ability of PAP1 to recognize 5′ monophosphorylated fragments, but could also be due to direct targeting of PAP1 to these fragments through its interaction with the degradosome. This would explain how 3′ fragments containing terminator hairpins or REP sequences protect primary transcripts, but are degraded once these mRNAs are cleaved by RNase E. A gene-array-based approach, combined with 3′ RACE (primed with oligo dT), was recently used to support the idea that Rho-dependent terminator hairpins are preferential sites of polyadenylation [8].

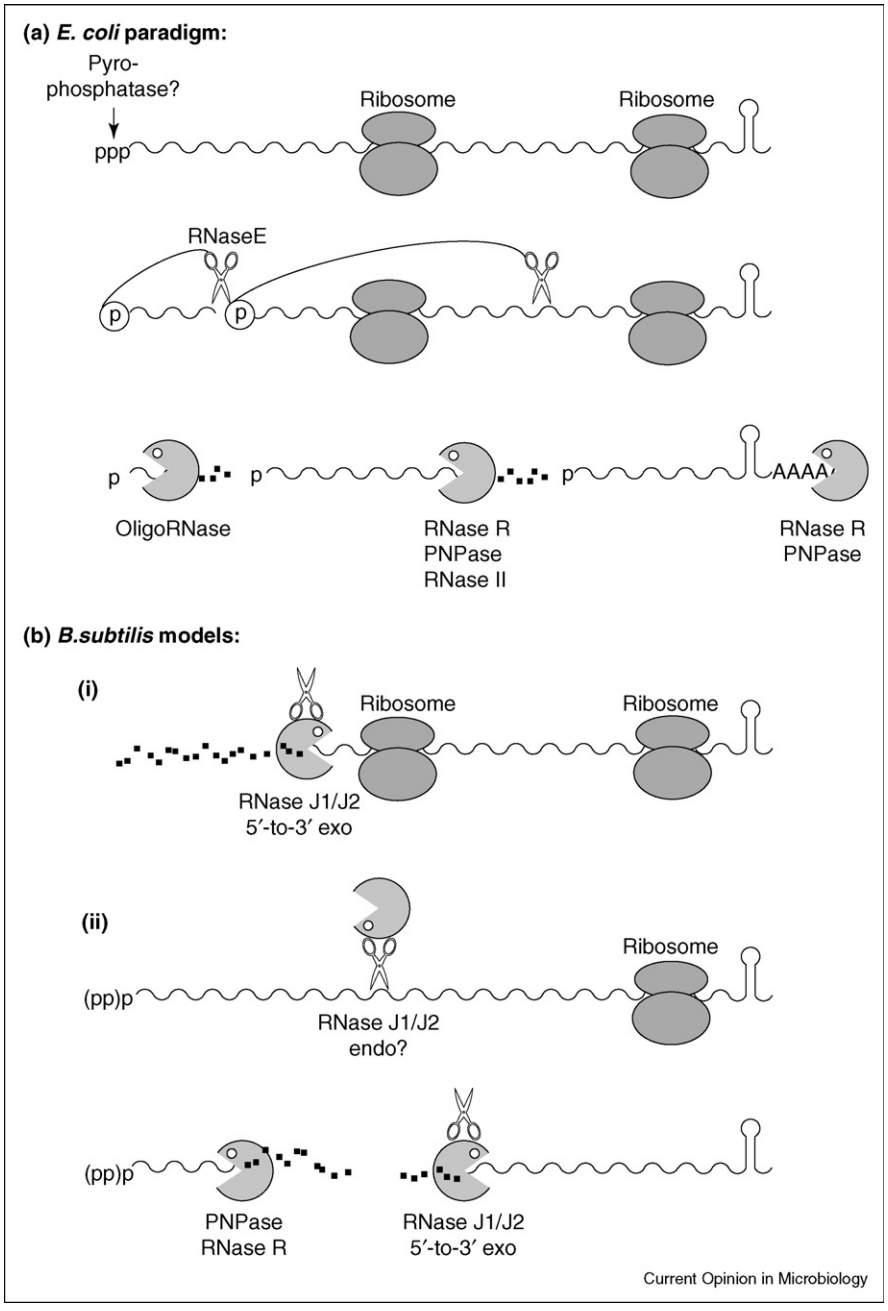
Although it was long assumed that the degradosome-associated helicase was dedicated to assisting PNPase degrade secondary structures, RhlB has also recently been shown to promote RNase E cleavage of transcripts synthesized by T7 RNA polymerase *in vivo* [9]. Translation is uncoupled from transcription of these RNAs because of the rapid elongation rate of T7 RNA polymerase, and RhlB functions perhaps by remodeling the RNA to reveal cleavage sites previously embedded in secondary structures. It will be interesting to see whether this observation also applies to native cellular mRNAs, under conditions of translation repression, for example, where transcription and translation are also uncoupled.

