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Bacterial ribonucleases and their roles in RNA metabolism

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Abstract

Ribonucleases (RNases) are mediators in most reactions of RNA metabolism. In recent years, there has been a surge of new information about RNases and the roles they play in cell physiology. In this review, a detailed description of bacterial RNases is presented, focusing primarily on those from *Escherichia coli* and *Bacillus subtilis*, the model Gram-negative and Gram-positive organisms, from which most of our current knowledge has been derived. Information from other organisms is also included, where relevant. In an extensive catalog of the known bacterial RNases, their structure, mechanism of action, physiological roles, genetics, and possible regulation are described. The RNase complement of *E. coli* and *B. subtilis* is compared, emphasizing the similarities, but especially the differences, between the two. Included are figures showing the three major RNA metabolic pathways in *E. coli* and *B. subtilis* and highlighting specific steps in each of the pathways catalyzed by the different RNases. This compilation of the currently available knowledge about bacterial RNases will be a useful tool for workers in the RNA field and for others interested in learning about this area.

Keywords

E. coli RNases; *B. subtilis* RNases; RNase structure; RNase mechanism; RNase function; RNase regulation; mRNA decay; RNA processing

Introduction

Ribonucleases (RNases) are essential participants in almost every aspect of RNA metabolism including RNA maturation, RNA degradation and turnover, RNA quality control, and even as mediators of regulation. The recognition of the widespread importance of RNases has led to an explosion in the study of these enzymes in recent years. In this review, we will provide a catalog of the known bacterial RNases, discussing their structures and mechanisms of action, their identified functions, their genetics and expression, and where known, their regulation. In addition, we include a broad overview of RNA metabolism

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and indicate the roles of the RNases in these processes. Our focus is primarily on *Escherichia coli* and *Bacillus subtilis*, as most studies have been carried out in these species. We mention examples from other organisms where the function of a ribonuclease may differ from what is known from the model organisms or where information from the model organisms is not available.

For many years, the importance of RNases for RNA metabolism was not well appreciated. RNases were considered to be nonspecific, degradative enzymes, and they were primarily studied as model proteins for structural analyses. RNases were also considered to be nuisance contaminants that often interfered with the ongoing studies of transcription and translation in the 1960s. The only *E. coli* RNases known at the time were RNases I, II, III and polynucleotide phosphorylase (PNPase). Although some of these enzymes showed a preference for either single-stranded (RNase II and PNPase) or double-stranded (RNase III) RNA, none of them was thought to serve any function *in vivo* other than RNA degradation.

The perception of RNases as purely degradative enzymes changed dramatically when it became clear that most RNA molecules were synthesized as precursors that required specific RNase action for their conversion to functional RNAs (see the Brookhaven Symposium in Biology, Number 26, 1974 for a historical perspective). This led to a search, particularly in *E. coli*, for purified RNases that could carry out the cleavage and trimming reactions that converted RNA precursors to mature RNAs. In relatively short order, RNases were discovered that acted at each end of tRNA precursors. These included the endoribonuclease, RNase P (Robertson et al. 1972), and the exoribonucleases, RNase D (Cudny and Deutscher 1980), BN (Asha et al. 1983), T (Deutscher et al. 1984), and PH (Deutscher et al. 1988). In addition, the known enzyme, RNase III, was found to be required for cleavage of double-stranded regions within the precursor rRNA transcript (Young and Steitz 1978; King et al. 1984). The discoveries of RNase E (Gegenheimer et al. 1977), G (Li et al. 1999c; Wachi et al. 1999), R (Cheng and Deutscher 2002), and oligoribonuclease (Niyogi and Datta 1975; Ghosh and Deutscher 1999) identified enzymes participating in mRNA breakdown and other aspects of RNA metabolism. More recently, the discovery that the protein YbeY has RNase activity, and is implicated in rRNA maturation and quality control, has expanded the *E. coli* repertoire of RNases even further (Jacob et al. 2013).

Following the initial studies in *E. coli*, work began in earnest on analysis of RNases in the Gram-positive organism, *Bacillus subtilis*. Although the processes of transcription and translation can be quite similar in diverse bacterial organisms, studies on RNA turnover and processing in *B. subtilis* over the last 25–30 years revealed substantial differences in the ribonuclease activities of this organism compared to those of *E. coli*.

The identification and characterization of ribonuclease activities in *B. subtilis* has historically lagged behind such studies in *E. coli*. Early interest in *B. subtilis* ribonucleases focused on the abundant extracellular enzymes that made Bacillus species an attractive source for industrial enzymes (Priest 1977). While these extracellular ribonucleases may play a role in the uptake of nucleic acids from the environment, they are likely irrelevant to the control of cellular RNAs. By the early 1980s, when 12 *E. coli* ribonucleases were already known, only three ribonucleases from vegetative *B. subtilis* cells had been

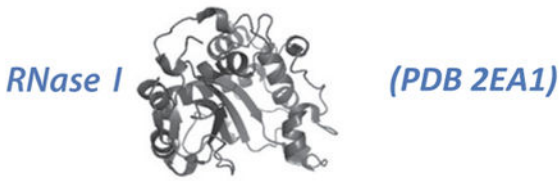
characterized to any extent, and these were all endonucleases that acted on stable RNAs: RNase M5 (5S rRNA maturation) (Sogin and Pace 1974), RNase P (tRNA maturation) (Gardiner and Pace 1980), and RNase III (rRNA processing) (Panganiban and Whiteley 1983a). RNase M5 is not present in *E. coli*, giving the first hint of major differences in ribonuclease activity between *E. coli* and *B. subtilis*.

On the one hand, in 1991, it was shown that ~90% of the degradative activity in an *E. coli* extract was hydrolytic and due to RNase II, while 10% was phosphorolytic and due to PNPase. In a *B. subtilis* extract, on the other hand, degradative activity was primarily phosphorolytic. Although the *B. subtilis* enzyme was not identified, it was suggested that this was a PNPase-like activity. Subsequently, the gene for PNPase was discovered (Luttinger et al. 1996), and the phosphate-dependent, 3′-to-5′ exoribonuclease activity of PNPase was identified (Mitra et al. 1996). This period of ribonuclease discovery in bacteria was accelerated greatly by the many genome sequences that started to appear in the late 1990s. The *B. subtilis* genome sequence (Kunst et al. 1997) revealed much about disparities with *E. coli*. Thus, the *B. subtilis* genome contained no sequences with homology to *E. coli* genes coding for RNase II, for oligoribonuclease, an essential 3′ exoribonuclease required for turnover of nanoRNAs, or for RNase E, the major decay-initiating endonuclease in *E. coli*. Consequently, much effort was expended to find these “missing” activities, or their functional equivalents, which were assumed to be required for life, in *B. subtilis*.

