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Enzymes Involved in Post-transcriptional RNA Metabolism in Gram-negative bacteria

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Abstract

Gene expression in Gram-negative bacteria is regulated at many levels, including transcription initiation, RNA processing, RNA/RNA interactions, mRNA decay, and translational controls involving enzymes that alter translational efficiency. In this chapter we discuss the various enzymes that control transcription, translation and RNA stability through RNA processing and degradation. RNA processing is essential to generate functional RNAs, while degradation helps control the steady-state level of each individual transcript. For example, all the pre-tRNAs are transcribed with extra nucleotides at both their 5′ and 3′ termini, which are subsequently processed to produce mature tRNAs that can be aminoacylated. Similarly, rRNAs that are transcribed as part of a 30S polycistronic transcript, are matured to individual 16S, 23S and 5S rRNAs. Decay of mRNAs plays a key role in gene regulation through controlling the steady-state level of each transcript, which is essential for maintaining appropriate protein levels. In addition, degradation of both translated and non-translated RNAs recycles nucleotides to facilitate new RNA synthesis. To carry out all these reactions Gram-negative bacteria employ a large number of endonucleases, exonucleases, RNA helicases, and poly(A) polymerase as well as proteins that regulate the catalytic activity of particular ribonucleases. Under certain stress conditions an additional group of specialized endonucleases facilitate the cell's ability to adapt and survive. Many of the enzymes, such as RNase E, RNase III, polynucleotide phosphorylase, RNase R, and poly(A) polymerase I participate in multiple RNA processing and decay pathways.

INTRODUCTION

All living organisms, including the Gram-negative bacteria, have two major classes of RNA molecules. Messenger RNAs (mRNAs) contain the information for the synthesis of the various proteins that are required for a living cell. The so-called non-translated RNAs that include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small regulatory RNAs (sRNAs) provide the RNA components for ribosome assembly, protein synthesis and the regulation of mRNA functionality based on RNA/RNA interactions. The highly diverse functions that these RNAs perform within the cell are possible due to numerous enzymes that are involved in post-transcriptional RNA metabolism. However, many of these enzymes have overlapping activities. Besides the normal cellular complement of enzymes that carry

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out the above functions, there are ribonucleases which are specifically associated with particular stress conditions as part of toxin/antitoxin systems.

Based on our current knowledge of bacterial RNases, there are no dedicated RNases for either RNA degradation or processing. Many of the ribonucleases and RNA helicases participate in more than one pathway, which is visually described in Fig. 1. For example, endoribonuclease E (RNase E) is involved in almost all aspects of RNA metabolism. RNase III is very important for initiation of rRNA processing, but it also participates in mRNA decay and sRNA degradation. Similarly, although RNase P is essential for tRNA 5′ end maturation, it also participates in tRNA processing and mRNA decay. The ribonuclease activities of most of the exonucleases are highly redundant, since they can complement each other. Furthermore, as single-stranded RNAs can rapidly fold into more complex forms containing secondary and tertiary structures, RNA helicases play an important role in various aspects of post-transcriptional processing and decay. In this chapter ribonucleases and RNA helicases found in various Gram-negative bacteria are discussed in the context of their *in vivo* biological functions. The basic properties of all the ribonucleases and RNA helicases are outlined in Table 1. Readers are encouraged to review a complementary chapter in this book entitled “RNases and helicases in Gram-positive bacteria” by Durand and Condon.

GENERAL MESSENGER RNA DECAY

Initiation of mRNA decay by endonucleases

The decay of mRNAs plays a major role in the post-transcriptional regulation of gene expression, since it helps to control the steady-state level of each individual mRNA and in turn the protein level. As a result, considerable effort has been devoted to document the mechanisms of mRNA decay in *E. coli,* which still serves as the model organism for Gram-negative bacteria. Apirion was the first to propose a model for mRNA decay in 1973 (1), which involved a combination of endoribonucleases and exoribonucleases. However, at that time the evidence supporting his hypothesis was not particularly strong. Subsequently, RNase E was initially identified based on its role in rRNA processing as discussed later in this chapter (2). At about the same time as RNase E was discovered, the *ams-1* allele (altered mRNA stability) was isolated and shown to affect cell viability. Strains carrying the *ams-1* mutation demonstrated an increase in the half-life of bulk mRNA at the nonpermissive temperature (3, 4). Subsequently, the *ams* and *rne* loci were shown to encoded the same protein, which is now called RNase E (5).

RNase E, an essential enzyme in *E. coli,* is one of its largest proteins. It is divided into an N-terminal catalytic region and a C-terminal scaffold region. Experiments have shown that the catalytic region of RNase E is located in the first 500 amino acids of the protein (6) and that it is associated with the inner membrane of the cell through a short amino sequence that is immediately downstream of the catalytic region (7). A very important advance in the analysis of mRNA decay pathways was the finding that RNase E was associated with several other enzymes involved in RNA degradation. First it was shown that RNase E forms a complex with polynucleotide phosphorylase (PNPase), a $3' \rightarrow 5'$ exonuclease (8). Subsequently, it was determined that the RhlB RNA helicase and the glycolytic enzyme

enolase were also included in this multiprotein complex, which was called the "degradosome" (8, 9). However, mRNA decay is not significantly affected in the absence of either degradosome assembly and membrane attachment, as shown by deletions of the carboxy terminal region that lack either the degradosome scaffold region (10) or both the degradosome scaffold region and the membrane attachment site (11). The degradosome is discussed in more detail in the Chapter by Bandyra and Luisi.

It appears that RNase E can recognize its substrates in more than one way. Mackie demonstrated that the enzyme was inhibited by the triphosphate moiety found at the 5′ terminus of RNA transcripts (12). Thus, RNase E processing is significantly enhanced by the removal of the pyrophosphate from the 5′ terminal triphosphate of many primary transcripts. While conversion of the 5′ triphosphate to a 5′ monophosphate was believed to be catalyzed by the RNA pyrophosphohydrolase (RppH) encoded by *rppH* (13), a recent report suggests that an unidentified enzyme converts the triphosphate to a diphosphate, which is the substrate of choice for the RppH protein (14). Although RNase E prefers to bind to substrates containing a 5′ monophosphate, a number of experiments have demonstrated that it can also cleave both mRNAs and tRNAs using a direct entry mechanism (15–19).

Several laboratories attempted to determine if there was sequence specificity associated with RNase E cleavages by comparing the sequences of known cleavage sites (20–22). These studies revealed that the enzyme prefers to cleave RNA in single-stranded A/U rich regions. Further work has shown that the cleavage sites are usually found either upstream or downstream of secondary structures. A recent RNAseq study suggests a minimal 5 nt RNase E consensus cleavage site as "RN↓WUU" (with R as G/A, W as A/U and N as any nucleotide) with a strong preference for uridine at +2 position (23).