E. coli exoribonucleases RNase II and RNase R accumulate fragments in the range 2–5 nt as end products. Another essential enzyme, called oligoribonuclease (Orn), is required to convert these short products to mononucleotides. It has recently been shown that Orn activity is inhibited by the high intracellular levels of 3′-phosphoadenosine-5′-phosphate (pAp) that accumulate in response to lithium stress [10]. Although a similar effect of pAp on the activity of the *Saccharomyces cerevisiae* 5′-to-3′ exoribonuclease Xrn1 has also been observed, the potential regulation of ribonuclease activity by products of intermediate metabolism has remained relatively unexplored.

Variations on the degradosome theme

Interesting variants of the degradosome have been found in other bacteria, usually by co-immunoprecipitation techniques. In *Streptomyces*, the catalytic domain of RNase E occupies a central position in the protein, with a scaffold domain on either side. Both scaffold domains

Figure 1



Principal mRNA decay pathways in *E. coli* and *B. subtilis*. **(a)** The *E. coli* paradigm. For a general description, see text. Endonucleolytic cleavage of mRNAs by 5′-monophosphate-dependent RNase E is indicated by scissors symbols. 3′-to-5′ exonucleolytic degradation of the cleaved intermediates by the major exoribonucleases RNase R, PNPase and RNase II is indicated by the 'Pacman' symbols. Fragments containing RNA structure, such as terminator hairpins, require polyadenylation before being degraded. Small fragments in the 2–5 nt range are degraded by oligoribonuclease. **(b)** *B. subtilis* models. **(i)** RNAs are degraded/matured from the 5′ end by the 5′-to-3′ exoribonuclease activity of RNase J1/J2. This enzyme is thought to have both exonucleolytic and endonucleolytic activities, indicated by the combined Pacman/scissors symbols. **(ii)** RNAs are first cleaved by an endoribonuclease, possibly also RNase J1/J2. The newly generated unprotected 3′ ends are degraded by 3′-to-5′ exoribonucleases, primarily PNPase and RNase R, while the newly generated monophosphorylated 5′ ends might be substrates of the 5′-to-3′ exoribonuclease activity of RNase J1/J2.

are thought to interact with PNPase. In the psychrotrophic bacterium *Pseudomonas syringae*, the degradosome has been shown to contain RNase R [11•], also known to be induced by cold and stress in *E. coli* [12]. The *Rhodobacter capsulatus* degradosome contains two DEAD-box helicases and the transcription termination factor Rho. The composition of the *E. coli* degradosome is also subject to variation in response to cold shock [13] or the

overexpression of two recently identified inhibitors of RNase E, called RraA and RraB [14].

Structures of the major players

Major advances have been made in the past few years in terms of the understanding of the functioning of ribonucleases at the molecular level. The crystal structures of PNPase, RNase III and oligoribonuclease were solved a number of years ago. The structures of RNase II [15••,16] and of the N-terminal catalytic domain of RNase E were solved more recently [17••]. The structures of most of the major ribonucleases involved in *E. coli* mRNA decay are therefore now known. The structure of RNase II bound to single-stranded RNA explains the processivity of the enzyme to the 10 nt range, after which it becomes distributive [15••]. An anchor domain is separated by about 10 nt from the catalytic site; once the RNA is too short to be bound by both domains, it is released at each catalytic cycle.