Although it was well known that eukaryotes were able to degrade RNA processively in the 5′-to-3′ direction (Muhlrad et al. 1994), the possibility of such an activity in bacteria was doubted, since no such activity is known in *E. coli*. However, in 2007, the first bacterial 5′-to-3′ exoribonuclease, *B. subtilis* RNase J1, was discovered (Mathy et al. 2007). The missing oligoribonuclease activity was also discovered in 2007 (Mechold et al. 2007), and, in fact, several *B. subtilis* ribonuclease activities can degrade nanoRNAs (Fang et al. 2009). Relatively recently, *B. subtilis* RNase Y, an endoribonuclease, was discovered and found to be highly conserved in the *Firmicutes* (Commichau et al. 2009). The enzyme is likely the functional equivalent of *E. coli* RNase E (Lehnik-Habrink et al. 2011). Of the dozens of RNases known to exist in *E. coli* and *B. subtilis*, relatively few of them are shared (Figure 1), allowing diverse mechanisms of RNA maturation and degradation.

E. coli endoribonucleases

At least nine endoribonucleases have been purified from *E. coli* and studied in some detail. These include RNases I, III, P, BN, E, G, YbeY, HI, and HII (Table 1). In addition, several toxins exhibiting ribonuclease activity, such as RelE and MazF that become active under stress conditions are also known, but will not be dealt with in detail here. The endoribonucleases generally require divalent cations to cleave RNA, generating products with 3′-hydroxyl and 5′-phosphate termini. However, RNase I functions differently; it does not require a divalent cation for activity, and produces fragments with 3′-phosphoryl termini. YbeY also generates 3′-phosphoryl-terminated products, but it is thought to require a divalent cation for activity. Some of the endoribonucleases are highly specific with regard to their substrates and to their cleavage sites, whereas others cleave RNA relatively indiscriminately, leading to extensive degradation.

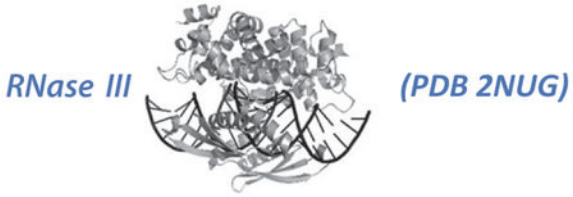


RNase I degrades all types of RNA molecules with a preference for single-stranded regions (Spahr and Hollingworth 1961). Initially, fragments with 2′, 3′-cyclic nucleotides are generated, which ultimately are converted to 3′-phosphates; limit digests consist primarily of 3′-mononucleotides. Based on the sequence of the *rna* gene encoding RNase I (Meador and Kennell 1990; Zhu and Deutscher 1992), RNase I is a protein of 245 amino acids with a calculated molecular mass of 27.2 kDa. The protein apparently functions as a monomer (Deutscher et al. 1984). RNase I is unusual with regard to its subcellular localization since almost 90% of the enzyme is released upon preparation of spheroplasts, indicating that it largely resides in the periplasmic space (Neu and Heppel 1964; Zhu and Deutscher 1992). However, upon preparation of extracts, RNase I is found bound to 30S ribosome subunits for which it has a high affinity (Spahr and Hollingworth 1961). RNase I is a member of the T2 superfamily of RNases, examples of which are found in most organisms (Irie 1997).

Over the years, multiple endoribonucleases with molecular masses and catalytic properties similar to RNase I have been reported to be present in *E. coli*. These activities have been referred to as RNase IV (Spahr and Gesteland 1968), RNase F (Gurevitz et al. 1982), RNase I* (Cannistraro and Kennell 1991), RNase M (Cannistraro and Kennell 1989), and RNase R (Srivastava et al. 1992). However, it is unlikely that these are distinct enzymes. Examination of the *E. coli* genome revealed no other genes related to *rna* (Subbarayan and Deutscher 2001). Thus, these enzymes either are altered forms of RNase I with slightly different catalytic properties or they are derived from multiple genes unrelated to *rna* despite the similarity of the proteins to RNase I. The latter possibility seems unlikely. In fact, it is already known that RNase I*, which is found inside the cell, is a partially reduced form of RNase I that differs in several properties from the fully oxidized form of RNase I present in the periplasm (Cannistraro and Kennell 1991). RNase I contains eight cysteine residues (Meador and Kennell 1990), and the possibility of multiple alternate structures, arising from the formation of different disulfide bonds, might account for some of the proteins apparently related to RNase I. In addition, the protein purified and characterized as RNase M (Cannistraro and Kennell 1989) is actually a multiple-mutated form of RNase I (Subbarayan and Deutscher 2001). RNase M has three amino acid changes compared to wild-type RNase I, and also is transcribed from an *rna* gene that contains a UGA nonsense codon at position 5, and is the result of a low level of readthrough (Subbarayan and Deutscher 2001).

The *rna* gene encoding RNase I, located at 13.9 min on the *E. coli* genetic map (Rudd 1998), has an unusual promoter. It contains a −35 region that is a poor match to the consensus sequence, and also is located within a stem-loop structure that may serve as a transcription termination site for an upstream gene (Zhu et al. 1990). The *rna* gene also encodes a 23-amino acid leader peptide that likely serves as the signal peptide for the transport of the protein to the periplasmic space (Meador and Kennell 1990).

The physiological role of RNase I is still unclear. Strains of *E. coli* in which RNase I is naturally absent or in which RNase I has been deleted, grow normally and show no major metabolic defects (Gesteland 1966; Zhu et al. 1990). Under certain stress conditions or conditions that damage the cell membrane, periplasmic RNase I may enter the cell, leading to extensive RNA degradation (reviewed in (Deutscher 2003)). However, it is unlikely that this is the primary role of RNase I since its entry would probably kill the cell. A recent paper has suggested that the intracellular portion of RNase I (~10% of the total) is responsible for generating 2', 3′ cNMPs in *E. coli*, known products of RNA degradation by this enzyme *in vitro*, and that RNase I and the cyclic nucleotides regulate biofilm formation (Fontaine et al. 2018). Further examination of this system will be of considerable interest. However, this finding still leaves unclear what role is played by the 90% of RNase I that resides in the periplasmic space.



RNase III is widely distributed in almost all bacteria (Condon and Putzer 2002). In *E. coli*, it is the primary enzyme that specifically hydrolyzes double-stranded RNA (dsRNA) (Robertson et al. 1968), and it has served as the prototype for the study of endoribonucleases that cleave double-stranded RNAs from other organisms. RNase III is active as a 50-kDa homodimer (Dunn 1976). Based on the sequence of the *rnc* gene, which encodes RNase III, the monomer is a polypeptide of 226 amino acids (March et al. 1985; Nashimoto and Uchida 1985) with a calculated molecular mass of 25.6 kDa.