High density tiling arrays as well as RNAseq experiments have demonstrated that RNase E is responsible for the initiation of mRNA decay for over 50% of all the transcripts generated during exponential growth in *E. coli* (18, 24). However, besides RNase E, there are a significant number of other endonucleases that initiate the decay of mRNAs as minor players. For example, RNase III, which will be discussed in more detail later in the context of rRNA processing, has been shown to affect the steady-state levels of up to 10% of the mRNAs in exponentially growing cells of *E. coli* resulting in either destabilization or stabilization of the transcripts (24, 25). RNase P, which is a ribozyme that contains an RNA catalytic subunit (26), specifically cleaves a small number of polycistronic mRNAs in intercistronic regions (27).

In addition, many Gram-negative bacteria have an RNase E ortholog called RNase G. A major distinction between the two enzymes is that RNase G lacks the degradosome scaffold region (28, 29). RNase G has been most studied in *E. coli*. It has been shown to target specific mRNAs and work in tandem with RNase E (30). Similar to RNase E, the enzyme prefers single stranded AU-rich sequences and is 5′ end dependent (31), but is present in much lower amounts than RNase E (32). *In vitro*, both RNase E and RNase G have similar substrate specificity. However, RNase G cleaves 5′ terminus of pre-16S rRNA precursors at different site than RNase E (28, 29). Although it was originally thought that RNase G could

complement the conditional lethality associated with RNase E mutants (32, 33), it has now been shown that complementation only takes place in the presence of a mutationally altered RNase G protein (34).

RNase Z, another endonuclease found in *E. coli* and other Gram-negative bacteria was initially identified in eukaryotes based on its ability to cleave tRNA precursors endonucleolytically to generate a 3′ terminus that was a substrate for tRNA nucleotidyl transferase, which adds the CCA determinant (35). In *E. coli* the enzyme seems to function primarily in mRNA decay (36), but can also serve in a backup role in some aspects to tRNA processing (see section on tRNA processing). Interestingly RNase Z, which was originally identified as RNase BN (37), also has 3′ → 5′ exonuclease activity and will be discussed later in the section on tRNA processing.

RNase LS, another endonuclease encoded by *rnlA*, has been shown to play a very limited role in the decay in both bacterial mRNAs and phage encoded mRNAs in *E. coli* (38) and is also part of the *rnlAB* toxin/antitoxin module (39).

The role of 3′ → 5′ exonucleases in mRNA decay

RNase II, RNase R and PNPase are the three major 3′ → 5′ exonucleases affecting mRNAs and rRNAs in most Gram-negative bacteria. RNase II and RNase R degrade single-stranded RNA employing a hydrolytic mechanism, releasing mononucleotides (40, 41). It has been shown that RNase II accounts for ~95% of the hydrolytic RNase activity in *E. coli* (42). RNase II is strongly inhibited by secondary structures (43), while RNase R can easily degrade RNA molecules containing secondary structures (44), but requires a single-stranded region of at least 7 nt in order to bind (45). Interestingly, RNase R is more important in stationary phase cells than RNase II (46).

In contrast, PNPase degrades RNA using a phosphorolytic mechanism that requires inorganic phosphate, releasing nucleoside diphosphates (47). Since the equilibrium constant for this reaction is one, the enzyme can also synthesize RNA in an untemplated reaction employing nucleoside diphosphates to generate single-stranded RNA containing all four nucleotides (47). In fact, the enzyme can work both biosynthetically and degradatively in *E. coli* (48) and other Gram-negative bacteria. PNPase exists in at least two multiprotein complexes. The degradosome, which contains RNase E, PNPase, the RhlB RNA helicase and enolase and the polyadenylation complex, which contains poly(A) polymerase (PAP I), PNPase and the RNA binding protein Hfq (49). RhlB is also reported to be associated with PNPase independent of the degradosome assembly helping its exonucleolytic activity *in vitro* (50), although such an effect has yet to be observed *in vivo* (51).

While none of these three ribonucleases are essential for cell viability by themselves, inactivation of both RNase II and PNPase results in synthetic lethality (52). Of most significance is that at the nonpermissive temperature (44°C) there is a large accumulation of partially degraded mRNAs in a *pnp-7 rnb-500* double mutant, demonstrating a significant role for these two enzymes in the mRNA turnover (52). It has also been shown that a *pnp-7 rnr* double mutant is a synthetic lethal, but a *rnb rnr* strain is viable (41).

While the decay of the majority of mRNAs is initiated via endonucleolytic cleavages employing a combination of RNase E, RNase III, RNase G, RNase P, RNase Z and RNase LS, the degradation of a significant number of transcripts is also initiated by exonucleases PNPase, RNase II and RNase R. A series of experiments employing either macroarrays (53) or RNAseq (54) have demonstrated that inactivation of any of the three exonucleases leads to significant changes in the steady-state levels of between 5–10% of the mRNAs. It is also thought that RNase R is more important in ribosome quality control by degrading nonfunctional rRNAs and tRNAs rather than mRNAs. In contrast, PNPase and RNase II are more involved in mRNA decay (53) and some aspects of tRNA processing (51) and rRNA degradation (55). It should also be noted that unlike Gram-positive bacteria (56), there do not appear to be any $5' \rightarrow 3'$ exonucleases in Gram-negative species.

A common feature of all three of these exonucleases is that they cannot degrade a RNA substrate completely, leaving short oligonucleotides of 2–4 in length (57–59). Many bacteria contain another $3' \rightarrow 5'$ exoribonuclease called oligoribonuclease, which specifically degrades these short oligonucleotides using a hydrolytic mechanism (60, 61). The complete degradation of the short oligonucleotides that remain after the action of RNase II, RNase R and PNPase seems to be essential, since there is evidence that oligoribonuclease is essential for cell viability (62). It is not clear at this time if an enzyme such as RNase T can also function on short oligoribonucleotides. Fig. 2 presents a model for general mRNA decay in *E. coli* initiated by RNase E and RNase G.

ENZYMES INVOLVED IN TRANSFER RNA PROCESSING

In *E. coli* and many other Gram-negative bacteria, all of the tRNAs are encoded with extra nucleotides at both their $5'$ and $3'$ ends. They occur as either monocistronic or polycistronic transcripts that contain only tRNAs, tRNAs and mRNAs, or tRNAs and rRNAs. Fig. 3 presents some of the pathways involved in tRNA processing. Many of these transcripts are terminated in a Rho-independent manner, which generates a stem-loop at the $3'$ terminus. The processing of the majority of these tRNA transcripts is initiated by RNase E, which either removes the Rho-independent transcription terminators (63–65) or cleaves in the intercistronic regions (11, 66). In some cases, RNase G and RNase Z can inefficiently substitute for RNase E (65) (Fig. 3A). In at least one well-documented case, the Rho-independent transcription terminator on the *leuX* primary transcript is removed exonucleolytically by PNPase (51) (Fig. 3B).