The structure of the catalytic domain of RNase E shows it forms tetramers held together by a pair of Zn atoms, the so-called 'Zn-link' [17••,18]. The Zn-link is not required for core catalytic activity, however [19]. The structure also provides a molecular explanation for the enzyme's preference for RNA fragments bearing 5′-monophosphate residues over 5′-triphosphorylated or 5′-hydroxylated termini [17••]. A number of specific interactions were observed between the terminal phosphate and residues of a 5′-sensor pocket. A model has been proposed whereby binding of the 5′-monophosphate by the 5′-sensor domain causes the neighboring S1 domain to clamp down on the RNA downstream [17••], increasing the catalytic power of the enzyme [20••]. The crystal structure of an enolase dimer bound to its binding site in the RNase E scaffold region has also been solved at high resolution [21]. The quaternary structures of RNase E and the other components of the core degradosome have inspired the proposal of a theoretical supramolecular degradation complex of >4 MDa (larger than the ribosome) that satisfies all of the available binding sites of the different partners [22••].

Targeting of mRNA degradation by non-coding RNAs

The translational regulation of gene expression by non-coding RNAs (ncRNAs) has, in many cases, been shown to lead to degradation of both the target and regulatory RNA. Although the primary effect of ncRNAs is thought to be inhibition of translation initiation [23], subsequent degradation of the target can play an important part in determining the sensitivity of control. Degradation can be initiated by RNase III (through cleavage of the duplex formed between the ncRNA and its target [24]) or by RNase E (in cases in which RNase E cleavage sites become exposed as a result of altered folding of the target or the lack of ribosomes). Hfq, which plays an important role as an RNA chaperone in bringing imperfectly paired target and ncRNAs together, has been proposed to help recruit RNase E to the targets through a direct protein–protein interaction [25•]. A parallel has thus been drawn between this system and the eukaryotic RISC complex involved in RNA interference. Although a classical RISC-mediated silencing phenomenon has not yet been demonstrated in bacteria, it is interesting to note that *Aquifex aeolicus* does have a homolog of argonaute, one of the key players in this phenomenon [26].

Messenger RNA degradation in *B. subtilis*

Although there were some early indications that mRNA degradation in *B. subtilis* differed somewhat from that in *E. coli*, this point was really driven home upon completion of the sequences of the two genomes. *B. subtilis* lacks homologues of some of the key enzymes of RNA degradation in *E. coli*, namely RNase II, oligoribonuclease and RNase E (and therefore cannot form *E. coli*-type degradosomes). In contrast to *E. coli*, where 3′-to-5′ exonucleolytic degradation is thought to be primarily hydrolytic, this is principally a phosphorolytic process in *B. subtilis*. PNPase has been established one of the primary 3′-to-5′ exonucleases in this organism, with RNase R also playing a significant back-up role in degrading decay intermediates ending in secondary structure [27••,28]. Polyadenylation has also been observed in *B. subtilis* [29], despite the lack of a PAP1 homolog; however, its role in mRNA stability has not yet been addressed.

A characterizing feature of *B. subtilis* mRNA degradation has been the often dramatic effect of 5′-stabilizers in this organism [30•]. Roadblocks such as bound or stalled ribosomes, bound proteins or strong secondary structures near the 5′ end of mRNAs have significant positive effects on the stability of the downstream portion (up to several kilobases) of the mRNA, whereas the upstream portion is usually undetectable (Figure 1). RNAs with similar features are unstable in *E. coli*. Whereas these results were best explained by a 5′-to-3′ exonucleolytic activity, up to very recently such an activity was not thought to exist in bacteria. It is now known, however, that the essential enzyme RNase J1 (and its paralog J2) of *B. subtilis* has both endonucleolytic and 5′-to-3′ exonucleolytic activity [31,32••]. Thus, exonucleolytic degradation in this organism can occur from either the 5′ or 3′ extremity, similar to what is observed in eukaryotes. 5′-to-3′ exonucleolytic degradation of the *cryIIIA* mRNA leader by RNase J1 as far as a bound 30S ribosomal subunit results in stabilization of the downstream portion of this mRNA. The 5′-to-3′ exonucleolytic activity of RNase J1 prefers single-stranded monophosphorylated 5′-extremities over double-stranded or triphosphorylated 5′ ends, and therefore could, in theory at least, degrade the products of its own endonucleolytic activity.