The *E. coli* enzyme consists of two domains: (1) the N-terminal RNase III domain of about 150 amino acids which contains a signature sequence of 10 conserved amino acids (Mian 1997) that are important for catalytic activity. This portion of the RNase III protein is catalytically active by itself retaining activity similar to that of intact RNase III under certain *in vitro* conditions (Sun et al. 2001). In fact, a shorter form of RNase III consisting of only the RNase III domain, termed Mini-III, is found in some organisms, such as *B. subtilis*, and participates in rRNA maturation (Redko et al. 2008); and (2) the C-terminal one-third of RNase III, which is a dsRNA-binding domain (dsRBD) (St Johnston et al. 1992; Kharrat et al. 1995; Tian and Mathews 2003). This portion of the protein contains a conserved $\alpha\beta\beta\beta\alpha$ fold involved in ds-RNA binding (Masliah et al. 2018). The isolated dsRBD by itself can bind dsRNA *in vitro*, but it is unable to cleave the substrate (Nicholson 1997). While the dsRBD is not essential for RNase III activity, it does play a role in determining substrate specificity and cleavage site location based on analysis of RNase III hybrid proteins (Conrad et al. 2001).

Several crystal structures of bacterial RNase III proteins have been reported. These include the RNase III domain of the enzyme from *Aquifex aeolicus* (Aa), both in a free form and in a complex with Mn^{2+} (Blaszczyk et al. 2001). This structure contains seven α-helices, but

no β-strands, and is a dimer. Dimerization of the monomers creates a valley that can accommodate the dsRNA substrate. Mn^{2+} binding to each subunit facilitates the formation of two potential RNA-cutting sites within each active center. Six negatively charged amino acid residues are present in the two sites some of which are known to be important for catalysis based on mutational analysis of the corresponding residues in the *E. coli* protein (Li and Nicholson 1996; Dasgupta et al. 1998; Blaszczyk et al. 2001). The structure of the nuclease domain of the protein from *Mycobacterium tuberculosis* has also been reported (Akey and Berger 2005), as has the structure of the dsRBD of *E. coli* RNase III (Kharrat et al. 1995). These structural analyses help explain why RNase III selectively recognizes dsRNA, why it displays an apparent lack of sequence specificity, and how it can cleave on both sides of the double strand (Ryter and Schultz 1998).

Cleavage on both strands of dsRNA by RNase III creates fragments with 5′-phosphate and 3′-OH groups and containing 2-nt 3′ overhangs (Dunn 1982; Nicholson 1996). The action of RNase III requires the divalent cation Mg^{2+}, but Mn^{2+}, Co^{2+}, or Ni^{2+} also support catalysis (Nicholson 1997). RNase III recognizes multiple features of its RNA substrates including at least two specific segments within the substrate that are important for recognition and positioning relative to the cleavage sites (Pertzev and Nicholson 2006). In addition, a common structural element required for cleavage is a dsRNA of about 20 bp (Robertson 1982; Court 1993). This structure may be generated by separate RNA strands or by intra-molecular base pairing and does not have to be perfectly complementary. However, the mechanism by which the site of cleavage is selected is not fully understood. For example, some known RNase III substrates contain an internal loop within the double-stranded region which can direct cleavage to one of the single strands rather than the ds region (Nicholson 1999). Further complicating a complete understanding of RNase III action are the highly specific cleavages within a ds region that are carried out by RNase III *in vivo*.

The primary role of RNase III in *E. coli* RNA metabolism is a direct reflection of its specificity for cleaving dsRNA substrates. The enzyme functions in the maturation of rRNA where it cleaves in the double-stranded regions that bracket the 16S and 23S rRNAs to convert the primary transcript into shorter intermediates (Young and Steitz 1978; Bram et al. 1980; Sirdeshmukh and Schlessinger 1985; Figure 2). These reactions, which are carried out on preribosomal particles (Srivastava and Schlessinger 1988; Allas et al. 2003), generate a 17S precursor to mature 16S rRNA containing 115 additional nucleotides (nt) at the 5’ terminus and 33 extra nt at the 3′ terminus. A P23S precursor to 23S rRNA with 7 or 8 extra 5’ nt and 7 extra 3′ nt, and a 9S precursor to 5S RNA are also generated (Deutscher 2009). Although RNase III is not absolutely essential for these reactions (cells can grow in the absence of RNase III), the absence of RNase III leads to the accumulation of a 30S rRNA precursor molecule, and aberrant processing and accumulation of incompletely processed, yet functional, 23S RNA species (King et al. 1984). 16S rRNA is matured normally in such a mutant strain (King et al. 1984).

RNase III also participates in the maturation or decay of cellular and phage mRNAs, of some tRNAs, and of transcripts from plasmids (Nicholson 1999; Conrad and Rauhut 2002). Approximately 12% of all mRNAs are affected by the absence of RNase III with some

increasing and some decreasing in abundance (Stead et al. 2011). However, since the absence of RNase III leads to slowed growth, it is possible that some of these effects may simply be a consequence of the change in growth rate. Nevertheless, it is clear that RNase III can affect mRNAs by cleaving stem-loop structures at either end of a message thereby influencing stability and translatability. For example, translation of *E. coli adhE* mRNA and several mRNAs derived from phage T7 are activated by RNase III cleavages in their 5′-UTR which remove sequences blocking the ribosome binding site (Court 1993; Aristarkhov et al. 1996; Nicholson 1996). Also, RNase III action is required for maturation of some combined tRNA-mRNA transcripts and some polycistronic mRNAs (Régnier and Grunberg-Manago 1989; Nicholson 1999).

RNase III is encoded by the *rnc* gene located at 58.2 min on the *E. coli* genetic map (Rudd 1998). It is the first gene in an operon that also contains *era* and *recO* downstream (Takiff et al. 1989). Interestingly, Era is a GTPase that also participates in ribosome biogenesis (Tu et al. 2009). Era-GTP binds to the anti-Shine–Dalgarno region on 16S rRNA and facilitates rRNA maturation (Tu et al. 2011). RNase III expression and activity are regulated at multiple levels. These include autoregulation of the *rnc* message by the action of RNase III (Bardwell et al. 1989; Régnier and Grunberg-Manago 1990), phosphorylation of RNase III protein following T7 infection, which stimulates its activity fourfold (Nicholson 1999), and association with YmdB, which prevents formation of the RNase III dimer (Kim et al. 2008).

RNase P is found in essentially all bacteria. It is primarily responsible for generating the mature 5’ terminus of tRNA molecules by endonucleolytically removing the 5’ precursor residues (Robertson et al. 1972; Figure 3), but it acts on other RNAs as well. The enzyme is unusual in that it contains both an essential RNA component and a protein subunit. The RNA component is responsible for catalysis (Guerrier-Takada et al. 1983). The *E. coli* RNA, termed M1 RNA (Stark et al. 1978), is 377 nucleotides in length with a mass of ~130 kDa. The protein component, termed C5, is a basic polypeptide of 119 amino acids with a molecular mass of 13.8 kDa. M1 RNA and C5 protein can be combined to reconstitute the holoenzyme *in vitro*. The RNase P action generates products with 5′-phosphate and 3′-hydroxyl termini in a reaction that utilizes Mg^{2+}, but other cations also can function. Under certain conditions *in vitro*, M1 RNA can digest tRNA precursors in the absence of C5 protein (Guerrier-Takada et al. 1983). However, the presence of C5 protein greatly enhances *in vitro* cleavage and is required for RNase P action *in vivo* (Altman 1990; Pace and Smith 1990)