Interestingly, many polycistronic tRNA transcripts do not utilize RNase E, but rather are dependent on RNase P for their initial processing (67) (Fig. 3C). Some require the initial removal of the Rho-independent transcription terminator by RNase E before RNase P can process the rest of the transcript (64, 65, 67). The RNase P cleavages occur at the mature $5'$ termini (65), while RNase E cleavages, with the exception of the three proline tRNAs (63), leave extra nucleotides downstream of the CCA determinant (11, 66). A surprising observation from the RNase P processed transcripts was that the enzyme cleaves the polycistronic transcripts starting from the $3'$ terminus and not the $5'$ terminus (64).

An interesting feature of tRNA maturation in Gram-negative bacteria is that there is only one ribonuclease, RNase P, that generates the mature 5′ terminus of all the tRNA species (26). In contrast, maturation of the 3′ terminus can be carried out by a variety of 3′ → 5′ exonucleases including RNase T, RNase PH, RNase D, RNase BN, and RNase II (42). PNPase and RNase II can also remove extra nucleotides from the 3′ terminus of a pre-tRNA but they cannot complete the final maturation process to expose the CCA determinant (51, 65).

The bulk (79/86) of the pre-tRNAs in *E. coli* employ a combination of RNase T and RNase PH for their final 3′ end maturation (68). However, RNase T appears to be the most important enzyme of the two based on its unique substrate specificity. Specifically, unlike other ribonucleases, it specifically stops at the terminal CCA because of its inhibition by the presence of C ribonucleotides within its catalytic site (69). Thus, tRNAs containing C residues downstream of CCA are more dependent on RNase PH, which is not inhibited by C residues (64). Inactivation of both RNase T and RNase PH leads to rapid accumulation of 3′ immature tRNAs which becomes substrates for PAP I (68). Contrary to mRNAs, PAP I adds short poly(A) tails (generally <5 nucleotides) to the immature tRNAs (68). However, instead of undergoing degradation as defective tRNAs (70), a majority of the polyadenylated tRNAs are matured slowly by inefficient exonucleases, such as RNase D and RNase BN/Z (68). These results suggest that PAP I helps regulate functional tRNA levels in *E. coli* (68). However, excess PAP I polyadenylates mature tRNAs inhibiting aminoacylation and protein synthesis resulting in rapid cell death (71).

Recently, it has been shown that the three proline tRNAs do not require exonucleolytic processing at their 3′ termini (63) (Fig. 3D). Rather, RNase E removes the Rho-independent transcription terminator by cleaving immediately downstream of the CCA determinant (63). It is not at this time what accounts for the altered substrate specificity of RNase E in the case of the proline tRNA transcripts.

It has now been shown that the 3′ → 5′ exonuclease RNase BN and that the endoribonuclease RNase Z are encoded by the same gene (72) and the enzyme does not remove the CCA determinant from pre-tRNAs (73). Although it has been shown that RNase D and RNase BN can participate in 3′ end maturation (37, 74), it is not clear at this time how significant a role they play in cells that contain RNase T and RNase PH. As described above, RNase Z has been shown to play a role in mRNA decay (36).

With the possible exception of RNase T and RNase BN, the other 3′ → 5′ exonucleases can conceivably partially degrade into the CCA determinant that is required for aminoacylation. The repair of such termini can be carried out by the enzyme tRNA nucleotidyl transferase, which can add C, CC or CCA in an untemplated reaction (75). Interestingly, this enzyme is not essential for cell viability in *E. coli* (76), most likely due to its overlapping biosynthetic activity with PNPase and PAP I (77). Recently, it has been suggested that the enzyme plays a role in tRNA quality control by adding CCACCA tag to defective tRNA molecules which are preferentially degraded by RNase R(78).

ENZYMES INVOLVED IN RIBOSOMAL RNA PROCESSING

All functional rRNAs in Gram-negative bacteria are matured from large (over 3 Kb) 30S rRNA polycistronic transcripts that contain the coding sequences for the 16S, 23S and 5S rRNAs as well as at least one tRNA and a significant amount of spacer sequences in between (Fig. 4). Inverted repeats lead to two large stem-loop structures containing the 16S and 23S species as part of large loops (Fig. 4). Initial processing by RNase III, an enzyme that is specific for double-stranded RNA (79, 80), within the double-stranded stems releases 17S (pre-16S), 25S (pre-23S), and 9S (pre-5S) rRNA precursors (81–83) as well as the embedded tRNAs.

The pre-16S species containing 115 extra nucleotides at its 5′ terminus is initially cleaved endonucleolytically by RNase E removing 60 nucleotides. Subsequently, RNase G endonucleolytically removes the remaining 55 nt to generate the mature 5′ terminus of the 16S rRNA (28, 29). The extra 33 nt found at the 3′ terminus of the pre-16S rRNA are removed either by the YbeY endonuclease or a combination of RNase II, RNase PH, PNPase, and RNase R (84–87).

The pre-5S species is cleaved by RNase E to within 3 nt on each side of the mature sequence (81, 88–91). The three extra nucleotides at the 3′ end of the pre-5S rRNA are removed by the 3′ → 5′ exonuclease RNase T. Nothing is currently known about how the three extra nucleotides at the 5′ end of 5S rRNA species are processed.

In the case of the pre-23S species, the RNase III cleavages leave 7–9 extra nucleotides at the 3′ terminus and either three or seven extra nucleotides at the 5′ terminus (89, 92). The final maturation of the 3′ terminus can be carried out either by RNase T alone or by a combination of PAP I, RNase II, RNase PH, and RNase T (91). In fact, the pre-23S rRNA species is an excellent target for PAP I in the wild type cells (49, 93). Although it is not clear at this time how the 5′ end is matured, since Gram-negative bacterium do not contain 5′ → 3′ exonucleases, this step most likely is carried out by an endonuclease. Recent experiments suggest that RNase III might in fact carry out this reaction (Chardhuri *et al.* 2018, submitted to *Molecular Microbiology*).

The tRNAs embedded in the rRNA operons are released as pre-tRNAs following RNase III cleavage of the primary transcript and RNase E action on the 9S precursor (Fig. 4). Presumably, these pre-tRNAs employ the various enzymes described in the section on tRNA processing for final maturation (See above).