Although there are 11 known endoribonucleases in *B. subtilis* (Table 1), it is not yet clear whether mRNA cleavage plays a major role in mRNA degradation in this

Table 1

Comparison of ribonucleases present in *E. coli* and *B. subtilis*.

Exoribonucleases				Endoribonucleases			
Enzyme	*E. coli* gene	*B. subtilis* gene	Homology[a]	Enzyme	*E. coli* gene	*B. subtilis* gene	Homology[a]
PNPase	***pnp***	***pnpA***	**52/69%**	**RNase III**	***rnc***	***rncS***[b]	**36/60%**
RNase R	***rnr***	***rnr***	**39/59%**	**RNase P**	***rnpAB***[b]	***rnpAB***[b]	**36/63%**
RNase PH	***rph***	***rph***	**56/73%**	**RNase Z**	***rbn/elaC***	***rnz***[b]	**47/64%**
RNase II	*rnb*	–		**MazF/EndoA**	***mazF***	***ndoA***	**32/48%**
RNase D	*rnd*	–		ChpBK	*chpBK*	–	
RNase T	*rnt*	–		YoeB	*yoeB*	–	
Oligo-RNase	*orn*[b]	–		RNase E	*rne*[b]	–	
YhaM	–	*yhaM*		RNase G	*rng*	–	
RNase J1	–	*rnjA*[b]		RNase I	*rna*		
RNase J2	–	*rnjB*		RNase LS	*rnlA*		
				RNase HI	*rnhA*	–[c]	
				RNase HII	***rnhB***	***rnhB***	**48/65%**
				RNase HIII	–	*rnhC*	
				RNase M5	–	*rnmV*	
				RNase Bsn	–	*yurI*	
				YhcR	–	*yhcR*	
				RNase J1	–	*rnjA*[c]	
				RNase J2	–	*rnjB*	

RNases in common between *E. coli* and *B. subtilis* are in bold font.
[a] Homology is given as % identity and % similarity.
[b] Essential genes.
[c] Although a protein with 46% similarity to *E. coli* RNase HI, YpdQ, exists in *B. subtilis,* it has been shown not to possess RNase H activity.

organism; many of the naturally occurring endonucleolytic cleavage events described thus far are actually maturation reactions. The endonuclease activity of RNase J1/J2 cleaves the *thrS* leader mRNA just upstream of a transcription attenuator [31], resulting in a processed RNA that is up to fivefold more stable than the primary transcript. Although the steady-state levels (and by inference, stability) of many RNAs are affected in RNase J1-depleted strains in transcriptome studies (C Condon, unpublished), it is not yet known whether these can be directly attributed to RNase J1 activity.

RNase J1 also uses its 5′-to-3′ exonuclease activity to generate the 5′-extremity of mature 16S ribosomal RNA in *B. subtilis* [2]. This reaction occurs on assembled ribosomes and requires another factor, perhaps a ribosomal protein bound near the beginning of 16S rRNA, to stop progression into the mature sequence. RNase J1 is a member of the metallo-β-lactamase family of enzymes and is widely distributed among prokaryotes, with a similar level of abundance to RNase E. RNase J1 orthologs are present in more than half of the fully sequenced bacterial genomes, often in multiple copies, and in about 80% of the Archaea. Interestingly, about half of those organisms containing RNase J1 and half of those containing RNase E have orthologs of both enzymes.