Extensive structural analysis of the RNA component of RNase P has led to a general understanding of its architecture. The RNA consists of two independent domains with

separate functions – a specificity domain (S domain) and a catalytic domain (C domain) (Pace and Smith 1990; Pace and Brown 1995; Massire et al. 1998; Kurz and Fierke 2000), one involved in substrate recognition and the other forming the active site (Torres-Larios et al. 2006). The RNase P RNAs have been divided into two groups, A and B, based on their structures, and each has a distinct distribution (Haas et al. 1996). *Escherichia coli* M1 RNA is a member of the A type. An RNA of the B Type can substitute for M1 RNA and preserve viability, but growth rates are reduced dramatically and many transcript levels are altered (Loveland et al. 2014). M1 RNA also plays a major role in the solubility of C5 protein *in vivo* and in its proper folding, which thereby prevents its proteolysis (Son et al. 2015). Interestingly, *E. coli* cells in which M1 RNA is not expressed are viable in the presence of the RNase P from *Arabidopsis*, an enzyme that normally lacks a catalytic RNA (Gößringer et al. 2017). However, while such cells retain viability, multiple tRNA substrates that do not have the normal tRNA structure are not processed. Nevertheless, these findings indicate that an RNA-based RNase P is not an essential component for *E. coli* viability.

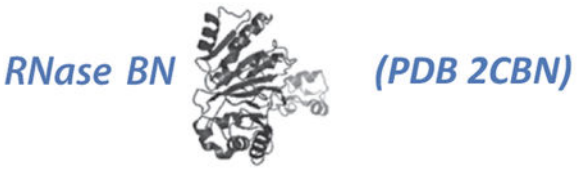
A major advance in our understanding of RNase P structure and its mechanism was the determination of the crystal structure of the *Thermatoga maritima* holo-enzyme in a complex with $tRNA^{phe}$ (Reiter et al. 2010). This structure confirmed much of the earlier biochemical, phylogenetic and theoretical work that had been carried out over many years (reviewed in Altman and Kirsebom 1999; Christian et al. 2002; Kirsebom 2002). Based on the *T. maritima* structure, there are no major changes in the RNA and protein components of RNase P upon binding of the tRNA substrate. The S-domain interacts with the TΨC and D loops of the tRNA through base stacking, and an unstacked A residue enters the minor groove of the tRNA acceptor stem. There is also an additional interaction between the 3′-CCA of the substrate and a loop of the RNase P RNA. The protein is adjacent to the 5’ end of the tRNA, and makes extensive contacts with the substrate leader sequence, but not the mature portion of the tRNA molecule. The active site contains parts of the phosphate backbone, at least two metal ions, and a universally conserved U residue. This detailed picture of the RNase P-substrate interaction provides important information about how the RNA and protein components of RNase P work together to cleave the pre-tRNA substrate. It is likely that this information is applicable to all RNA-containing RNase P’s.

The primary function of RNase P is the maturation of the 5′ end of tRNAs (Figure 3), and no other enzyme can substitute for RNase P in this process. Consequently, RNase P is essential for cell viability, and temperature-sensitive mutants of either M1 RNA or C5 protein accumulate high levels of tRNA precursors at non-permissive temperatures (Sakano and Shimura 1978). RNase P also participates in maturation of other RNAs. These include the 5′ ends of 4.5S RNA (Peck-Miller and Altman 1991), tmRNA (Komine et al. 1994), and C4 antisense RNAs of bacteriophages P1 and P7 (Komine et al. 1994). In some cases, RNase P acts at the 3′ end of tRNA precursors. For example, it is involved in the maturation of the 3′ end of tRNA-leuX (Nomura and Ishihama 1988), and it removes Rho-independent transcription terminators from valU and lysT transcripts (Agrawal et al. 2014). RNase P displays only limited action on mRNAs (Alifano et al. 1994; Li and Altman 2003).

M1 RNA is the product of the *rnpB* gene located at 70.4 min of the *E. coli* chromosome (Rudd 1998). The initial RNA transcript contains 36 extra nucleotides at its 3′ end

(Lundberg and Altman 1995), which are removed by an RNase E cleavage 1 or 2 nucleotides downstream of the mature 3′ end, followed by trimming by any one of several exoribonucleases (Li et al. 1998). Processing of the 3′ end of M1 RNA is essential for cell viability and for protecting the transcript from degradation (Kim et al. 2005). M1 RNA is transcribed from multiple promoters, but the strongest is the most proximal one that leads to the pM1 precursor (Sakamoto et al. 1983; Motamedi et al. 1984). Transcription from upstream start sites results in transcripts having various lengths of 5′ extra sequences, the longest of which has four additional open reading frames upstream of the transcript of *rnpB* (Motamedi et al. 1984; Lundberg and Altman 1995). The function of these longer transcripts is unclear. In fact, one such a transcript was degraded by RNase E rather than being processed (Ko et al. 2008). The *rnpA* gene, encoding the C5 protein, maps at83.7 min (Rudd 1998). It is co-transcribed with the upstream *rpmH* gene encoding the ribosomal protein L34 (Hansen et al. 1985), but whether this has any functional significance is not known.

Very little is known about the regulation of RNase P expression. The fact that M1 RNA can be transcribed from multiple promoters and that it must be processed raises the possibility of regulation at these steps. RNase P is down-regulated by the *relA* locus and ppGpp (Dong et al. 1996; Altman and Kirsebom 1999; Park et al. 2002), and a consensus sequence responsive to stringent conditions is present in the promoter of the *rnpB* gene. Mutation of this sequence (Jung and Lee 1997), or of the flanking region (Park et al. 2002), alters the response of M1 RNA production to stringent conditions.



RNase BN was originally discovered as the enzyme responsible for 3′ processing of the four bacteriophage T4-encoded tRNAs that lacked an encoded -CCA sequence and required a host nuclease for their maturation (Seidman et al. 1975). This nuclease was identified in wild-type cells (Asha et al. 1983), but was missing in the *E. coli* mutant strains, BN and CAN (Maisurian and Buyanovskaya 1973), which resulted in the inability of these strains to support growth of a mutant T4 phage that depended on the synthesis of one of these tRNAs, a suppressor $tRNA^{Ser}$ (Seidman et al. 1975). Subsequent work (Ezraty et al. 2005) showed that RNase BN is a member of the RNase Z superfamily of nucleases that are widespread in eukaryotes, archaea and bacteria (Minagawa et al. 2004; Li de la Sierra-Gallay et al. 2005). The RNase Z homolog in *E. coli* is encoded by the *rbn* gene (originally called *elaC*), located at 51.3 min on the chromosome. Members of the RNase Z family are endoribonucleases that cleave tRNA precursors lacking an encoded -CCA sequence right after the discriminator nucleotide (Schiffer et al. 2002; Pellegrini et al. 2003), followed by -CCA addition catalyzed by tRNA nucleotidyltransferase (Deutscher 1990). The presence of an RNase Z homolog in *E. coli* has been surprising in light of the fact that all its tRNA genes encode a -CCA sequence (Deutscher 1990), obviating a need for this activity. Moreover, mutant strains lacking this enzyme have no obvious growth phenotype (Schilling et al. 2004).