The ribosome, an essential component of protein synthetic machinery, is assembled using mature 23S, 16S rRNA and 5S rRNAs. While it has been shown that pre-23S rRNAs can be incorporated to form functional ribosomes (81), incorporation of pre-16S species do not result in functional ribosomes (94). Thus, it was predicted that RNase III would be an essential enzyme because of the need to separate the 16S, 23S and 5S species from the larger polycistronic transcript. However, it has been shown that *rnc* deletion mutants are viable and show only small defects in their growth rates (90, 95). Clearly there is an alternative mechanism for generating rRNAs that can be incorporated into functional ribosomes in the absence of RNase III.

PROCESSING AND DECAY OF SMALL REGULATORY RNAS

Recent experiments have shown that in a wide range of organisms small RNAs (sRNA) play more widespread regulatory roles than previously envisioned. In prokaryotes, sRNAs generally range in size from ~ 50 to 400 nt in length. Gram-negative bacteria contain > 100 sRNAs that are encoded on plasmids or the genome (96–99). In fact, high density tiling array analysis of RNase E and RNase III mutants have suggested that there may in fact be as many as 300 sRNAs in *E. coli* (24, 100). While sRNAs that do not undergo any processing to be functional cannot be ruled out, all sRNAs identified to date require some initial processing by RNase E processing of many sRNAs for them to be functional (101–103). In case of MicC, conversion of 5′ triphosphate to 5′ monophosphate enhanced RNase E mediated decay of *ompD* (104).

Based on their interactions with their target mRNAs, sRNAs can inhibit translation by masking the ribosome binding site (RBS) (105) or in some cases binding outside of the RBS (106) followed by rapid turnover of the transcripts. Other sRNAs control Rho-dependent termination at the 5′ UTR of the transcripts by blocking the action of Rho and resulting in increased translation of the transcripts (107). The association of many sRNAs with a functionally important cognate RNA-binding protein (Hfq) is critical for its functionality. For example, RyhB directly promotes mRNA instability by forming a RNA/protein complex containing Hfq that attracts RNase E (108). A recent study suggests that a large number of sRNAs in *Salmonella enterica* are associated with a conserved RNA-binding protein ProQ (109). In some cases, RNase III is responsible for the decay of an mRNA targeted by a sRNA (110).

An interesting question relates to the degradation of sRNAs. Since these are relatively small molecules and highly structured, it was not expected that their degradation would be initiated by endonucleolytic attack. However, both RNase E and RNase III play important roles in degrading sRNAs when they are bound to their target mRNAs (111, 112). The association of Hfq with sRNA-mRNA complexes facilitates this degradation process (113), but the experiments of Andrade *et al.* (114) have shown that sRNAs not associated with Hfq were not substrates for either RNase E or RNase III. Rather, PNPase seemed to be required for their degradation (114), a surprising result since these sRNAs are highly structured. However, it has previously been shown that PNPase can degrade the Rho-independent transcription terminator associated with the *leuX* primary transcript (51). The recent demonstration of that PNPase can both protect and degrade sRNAs is consistent with these observations (115). However, since PNPase has been shown to be inhibited by strong secondary structures (43), it is likely that either polyadenylation by PAP I or unwinding of the secondary structures by one of the DEAD-box RNA helicases (see below) facilitate degradation of sRNAs by PNPase (114, 116). In contrast, sRNAs, such as ryhB, sgrS and cyaR, are destabilized in the absence of PNPase, presumably by RNase E (117).

RNA HELICASES

Although RNAs are transcribed as single-stranded molecules, both nontranslated and translated transcripts readily form secondary and tertiary structures, which are critical for

their proper functioning and stability. For example, many mRNAs are terminated with Rho-independent transcription terminators, a double-stranded stem-loop structure, which also serve as stability elements for mRNAs. All tRNAs assume their tertiary cloverleaf structures as soon as they are transcribed, a step that is crucial for their maturation by various ribonucleases, post-transcriptional modification, and their functionality as amino acid carriers. The formation of large stem-loops in rRNA precursors generate cleavage sites for RNase III. The mature 16S and 23S rRNAs form complex arrays of secondary and tertiary structures in the generation of functional ribosomes. In addition, changes in growth temperatures, such as cold and heat shock, result in altered mRNA structure, which has a significant effect on translation efficiency. It is thus not surprising that bacteria contain helicases that use the energy derived from the hydrolysis of ATP to alter the structure of various RNA molecules. This class of enzyme is characterized by the DEXD/H (usually referred to as the DEAD-box) amino acid motif.

The best characterized of the DEAD-box RNA helicases are the five paralogs (*rhlB, rhlE, srmB, dbpA* and *csdA*, formerly called *deaD*) found in *E. coli.* A strain lacking all the helicases is still viable (118). Only loss of DeaD or SrmB causes significant growth defects and alterations in ribosomal RNA processing and maturation at both 30°C and 37°C (118–120).

RhlB is involved in mRNA decay through its association with the RNase E-based degradosome (9, 121) as well as separately with PNPase (50) and is discussed in more detail in the chapter by Bandryra and Luisi. It has also been shown that RhlE can also associate with the RNase E-based degradosome under certain conditions (122). It aids in the degradation of mRNAs and seems to regulate the roles of other RNA helicases associated with ribosome maturation (122, 123). The SrmB and CsdA helicases function in the process of ribosome biogenesis (119, 120, 124). CsdA has also been associated with a “cold shock degradosome” (125). The DbpA helicase activity is dependent on 23S rRNA (126–128).

SPECIALIZED RIBONUCLEASES

Toxin/antitoxin systems

The phenomenon of bacterial persistence was originally discovered by Bigger in 1944 (129). Simply put in any bacterial population some cells grow considerably slower than others. These slow growing cells are inherently more resistant to antibiotics, which are more effective in killing rapidly growing bacteria. In 1983 Moyed *et al.* (130) showed that mutations in the *hipA* locus led to increased levels of persister cells. These mutations were part of the *hipBA* toxin-antitoxin (TA) locus. Since then multiple TA systems have been identified which are broadly classified into five types. Generally, most toxins target an mRNA to inhibit translation either in a ribosome dependent or independent manner. These are sequence specific endonucleases and have been called “mRNA interferases.” Readers are encouraged to study the chapter: “Leaderless mRNA: Novel Aspects of Ancestral transcripts” in this book for more details.