Messenger RNA decay under stress conditions

Severe stress conditions, such as starvation for amino acids, can lead to the activation of toxin–antitoxin systems in *E. coli*. At least four of the toxins, MazF, ChpBK, YoeB and RelE, are known to be RNases or facilitators of RNase activity. In each case, the toxin and antitoxin are encoded by the same operon; the antitoxin tightly binds the toxin, blocking its activity (for recent reviews, see [33••,34,35]). However, the antitoxin is much more labile than the toxin and requires constant *de novo* synthesis to keep the toxin under wraps. As such, it serves as a very accurate sensor of the cell's translational capacity. An arrest of translation will lead to activation of RNase activity. The RNase toxins generally recognize and cleave short single-stranded sequences (often as short as triplets) in the RNA, and thus most cellular RNAs become targets for destruction, a phenomenon that has been called shut-down decay (SDD) [35]. MazF, for example, cleaves ACA triplets, whereas EndoA, its *B. subtilis* homologue, cleaves preferentially in a (U/A)UAC(U/A) context [36]. Current models suggest that SDD serves to rapidly wipe the slate clean, so that cells can reprogram ribosomes to the synthesis of mRNAs better adapted to new growth conditions. Although MazF, EndoA, ChpBK and YoeB can all cleave mRNAs directly [36–38], RelE can only function when bound to the A-site of the ribosome. Thus, it is not yet clear whether RelE has RNase activity itself or whether it stimulates the ribosome to cleave RNAs. RegB, encoded by bacteriophage T4, has also recently been shown to be a member of the RelE family of toxins [39]. The degradation pathway of RNAs cleaved by toxins, which have been shown to bear a 3′-phosphate group [36,40] rather than the 3′-hydroxyl generated by the so called processing endonucleases such as RNase E, has not yet been studied in any detail.

Stable RNA maturation and decay

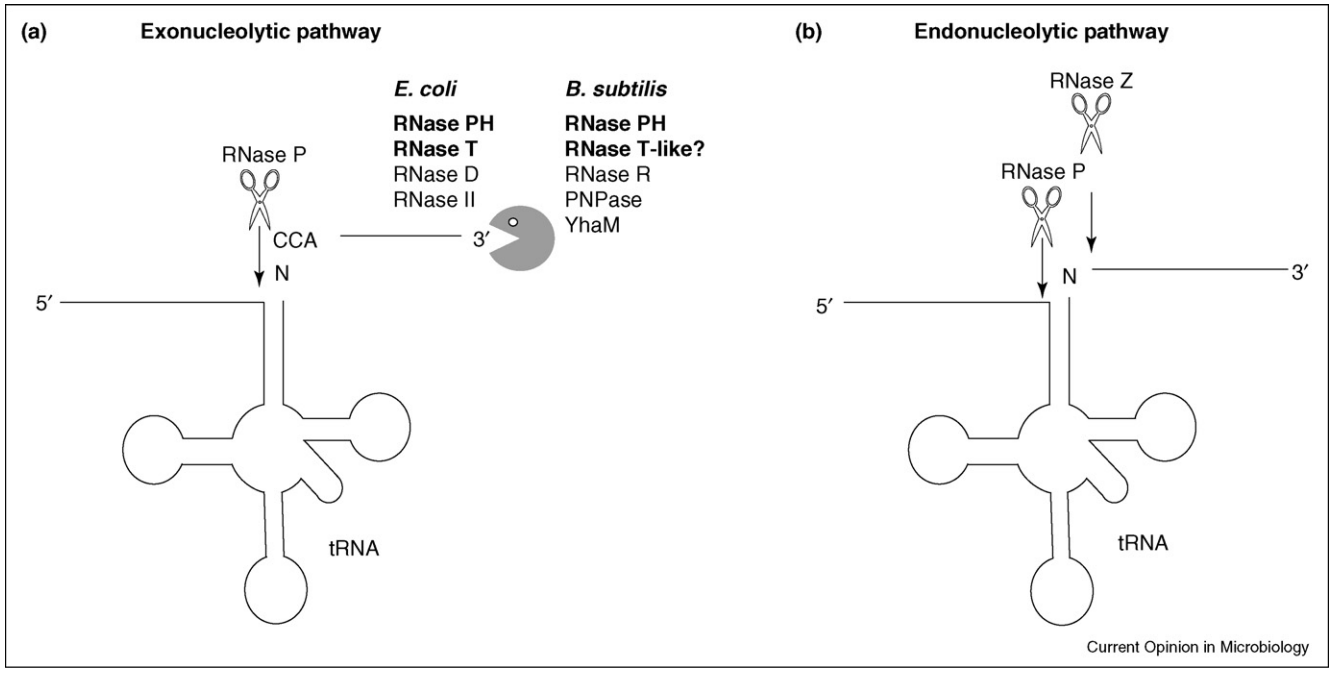
Ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are generally synthesized as larger precursors that require processing of both their 5′ and 3′ ends to become functional molecules, and there is significant overlap between the enzymes involved in mRNA degradation and stable RNA maturation [41•]. As most of the recent progress has been made on tRNA maturation, I focus on this area for the purposes of this review.