Consequently, it has been of considerable interest to determine the physiological role in *E. coli* of this apparently unnecessary enzyme.

Purified RNase BN is an α_2-dimer with a molecular mass of ~65–70 kDa (Vogel et al. 2002; Ezraty et al. 2005), in excellent agreement with the monomer mass calculated from the *rbn* gene of 32.9 kDa (Ezraty et al. 2005). This information was confirmed by the crystal structure of the enzyme that showed it is a dimer with a core zinc-dependent β-lactamase domain containing a H*X*H*X*DH metal binding motif and additional His and Asp residues that also coordinate to the metal ions (Kostelecky et al. 2006). The metal binding site is believed to be the catalytic site. Each subunit of the protein contains a flexible arm thought to be involved in tRNA binding. A channel leads to the catalytic site which facilitates stable RNA binding, and a channel leading from the metal-binding site binds the 3′ extra residues that have been cleaved by the action of the enzyme (Dutta et al. 2013).

Escherichia coli RNase BN is unusual among RNase Z family enzymes in that it is an efficient phosphodiesterase active against bis(*p*-nitrophenyl) phosphate and thymidine-5'-*p*-nitrophenyl phosphate (Vogel et al. 2002; Dutta and Deutscher 2009). This is in keeping with the observation that RNase BN has both exo- and endoribonuclease activity (Dutta and Deutscher 2009). RNase BN is also unusual in that its activity is the highest in the presence of Co^{2+} (Asha et al. 1983; Dutta and Deutscher 2009). In addition, Co^{2+} promotes exoribonucleolytic activity against certain substrates, whereas Mg^{2+} favors endoribonucleolytic activity (Dutta and Deutscher 2010). RNase BN is active on both double-and single-stranded RNA molecules, although the former are preferred (Dutta and Deutscher 2009). The enzyme displays a dramatic base specificity, being most active on runs of A residues and essentially inactive on runs of C. RNase BN acts as a distributive exoribonuclease on some substrates, releasing mononucleotides, and as an endoribonuclease on others, releasing fragments as short as 4 nt (Dutta and Deutscher 2009). The presence of a 3′-phosphoryl group inhibits the exoribonuclease activity, but has no effect on endonucleolytic cleavages. RNase BN is also strongly inhibited by a -CCA sequence Many of these properties are quite distinct from other members of the RNase Z family.

RNase BN displays a narrow specificity on tRNA-type and other RNA molecules. It is active on some artificial tRNA substrates in which residues within the -CCA sequence have been altered, such as tRNA-CU and tRNA-CA, but tRNA-CC, intact tRNA-CCA, diesterase-treated tRNA, rRNA, and poly (A) are poor or inactive substrates (Asha et al. 1983; Ezraty et al. 2005). RNase BN also acts on the tRNA precursor analog tRNA-CCA-C_n. Purified RNase BN can process tRNA precursors *in vitro* containing or lacking a -CCA sequence (Dutta and Deutscher 2010). On precursors lacking a -CCA sequence, RNase BN endonuceolytically cleaves the molecule immediately after the discriminator nucleotide to generate a substrate for -CCA addition by tRNA nucleotidyltransferase. On precursors in which the -CCA sequence is present, RNase BN can act as either an exo-or endoribonuclease dependent on the cation present. In the presence of Co^{2+}, RNase BN removes the extra 3′ nt predominantly in an exonucleolytic manner to generate a mature 3′ end. In contrast, when Mg^{2+} is present, the extra nucleotides are removed by an endonucleolytic cleavage right after the -CCA sequence. In no case is the -CCA removed.

The action of RNase BN on tRNA precursors *in vitro* agrees very well with how it acts *in vivo*. RNase BN can participate in the maturation of cellular tRNAs, but its action only becomes evident when the exoribonucleases, RNases II, D, T, and PH, which are the preferred tRNA maturation enzymes, are removed (Kelly and Deutscher 1992b). The mutant cells remain viable, but grow poorly; their slow growth is dependent on RNase BN as its removal leads to inviability (Kelly and Deutscher 1992b). Mature tRNAs are made, but generally at low levels; for example, a suppressor $tRNA^{Tyr}$ is made at only ~10% of the wild-type level (Reuven and Deutscher 1993). Nevertheless, these data indicate that all essential cellular tRNAs can be processed by RNase BN. Likewise, RNase BN is essential for the maturation of certain bacteriophage-encoded tRNAs, even when all other RNases are present (Callahan and Deutscher 1996). How RNase BN acts on these various tRNAs *in vivo* then becomes of considerable interest.

Utilizing a strain carrying a chromosomal copy of a mutant *rbn* gene (P142G) whose RNase BN lacks exoribonuclease activity (Dutta et al. 2012), it was found that the exonuclease activity was not needed for maturation of those phage T4 tRNA precursors that lack an encoded -CCA sequence, in keeping with the finding that such tRNAs are processed endonucleolytically *in vitro*. On the contrary, in a strain lacking other processing exoribonucleases, removing the exonuclease activity of RNase BN results in slower growth and poorer maturation of multiple cellular tRNAs, indicating that both the exo- and endonuclease activities of RNase BN can function *in vivo*.

One interesting feature of RNase BN is that it does not remove the -CCA sequence from mature tRNAs or from tRNA precursors in either the exo- or endonucleolytic mode of action (Dutta and Deutscher 2010). This unusual specificity avoids a futile cycle of removing and repairing this essential sequence. Both the adjacent C residues of the -CCA sequence and an Arg residue (Arg^{242}) present within a highly conserved motif in the catalytic channel are required for sparing the -CCA sequence. When these determinants are present, CCA-containing tRNAs present in the catalytic channel are unable to move into the catalytic site. Conversion of the Arg residue to Ala or substitution of either C residue by A or U enables RNA movement and allows catalysis to proceed. This novel mechanism preserves the stability of mature tRNAs from the action of RNase BN which otherwise would cleave after the discriminator nucleotide requiring re-addition of the -CCA sequence.