E. coli contains a large number of TA loci including *mazEF* (2 copies), *relBE* (6 copies), *hipAB*, *rnlAB,* and *hicAB* (131). In the case of RelE, the protein cleaves an mRNA

positioned at the ribosomal A site, between the second and third base of the A-site codon (132–134) leading to translation inhibition. The MazF endonuclease probably has the most sequence specificity of any *E. coli* endonuclease, with the possible exception of the ribonucleases (bacteriocins) that target tRNAs (see next section). It cleaves mRNAs site-specifically at ACA base motifs independent of ribosomes (135). In contrast, the HicA ribonuclease, a protein of only 58 amino acids, cleaves mRNAs without any ribosomal involvement in an apparent random fashion (136).

RNase LS, which was discussed in the section of mRNA decay, is part of the *rnlAB* TA module (39). It is not clear whether there is any cleavage specificity associated with this enzyme or whether it has specific targets or whether it plays any role in persistence.

Bacteriocins function as ribonucleases

Bacteria can produce bacteriocins that can kill other bacteria that are living in close proximity. Colicin, the earliest discovered bacteriocin is encoded by the Col-plasmids. *E. coli* cells not carrying the same or cognate plasmid are killed by the released colicin (137). Colicin production is induced by nutrient starvation, the stringent response, the SOS response and various other stress responses (137). Many of the colicins use the BtuB receptor for cell entry, which is involved in vitamin B12 uptake. There are several types of Colicins, but here we only focus on those encoding an RNase activity.

Of the RNase type, colicin E3 is the most studied. It interacts with intact ribosomes, cleaving the 3′ region of the 16S rRNA between A1493 and G1494 in the decoding A-site, leading to functional inactivation (138–140). In contrast, colicin E5 and colicin D use different cell surface receptors and specifically cleave selected tRNAs. For example, colicin E5 cleaves between the Q and U residues (positions 34 and 35) in the anticodon arm of $tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Asp}$, and $tRNA^{Tyr}$ (141). Colicin D cleaves four of the six $tRNA^{Arg}$ species between positions 38 and 39 at the 3′ end of the anticodon loop.

Additional tRNA cleaving ribonucleases have been identified in *Shigella* and *Salmonella* as part of the *vapBC* toxin/antitoxin system (142). In this case, translation is inhibited by cleavage of the initiator $tRNA^{Met}$ at the 3′ end of the anticodon loop (143). While it had been suggested that the tRNA cleavages lead to cell death, recent work has shown that cells are not immediately killed (141). Rather the tRNA cleavages serve to help regulate cell growth in order to survive a variety of stresses conditions (141).

CRISPR/Cas systems

Unlike the well-characterized restriction/modification systems that provide bacteria broad range protection from phage attack (144), CRISPR/Cas systems provide adaptive immunity to the bacterial strains that carry them. The term CRISPR was first used by Jansen *et al.* (145). These systems are found in almost all archaea and about half of the bacteria that have been examined (146). The pathway found in *E. coli* K12 falls into the Type IE system. Simply put, bacteria carrying a CRISPR/Cas system have one or more CRISPR arrays, which contain short spacer sequences derived from previous bacteriophage infections or exposure to foreign bacterial plasmids, that are separated by identical repeat sequences (147). For any of the spacers to function properly in terms of targeting an invading

bacteriophage, the CRISPR array must be transcribed and processed. In *E. coli*, five Cas proteins form a multiprotein complex called Cascade (148). The *cas6a* gene encodes a metal independent endoribonuclease that cleaves in the repeat sequences to yield 61–62 nt crRNAs that have extra nucleotides at both their 5′ and 3′ ends. These species remain bound to the Cascade complex to facilitate its interaction with the target DNA (147). Interestingly, the Cas6a ribonuclease is normally not expressed in *E. coli* (147). Readers are encouraged to read the excellent recent review by Mohanraju *et al.* (149) on diverse CRSPR/Cas systems for more details.

CONTROL OF RIBONUCLEASES

There are many different ways that bacteria respond to the ever changing environments in which they live. The regulation of various ribonuclease activities is one of them. The levels of RNase E, RNase III and PNPase are autoregulated based on specific circumstances by modulating the stability of their respective mRNAs (150–152). Under certain stress conditions the activity of RNase E is also controlled by global protein regulators, such as RraA and RraB (153, 154). RraA has also been shown to interact with RhlB helicase, a component of the degradosome, to inhibit its RNA-binding and helicase activities (155). The protein YmdB, which is expressed upon cold shock or entry into stationary phase, has been shown to inhibit RNase III activity by preventing its dimerization (156). The level of the 3′ → 5′ exoribonuclease RNase R increases significantly upon entry into stationary phase or cold shock compared to the exponential phase cells due to reduced proteolysis (157).

CONCLUSIONS

Although there has been tremendous progress in our understanding of the mechanisms of post-transcriptional RNA processing and decay over the past 25 years, many questions remain to be answered. With the discovery of new enzymes some aspects of RNA processing have gotten closer attention and some conventional models have had to be significantly revised. However, even after the complete sequencing of multiple *E. coli* genomes, the functions for over one third of the genes are still unknown. Are there additional ribonucleases present in the cell that have not yet been identified?

Perhaps all of the enzymes involved in mRNA decay have been identified, but the regulation of the process is still not well-understood (158) and all the targets of the various enzymes have yet to be identified. For example, RNase III initiates the processing of the 30S rRNA, but the enzyme is not essential for cell viability. Clearly there must be an alternative processing pathway to generate the 16S, 23S and 5S species that are required to produce functional ribosomes. In addition, the ribonuclease(s) required for the 5′ end maturation of both 23S and 5S rRNA are still a mystery.

Our understanding of the role of PAP I and PNPase as polyadenylating enzymes in Gram-negative bacteria is also still limited, especially the function of the polynucleotide tails synthesized by PNPase. First of all, it is not yet clear how these two enzymes select their substrates. The available data (49, 159, 160) suggest that PAP I can add poly(A) tails to both the full-length and decay intermediates. However, in the case of full-length transcripts, those

terminated with a Rho-independent transcription terminator are the preferred substrates and the poly(A) tails are mostly added downstream of the terminator. In contrast, PNPase adds polynucleotide tails to both full-length transcripts terminating in a Rho-dependent fashion and decay intermediates. While poly(A) tail addition clearly targets transcripts for decay (161), no such data is available for polynucleotide tails. However, the extremely long unstructured polynucleotide tails added by PNPase (49, 160) suggest that they also facilitate degradation of its substrates by exonucleases by providing them unstructured substrates.

The notion of cellular compartmentalization in bacteria (162), similar to what occurs in eukaryotes, has further complicated our understanding of the process of post-transcriptional regulation by various enzymes. Since the degradosome is associated with the inner membrane, does that mean that all mRNA decay has to occur at this location? What about the transcripts that are decayed by enzymes other than RNase E? In fact, limited dispersion of some transcripts from the site of transcription was observed in a recent study (163). Clearly, identification of new player(s) and their regulatory processes will help us develop a better understanding of RNA processing and degradation.