The pathways for tRNA maturation are fairly well understood in both *E. coli* and *B. subtilis* (Figure 2). Processing at the 5′ side is catalyzed by the quasi-universal ribozyme RNase P (this enzyme has been found in every organism studied thus far but *A. aeolicus*). Processing at the 3′-extremity is essentially an exonucleolytic process in *E. coli*, catalyzed by any one of a number of redundant 3′-to-5′ exonucleases, including RNase PH, RNase T, RNase D and RNase II, with the first two playing key roles. Processing of the 3′ end of tRNAs in *B. subtilis* can be either endonucleolytic or exonucleolytic, depending on whether the tRNA gene directly encodes the CCA motif present at the 3′-extremity of all mature tRNAs [42]. In contrast to *E. coli*, where all tRNAs have an encoded CCA motif, this sequence is lacking from about one third of *B. subtilis* tRNA genes and must be added post-transcriptionally by tRNA nucleotidyl transferase. Transfer RNAs with an encoded CCA are matured by an exonucleolytic pathway involving redundant enzymes, much like in *E. coli*. One of the main enzymes involved is RNase PH, with minor roles associated with RNase R, PNPase and YhaM. Another key component of this pathway, thought to play a role analogous to RNase T in removing the nucleotide 3′ to the CCA motif, remains to be identified.

tRNAs lacking an encoded CCA motif are primarily matured by a widely conserved and essential endonuclease in *B. subtilis* called RNase Z. RNase Z cleaves these tRNAs just downstream of the discriminator base in most cases. It is inhibited by the presence of a CCA motif, thus preventing futile cycles of CCA removal and addition in the cell. A particular form of RNase Z has been identified in *Thermotoga maritima*, however, that can also cleave tRNAs downstream of the CCA [43]. RNase Z is also present in *E. coli* (also known as RNase BN [44]), where it can provide sufficient tRNA maturation activity for cell survival in the absence of all of the components of the exonucleolytic pathway, despite inhibition of enzyme activity by the CCA motif.

The structures of RNase Z from *E. coli*, *T. maritima* and *B. subtilis* (both free and tRNA-bound forms) have been solved in the past two years [45–47,48••]. The enzyme consists of a core dimer of β-lactamase domains, with each monomer having a long flexible arm that extends from the protein to clamp the tRNA into position. The properties

Figure 2



Exonucleolytic and endonucleolytic tRNA maturation pathways in *E. coli* and *B. subtilis*. **(a)** Maturation of the 3′ end of tRNAs by exoribonucleases (Pacman symbols) in *E. coli* and *B. subtilis*. The principle enzymes identified thus far are in bold type. Maturation of the 5′ end by RNase P is indicated by the scissors symbol. **(b)** Maturation of the 3′ end of tRNAs by the endonucleolytic pathway. Endonucleolytic cleavages by RNase P and RNase Z are indicated by scissors symbols. RNase Z cleaves downstream of the discriminator nucleotide (N) of the precursor tRNA.

of RNase Z have been reviewed in some detail recently [49,50•].

Stable RNAs are generally only turned over under stress conditions or when they are incorrectly folded. This latter process has been termed quality control and the principal pathway in *E. coli* is very similar to that observed for mRNAs, that is, polyadenylation of the fragments and degradation by 3′-to-5′ exoribonucleases, principally RNase R and PNPase [41•].

Conclusions and perspectives

Although we have come a long way towards understanding RNA maturation and decay in *E. coli*, this organism clearly has not yet revealed all its secrets; the recent discovery of toxin–antitoxin systems with RNase activity, just to cite one example, came as a surprise to many. It is also evident from the discovery of 5′-to-3′ exoribonuclease activity in *B. subtilis* that we should consider other model organisms before we can claim even a remote understanding of these processes in the bacterial kingdom as a whole. One cannot help feeling that only the tip of the proverbial iceberg has yet been revealed!

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