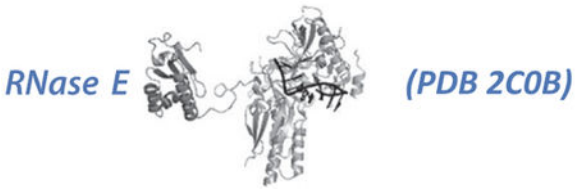
Although RNase BN can process tRNA precursors *in vivo* (Figure 3), it is unlikely that this is a significant function of the enzyme in *E. coli*. Maturation of tRNAs by RNase BN is extremely inefficient compared to other exoribonucleases, and, moreover, removal of RNase BN has essentially no effect on cell growth. These findings raise the question of what RNAs might be the primary targets of RNase BN. One hint came from the finding that RNase BN levels are high in exponential phase cells, but decrease as much as 90% in stationary phase (Chen et al. 2016). This led to an examination of the effect of RNase BN on multiple small RNAs that are low in exponential phase and increase in stationary phase. The analysis revealed that RNase BN had a direct effect on 6S RNA, a global regulator of transcription that acts by binding to sigma 70-containing RNA polymerase. The presence of RNase BN keeps 6S RNA levels low by decreasing its stability in the exponential phase, and as RNase BN decreases, 6S RNA in stationary phase cells increases (Chen et al. 2016). Removal of

RNase BN elevates 6S RNA in exponential phase cells as well. The action of RNase BN on 6S RNA is dependent solely on its endonuclease activity (Chen et al. 2016). These findings raise the possibility that the primary role of RNase BN in *E. coli* may be to down-regulate certain sRNAs in exponential phase cells, although it has also been implicated in mRNA decay (Perwez and Kushner 2006).

The findings with 6S RNA indicate that RNase BN itself must be subject to growth phase-dependent regulation. Since RNase BN and *rbn* message both decrease concomitantly in stationary phase (Chen et al. 2016), it is thought that regulation is post-transcriptional. In fact, further analysis revealed that *rbn* mRNA is much more unstable in stationary phase cells, and that the relative stability of the message in exponential phase cells is due to binding of the small RNA, GcvB, and the protein, Hfq, that together protect *rbn* mRNA against cleavage by RNase E (Chen and Deutscher, unpublished). Reduction of GcvB in stationary phase cells lowers the protection, rendering *rbn* mRNA sensitive to the action of RNase E thereby reducing the amount of RNase BN.

RNase E/G family

RNase E and RNase G can be considered to be members of the same family of endoribonucleases. Members of this family are widely distributed among Gram-negative bacteria, some containing only one of the enzymes, and others containing both. RNase G is the smaller protein, and is highly homologous to the catalytic, N-terminal half of RNase E, but it lacks the additional domains that are responsible for the interaction of RNase E with other proteins to generate the RNA degradosome.



RNase E is a multifunctional enzyme that participates in many aspects of RNA metabolism in *E. coli*. It was first discovered as an activity responsible for converting 9S RNA to a precursor of 5S rRNA (Gegenheimer et al. 1977; Ghora and Apirion 1978; Figure 2). Subsequently, it was found to also participate in mRNA degradation (Ono and Kuwano 1980), and is now known to be the primary enzyme initiating mRNA decay in this organism (Mackie 2013; Bandyra and Luisi 2018; Figure 4). Over the years, the role of RNase E has expanded to include maturation of 16S rRNA (Li et al. 1999b; Figure 2), tRNA (Li et al. 1999b; Figure 3), tmRNA (Lin-Chao et al. 1999), and M1 RNA (Lundberg and Altman 1995; Sim et al. 2002). It also is involved in the cleavage of the anti-sense RNAI of pBR322 and many other small RNAs with regulatory functions (e.g. Lin-Chao and Cohen 1991; Schmidt and Delihas 1995; Dam Mikkelsen and Gerdes 1997; Jerome et al. 1999; Briani et al. 2002; Moll et al. 2003). Most recently, RNase E has been found to have a critical role in the degradation of rRNA (Sulthana et al. 2016). Considering its many functions, it is not surprising that RNase E is an essential enzyme in *E. coli*.

RNase E is a polypeptide of 1061 amino acids with a calculated molecular mass of 118 kDa (Casarégola et al. 1992), making it one of the largest *E. coli* proteins. The approximately N-terminal half of the protein contains the RNase E active site and multiple sub-domains including an S1 RNA-binding domain and a 5' monophosphate sensor domain, among others (McDowall and Cohen 1996; Bycroft et al. 1997; Callaghan et al. 2005). There are also two aspartic acid residues at positions 303 and 346 required for metal ion coordination at the active site and essential for phosphate backbone cleavage (Thompson et al. 2015). The N-terminal portion of RNase E organizes into the catalytically active tetramer in solution dependent on a sub-domain in the 410–510 region of the protein (Callaghan et al. 2005). The C-terminal half of RNase E serves as a scaffold for binding protein partners that together with RNase E constitute the RNA degradosome. In *E. coli* during exponential growth, these partners include the RNA helicase, RhlB, the exoribonuclease, polynucleotide phosphorylase, and the glycolytic enzyme, enolase (Vanzo et al. 1998; Marcaida et al. 2006).

RNase E preferentially cleaves at single-stranded, AU-rich sequences (Ehretsmann et al. 1992; Mackie 1992; Lin-Chao et al. 1994; McDowall et al. 1994), generating products with 5′-monophosphate and 3′-hydroxyl termini. Monovalent and divalent cations are required for activity (Nanbu-Wakao et al. 2000). Although no universal consensus sequence for cleavage by RNase E has been defined, detailed analysis of thousands of cleavage sites suggests that the consensus sequence (A/G)N-(A/U)UU is a preferred site of cleavage, and that there is strong preference for a U residue in the +2 position relative to the cleavage site (noted by the dash) (Chao et al. 2017). The cleavage sites are often flanked by secondary structures (Del Campo et al. 2015). The enzyme also displays a strong preference for a 5' monophosphate terminus (Mackie 2000) that sits in a 'sensing pocket' allowing catalysis to proceed (Koslover et al. 2008). Very recent work has shown that RNase E locates cleavage sites by scanning linearly from the 5' terminus along single-stranded regions of the message (Richards and Belasco 2019). For some substrates, RNase E bypasses 5' end recognition and relies on internal entry instead (Kime et al. 2010; Bouvier and Carpousis 2011). What structural features determine which mode of cleavage will be utilized is not fully understood.

As noted above, RNase E serves many important functions in *E. coli* cells, acting to process or degrade essentially every type of RNA molecule. RNase E also is an essential enzyme, indicating that no other RNase is able to take over at least one of its many functions in its absence. This raises the question of which functions of RNase E are essential for cell viability. One would assume that its roles in tRNA and rRNA maturation would be essential processes inasmuch as a full complement of these components is required for protein synthesis. In fact, it was shown, using suppressor analysis, that maturation of stable RNAs is essential for *E. coli* viability (Perwez et al. 2008). On the contrary, it was also found that overexpression of an extended form of RNase G restores growth of *rne* mutant cells without affecting tRNA processing (Deana and Belasco 2004), implying that the effect of RNase E on tRNA maturation is not responsible for the inability of *rne* mutant cells to grow.

RNase E also is responsible for the initiation of decay of over 50% of *E. coli* mRNA transcripts during exponential growth (Stead et al. 2011; Clarke et al. 2014), raising the issue if any of these reactions were also essential for viability. Recently, it was shown that overexpression of RelE could suppress the lethality due to inactivity of RNase E (Hughes

2016). Inasmuch as the only known function of RelE is cleavage of mRNA in the A-site of the ribosome, its ability to suppress the *rne* mutation provides strong evidence that initiation of mRNA decay is another essential function of RNase E.