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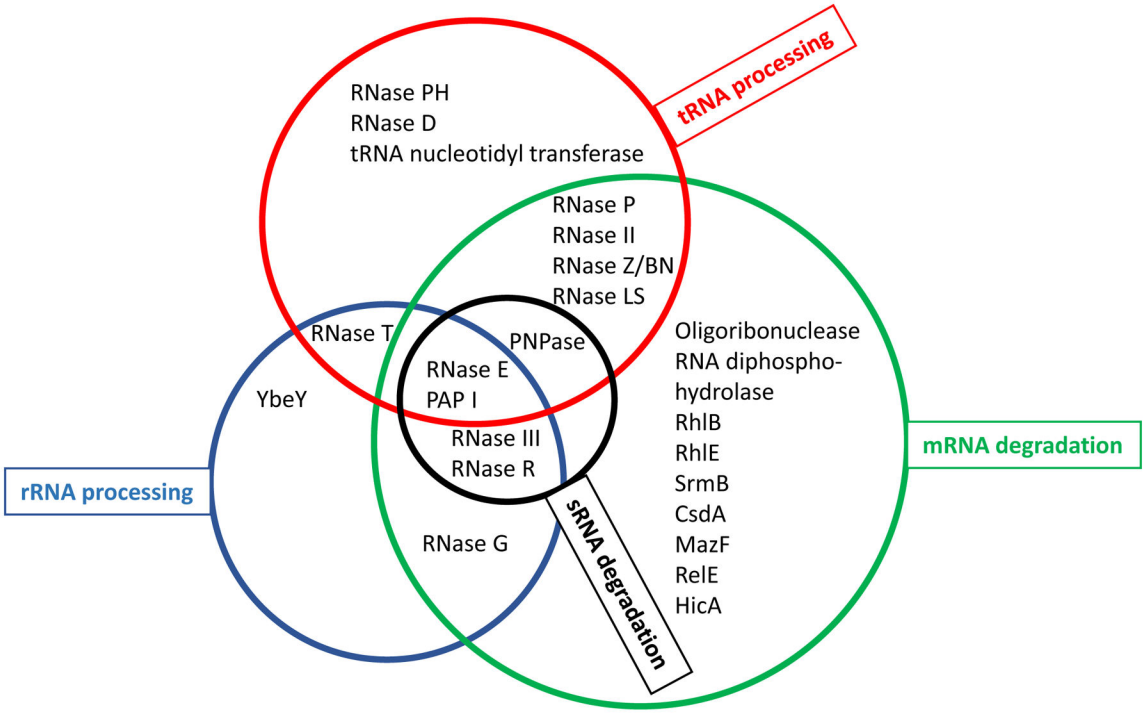

Fig. 1.

Venn diagram of ribonucleases in *E. coli* showing their involvement in the four major RNA metabolic pathways in Gram-negative bacteria. The participation of the various proteins is only included in pathways where it has been established that they play a significant role. In addition, it is possible that some proteins, such as YbeY, are involved in additional pathways.

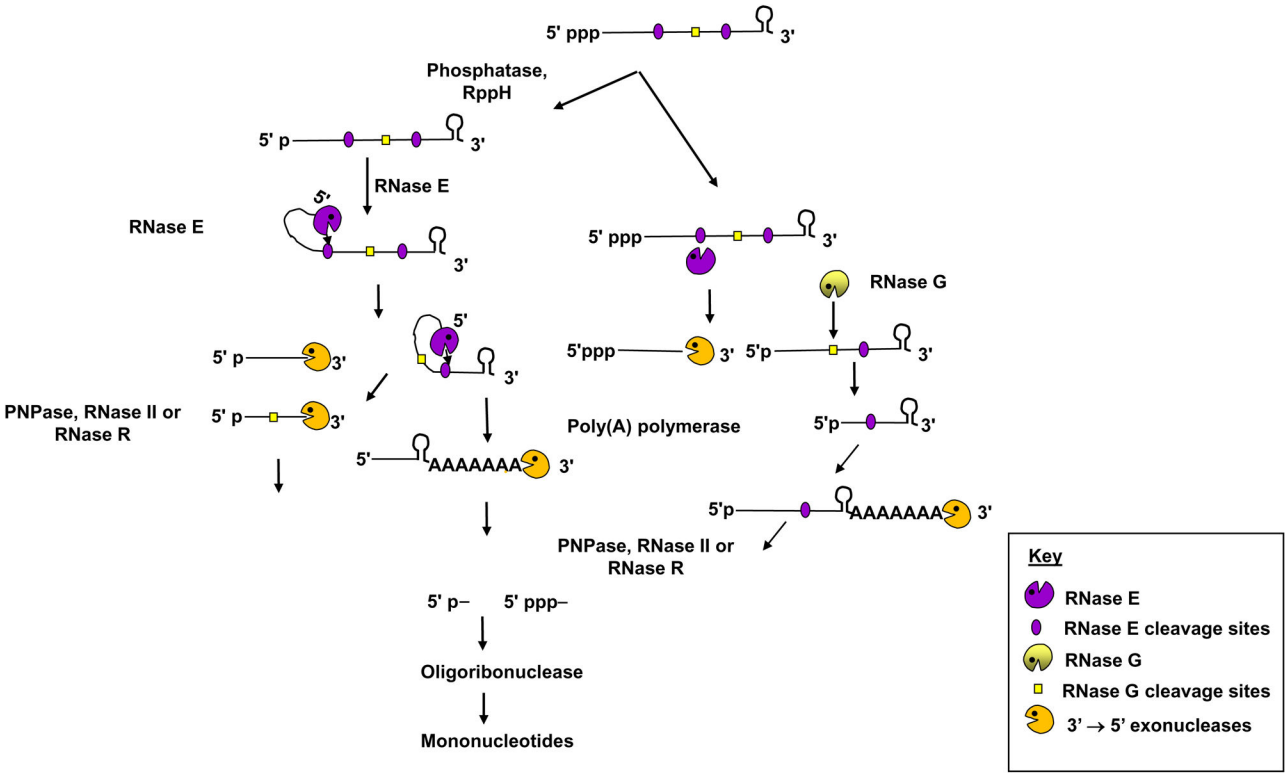

Fig. 2.

Model for the initiation of mRNA decay by RNase E. For the sake of simplicity, the other proteins associated with the RNase E-based degradosome are not shown. In addition, this model is independent of whether RNase E is associated with the inner membrane of *E. coli*. 5′ monophosphate RNA, a preferred substrate for RNase E is degraded via 5′ end dependent pathway. In contrast, 5′ triphosphate RNA is degraded via RNase E internal entry mechanism. Any endonucleolytically cleaved fragments with strong secondary structures, such as one containing a Rho-independent transcription terminator shown here, undergoes polyadenylation by PAP I. Subsequently, all decay intermediates are degraded by 3′ → 5′ exonucleases (PNPase, RNase II and RNase R) followed by oligoribonuclease to mononucleotides. Figure is not drawn to scale.

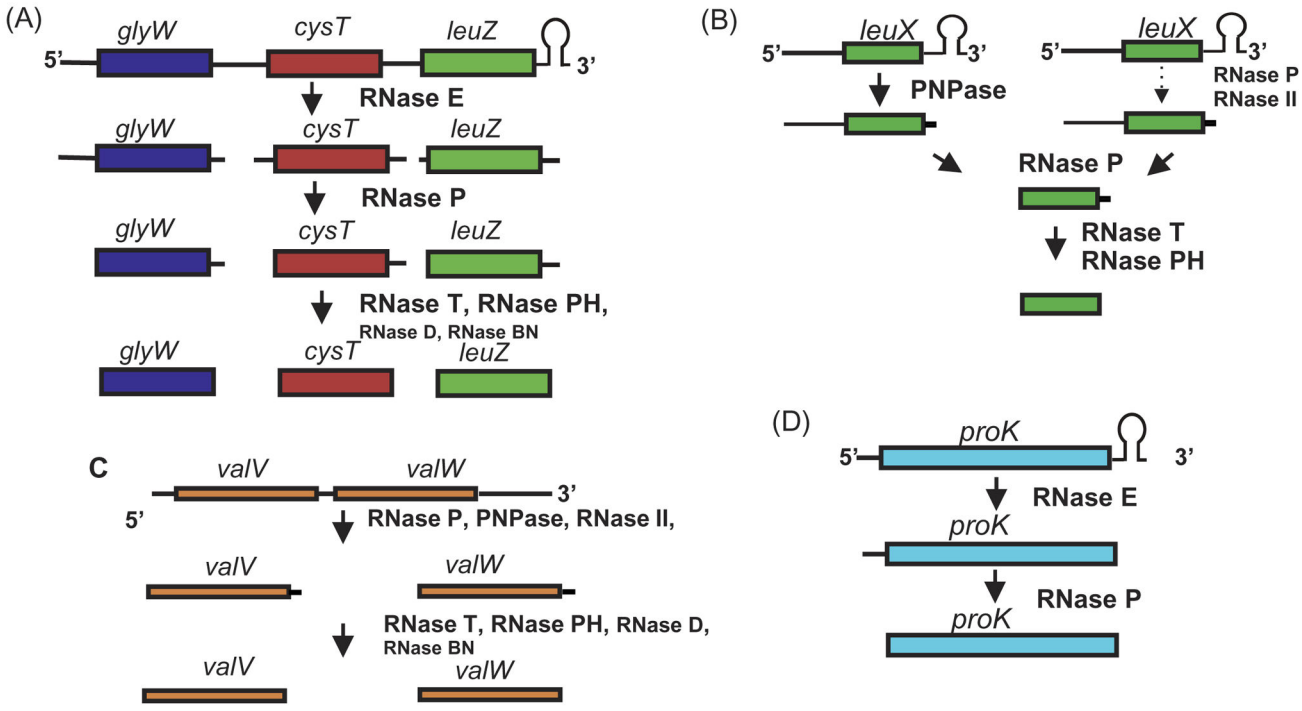

Fig. 3.

Diagrammatic representation of four independent pathways of tRNA processing. (A). Processing of the *glyW cyst leuZ* polycistronic operon. RNase E initiates processing by cleaving the polycistronic transcript to release pre-tRNAs (11). Processing at the 5′ termini is carried out by RNase P. Maturation of the 3′ termini is usually carried out by RNase T and/or RNase PH. If these two enzymes are not present, RNase D and/or RNase BN can complete the process. (B) Processing of the monocistronic *leuX* transcript (51). The Rho-independent transcription terminator is removed exonucleolytically by PNPase. In the absence of PNPase, a combination of RNase P and RNase II can digest the terminator. Subsequently, RNase P matures the 5′ terminus, while RNase T and RNase PH complete the process at the 3′ terminus. (C) Processing of the *valV valW* polycistronic operon (67). RNase P separates *valV and valW* pre-tRNAs by cleaving at their respective mature 5′ ends while PNPase and RNase II shorten the 3′ Rho-depended terminator. Subsequently, 3′ → 5′ exonucleases (RNase T/RNase PH/RNase D/RNase BN) matures the 3′ ends. (D). Processing of the monocistronic *proK* transcript (63). RNase E removes the Rho-independent transcription terminator to generate the mature 3′ terminus without the need of any of the 3′ → 5′ exonucleases. RNase P cleaves at the mature 5′ end. Figure is not drawn to scale.