The *rne* gene encoding RNase E is located at 24.6 min on the genetic map of *E. coli* (Ghora and Apirion 1978; Rudd 1998). The 3.6-kb rne mRNA is transcribed from three promoters (Ow et al. 2002), and is itself a substrate of RNase E, providing a mechanism for RNase E to autoregulate its own expression (Mudd and Higgins 1993; Jain and Belasco 1995). It is not at all surprising that RNase E is subject to autoregulation given its important role in cell physiology and the need to keep its activity within certain boundaries (Jain and Belasco 1995; Sousa et al. 2001). The autoregulation process limits RNase E overexpression that could needlessly lead to degradation of important RNAs (Jain et al. 2002). Likewise, autoregulation could lead to stabilization of the *rne* message when RNase E levels are too low. The autoregulation process is mediated by a stem-loop structure located in the 5' UTR of *rne* mRNA (Schuck et al. 2009). Presumably, binding of RNase E to this structure leads to cleavage within the 5' UTR, inactivating the message, although details of the process are not fully understood.

Several other mechanisms of RNase E regulation have also been identified. One involves phosphorylation of the C-terminal half of RNase E upon T7 bacteriophage infection that inhibits RNase E and thereby stabilizes T7 messages (Marchand et al. 2001). In contrast, the Srd protein of bacteriophage T4 stimulates RNase E leading to degradation of host mRNAs upon phage T4 infection (Qi et al. 2015). RNase E activity also is affected by several trans-acting proteins including RraA and RraB (Gao et al. 2006) and ribosomal protein L4 (Singh et al. 2009). These proteins inhibit RNase E activity by binding to the C-terminal half of the protein, and RraA and RraB also alter the composition of the degradosome; however, it is not yet clear whether they actually regulate RNase E *in vivo*.

Another extremely interesting feature of RNase E that also has the potential to serve a regulatory purpose (Mackie 2013) is that in *E. coli* the enzyme is bound to the cell's inner membrane (Khemici et al. 2008). Association with the membrane is mediated by an amphipathic helix near the catalytic domain, and mutations within the helix that abrogate binding to the membrane lead to poor growth (Khemici et al. 2008). RNase E may also come off the membrane under certain environmental conditions (Murashko and Lin-Chao 2017). Removal of the membrane attachment site leads to a cytoplasmic form of RNase E and to a cytoplasmic degradosome (Hadjeras et al. 2019). However, the mutant RNase E is less stable and the turnover of ribosome-free transcripts increases, emphasizing the importance of the membrane attachment. Recent microscopic evidence revealed that RNase E rapidly diffuses over the entire inner membrane, and also forms short-lived foci (Strahl et al. 2015). Based on these findings, it was suggested that the foci limit diffusion and act as degradative bodies. These observations raise important questions about how the many RNA substrates reach RNase E molecules that are localized to the membrane, how important is the membrane localization to RNase E function, and does membrane localization increase the efficiency of RNase E action? There is no doubt that answers to these questions will profoundly affect our understanding of RNA metabolism.

RNase G

The protein now known as RNase G was first named CafA protein because upon overexpression it caused the formation of Cytoplasmic Axial Filaments (Okada et al. 1994). Subsequently, the protein was found to have endoribonuclease activity and was re-named RNase G (Wachi et al. 1999; Li et al. 1999b). The protein is closely related to RNase E, sharing ~35% identity and 50% similarity with the N-terminal, catalytic domain of RNase E (McDowall and Cohen 1996). The RNase G protein retains many of the residues important for substrate binding and catalysis, and it also overlaps with RNase E in certain catalytic and functional properties (Mackie 2013). However, while RNase E is essential for *E. coli* viability, strains devoid of RNase G activity are still able to grow. Overexpression of RNase G can partially suppress the temperature-sensitive growth phenotype of *rne* mutants (Lee et al. 2002), although more recent work has suggested that complementation occurs only with a mutant form of RNase G (Chung et al. 2010). Nevertheless, these findings indicate that RNase G has the capability to take over functions of RNase E, although it cannot do so under normal physiological conditions.

RNase G polypeptide is 489 amino acids in length with a predicted molecular mass of 53.8 kDa. Structural analyses revealed that it is translated from the second possible start codon in its mRNA and that the N-terminal methionine residue is removed (Briant et al. 2003). The purified enzyme exists primarily as a dimer that is in equilibrium with monomers and higher multi-mers (Briant et al. 2003). The dimer form is required for activity. As was found for RNase E, RNase G also senses and its activity is stimulated by a 5′ monophosphate end on the RNA substrate (Jiang et al. 2000; Briant et al. 2003; Jourdan and McDowall 2007; Garrey et al. 2009). *In vitro*, RNase G is most active at pH 7.5 in the presence of 10 mM Mg^{2+} and ~100 mM monovalent cations (Li et al. 1999b; Jiang et al. 2000; Tock et al. 2000). Like RNase E, purified RNase G also favors single-stranded AU-rich sequences (Jiang et al. 2000; Tock et al. 2000). However, there are significant differences in the catalytic activities of the two enzymes. For example, RNase G acts on the 5′-RNase E cleavage site of 9S RNA poorly and cleaves the 5’ UTR of *ompA* RNA at a different site. Poly (A) is cut much less efficiently by RNase G than by RNase E (Tock et al. 2000).

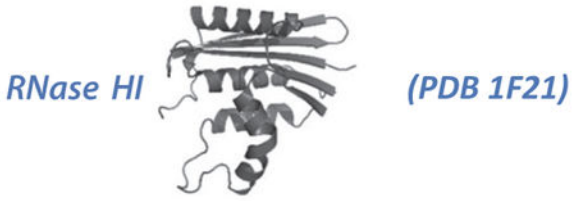
In vivo, the role of RNase G differs from that of RNase E. RNase G is responsible for the cleavage that generates the mature 5′ terminus of 16S rRNA (Wachi et al. 1999; Li et al. 1999b) (Figure 2). Efficient production of the mature 5′ end by RNase G depends on a prior cleavage at the +66 position by RNase E. In the absence of RNase G, the +66-nt 16.3S intermediate accumulates. It can be matured to 16S RNA by additional RNase E cleavages, but more slowly, and not exactly at the mature 5′ end. When both RNase E and RNase G are inactive, processing of the 17S precursor ceases (Li et al. 1999b). By itself, the absence of RNase G has little effect on the maturation of 9S RNA to 5S RNA, but its removal has a major effect if cells are already lacking RNase E (Ow et al. 2004) (Parambil and Deutscher, unpublished). It has been suggested that RNase G participates in the maturation of the 5’ end of 23S RNA (Song et al. 2011), but this has only been shown with an altered form of 23S RNA, and has not been substantiated.