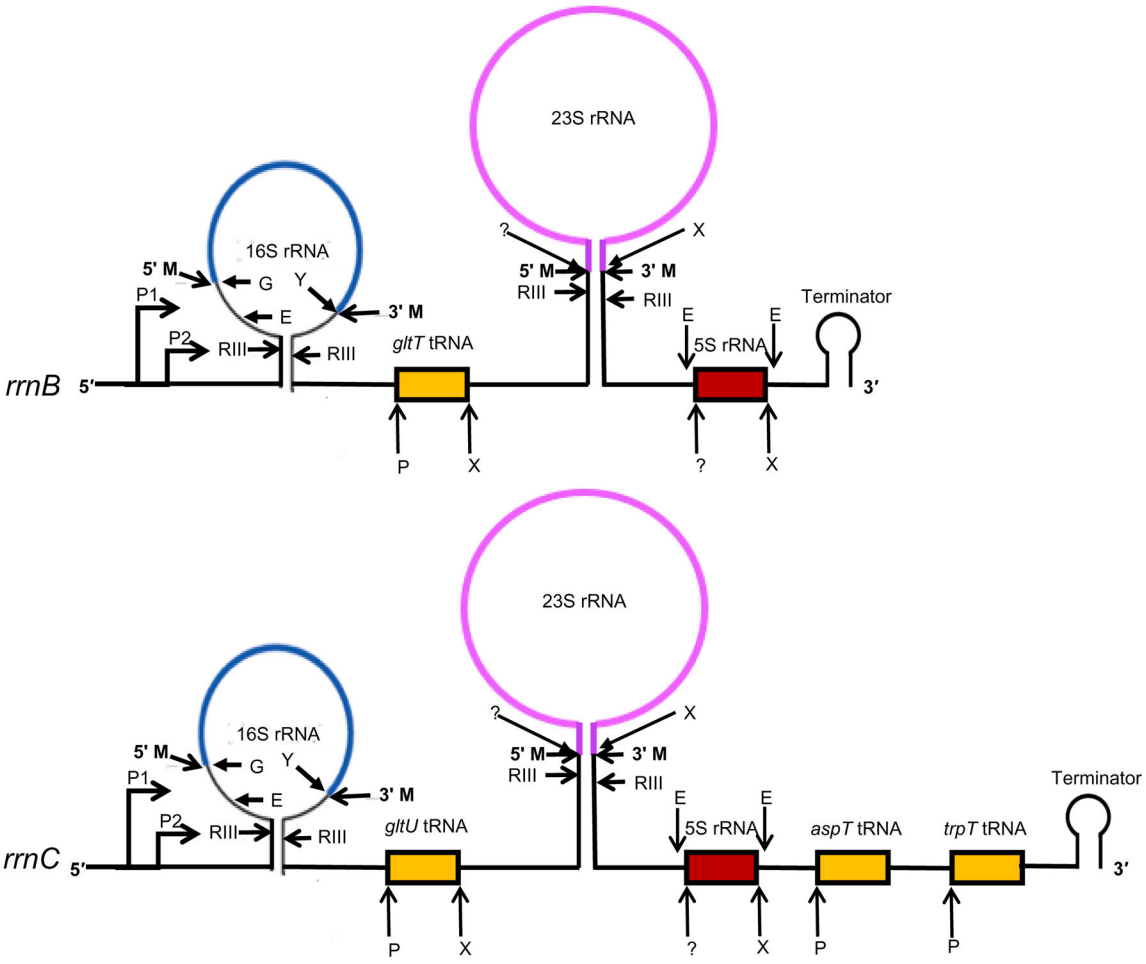

Fig. 4.

Processing of rRNA operons in *E. coli.* The *rrnB* and *rrnC* operons are shown as model operons. RNase III (RIII) cleaves the 30S rRNA transcript first within the double-stranded stems formed by the spacer sequences adjacent to the mature 16S and 23S rRNAs, generating 17S, 25S, and 9S pre-rRNAs. The functional mature 16S rRNA is generated from 17S pre-rRNA after initial RNase E (E) cleavage followed by RNase G (G) at the mature 5′ end and removal of extra 33 nts at the 3′ ends by YbeY (Y) along with multiple exoribonucleases (not shown). A p5S precursor is generated from the the 9S precursor by initial RNase E cleavage at three nt upstream (E) and downstream (E) of the mature termini of the mature 5S rRNA. The mature 5′ end of the tRNAs are generated by RNase P (P) cleavage. Exoribonucleases (X) (primarily RNase T) are responsible for the 3′ end maturation of the tRNAs, 23S rRNA, and 5S rRNA, but the ribonuclease(s) (?) responsible for the maturation of 5′ ends of 23S and 5S rRNAs remain unidentified. The model is not drawn to scale.

Table 1

Proteins involved in post-transcriptional RNA metabolism in *E. coli*.

Name	Gene	Mode of Action	Substrate Preference	Functions*
RNase E	*rne*	Endonuclease	Single-stranded RNA	mRNA decay, tRNA processing, rRNA processing, sRNA degradation
RNase III	*rnc*	Endonuclease	Double-stranded RNA	rRNA processing, mRNA decay, sRNA degradation
RNase P	*rnpA, rnpB*	Endonuclease	Single-stranded RNA	tRNA processing, mRNA decay, rRNA processing
RNase G	*rng*	Endonuclease	Single-stranded RNA	rRNA processing, mRNA decay, tRNA processing
YbeY	*ybeY*	Endonuclease	Single-stranded RNA	rRNA processing
RNase Z/BN	*rnz*	Endonuclease/3′ → 5′ exonuclease	Single-stranded RNA	mRNA decay, tRNA processing
RNase LS	*rnlA*	Endonuclease	Single-stranded RNA	mRNA decay
RNase T	*rnt*	3′ → 5′ exonuclease	Single-stranded RNA	tRNA processing, rRNA processing
RNase PH	*rph*	3′ → 5′ exonuclease	Single-stranded RNA	tRNA processing
RNase D	*rnd*	3′ → 5′ exonuclease	Single-stranded RNA	tRNA processing
PNPase	*pnp*	3′ → 5′ exonuclease	Single-stranded RNA	mRNA decay, sRNA degradation, tRNA processing
RNase II	*rnb*	3′ → 5′ exonuclease	Single-stranded RNA	mRNA decay, tRNA processing
RNase R	*rnr*	3′ → 5′ exonuclease	Single-stranded RNA	rRNA processing, sRNA degradation
Oligoribonuclease	*orn*	3′ → 5′ exonuclease	Single-stranded RNA	mRNA decay
Poly(A) polymerase	*pcnB*	polymerase	Single-stranded RNA	mRNA decay, tRNA maturation
Hfq	*hfq*	RNA binding protein	Single-stranded RNA	mRNA decay, polyadenylation, sRNA metabolism
tRNA nucleotidyl transferase	*cca*	polymerase	tRNAs	tRNA maturation
RNA diphosphohydrolase	*rppH*	phosphatase	5′ triphosphate or 5′ diphosphate	mRNA decay
RhlB	*rhlB*	Helicase	Double-stranded RNA	mRNA decay
RhlE	*rhlE*	Helicase	Double-stranded RNA	mRNA decay
SrmB	*srmB*	Helicase	Double-stranded RNA	mRNA decay
CsdA	*csdA*	Helicase	Double-stranded RNA	mRNA decay under low temperature
DbpA	*dbpA*	Helicase	Double-stranded RNA	Ribosome biogenesis
Enolase	*eno*	Enolase	?	RNA metabolism ?
MazF	*mazF*	mRNA interferase	Single-stranded RNA	mRNA decay, stress management
RelE	*relE*	mRNA interferase	Single-stranded RNA	mRNA decay, stress management

Name	Gene	Mode of Action	Substrate Preference	Functions*
HicA	*hicA*	mRNA interferase	Single-stranded RNA	mRNA decay, stress management
Colicin E5		tRNA targeting enzyme	Single-stranded RNA	Stress management
Colicin D		tRNA targeting enzyme	Single-stranded RNA	Stress management
Colicin E3		tRNA targeting enzyme	Single-stranded RNA	Stress management
Cas6A	*cas6*	Endonuclease	Single-stranded RNA	Immune defense

*Listed in order of the importance of the enzyme

?: Role of enolase in RNA metabolism is yet to be defined.