RNase G also plays a limited role in mRNA decay. It is involved in the degradation of *adhE* and *eno* mRNAs (Umitsuki et al. 2001; Wachi et al. 2001; Kaga et al. 2002), and in the

former case, RNase G was shown to specifically cleave between residues 18 and 19 in the 5' UTR (Ito et al. 2013). A genome-wide survey identified 18 mRNAs that were elevated more than 1.5-fold in the absence of RNase G, suggesting that these mRNAs might be targets of RNase G action (Lee et al. 2002). Eleven of the mRNAs were decreased in abundance when RNase G was overexpressed. Generally, the effects of RNase G removal only become evident when RNase E is already absent (Ow et al. 2004), suggesting it has a secondary role in wild-type cells. Perhaps, under certain physiological conditions, its role may become more important. Its limited role is supported by the fact that the cellular amount of RNase G is only 3% that of RNase E (Lee et al. 2002). This finding is actually quite surprising when one considers its critical role in the maturation of 16S rRNA, a molecule that is produced in very large amounts when cells are growing in rich media. However, very little is known about the expression of the *rng* gene (located at 73.2 min on the *E. coli* chromosome, Rudd 1998; Wachi et al. 1999), or how it might be affected by environmental conditions.

RNase H family

Members of the RNase H family, which are widely distributed among prokaryotes and eukaryotes (Crouch 1990; Condon and Putzer 2002), specifically hydrolyze the RNA strand of DNA-RNA hybrids (Crouch 1990; Kanaya and Ikehara 1995). These enzymes are found in three separate classes, termed RNase HI, HII, and HIII (Ohtani et al. 1999; Ow et al. 2004), but only RNases HI and HII are found in *E. coli*. While RNases HII and HIII are homologous and likely arose from a common ancestor, RNase HI is distinct (Itaya 1990; Lai et al. 2000). The classes are distinguished by the fact that RNase HI enzymes require at least four ribonucleotides to initiate cleavage, whereas RNases HII and HIII can cleave when only a single ribonucleotide is present within a DNA strand (Tadokoro and Kanaya 2009).



RNase HI was identified based on its cleavage of the RNA strand in a DNA-RNA duplex (Hausen and Stein 1970). It is a protein of 155 amino acids with a calculated mass of 17.6 kDa, and functions as a monomer (Kanaya and Crouch 1983). Cleavage by RNase HI produces 5′-phosphate and 3′-hydroxyl termini. The enzyme requires Mg^{2+} for activity, although Mn^{2+} can partially substitute (Berkower et al. 1973). Molecular dynamics simulations indicate that the active site is highly rigid due to the distinctive RNase H protein fold that places it in a conformation that favors binding of Mg^{2+} (Stafford and Palmer III 2014). Based on high-resolution crystal structures (Yang et al. 1990; Katayanagi et al. 1992), and an NMR solution structure (Fujiwara et al. 2000), RNase HI contains five α-helices, five β-strands, and five reverse turns. A conserved group of three carboxylate side chains from Asp10, Glu48, and Asp70 are essential for catalysis since mutagenesis of these residues abolishes catalytic activity (Katayanagi et al. 1993). The divalent metal ion binds in the vicinity of these residues (Huang and Cowan 1994). In addition, two other conserved residues, Asp134 and His124 also are important for catalysis (Oda et al. 1993; Nowotny and

Yang 2006). The mechanism of RNase HI catalysis is still controversial as there is evidence supporting both a two-metal ion mechanism (Yang et al. 2006) and a general acid-base mechanism (Bastock et al. 2007).

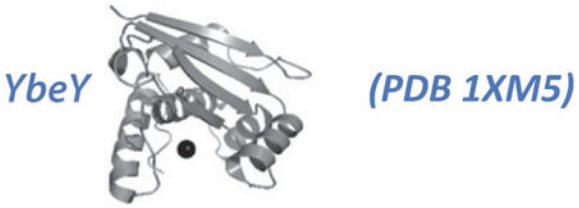
Based on the close structural resemblance of *E. coli* RNase HI to its human counterpart, whose interaction with substrate has been determined (Nowotny et al. 2007), the DNA-RNA substrate binds such that the RNA strand fits into one groove that contains the active site, and the DNA strand binds to a second groove. A ridge between the two grooves interacts with the minor groove of the substrate. The 2'-OH groups of the required four consecutive ribonucleotide residues, situated such that two are on either side of the bond to be cleaved, interact with Glu48 and several other residues including Cys13, Gly15, Asn16, and Gln 72 in the RNA-binding groove. The DNA strand is bound at two sites within the DNA-binding groove, one a pocket of Arg41, Thr43, and Asn100, and the second, a channel formed by Trp81, Trp85, and Ala93. These interactions are the major contributors to the specificity for a DNA–RNA hybrid. In addition, RNase HI forms a complex with the C-terminal region of single-stranded DNA-binding protein (SSB) that results in a lowering of K_m and stimulation of RNase activity (Petzold et al. 2015).

The fact that *E. coli* and most other organisms contain multiple RNase H's suggests that they may have different physiological functions, and this is supported by their different substrate specificities. During replication, the RNA of DNA–RNA hybrids act as primers and are continually made and degraded in each round of replication. DNA–RNA hybrids also form transiently during transcription, and have the potential to invade duplex DNA forming R-loops that can affect genome stability (Aguilera and Garcia-Muse 2012). The substrate specificity of RNase HI makes it ideally suited to remove the RNA strand in each of these situations. It is known that RNase HI participates in the replication of ColE1 plasmids and in the replication of chromosomal DNA from *oriC* (Hostomsky et al. 1993; Kogoma 1997). The role of RNase HI includes removal of RNA primers from Okazaki fragments (Ogawa and Okazaki 1984) and degradation of RNA in R-loops (von Meyenburg et al. 1987; Drolet et al. 1995). Interestingly, RNase HI-deficient cells are extremely sensitive to the Rho inhibitor, bicyclomycin, since Rho deficiency leads to an increase in R-loops, and in the absence of RNase HI, the R-loops are removed poorly (Raghunathan et al. 2018). Despite its important roles, RNase HI is not essential since R-loops can be removed by other pathways that involve *recBCD* (Kogoma et al. 1993). However, inactivation of components of *recBCD* in a background lacking RNase HI is lethal (Itaya and Crouch 1991).

RNase HI is encoded by the *rnhA* gene located at 5.1 min on the *E. coli* genetic map (Rudd 1998). The promoter of *rnhA* overlaps with that of *dnaQ*, which runs in the opposite direction (Maki et al. 1983). It appears that the two genes are inversely regulated (Quiñones et al. 1987), but little is known about the regulation of the expression of RNase HI.

RNase HII was identified based on its ability to rescue the lethality of a mutant strain lacking *rnhA* and *recC* (Itaya 1990). RNase HII is a polypeptide of 198 amino acid residues with a calculated mass of 21.5 kDa (Itaya 1990; Ohtani et al. 2000), and it likely functions as a monomer. Its sequence shows only 17% identity with that of *E. coli* RNase HI (Itaya 1990; Ohtani et al. 2000). RNase HII activity differs from that of RNase HI in that it can remove single ribonucleotides from DNA chains at the DNA–RNA junction that might arise from misincorporation by polymerases (Lazzaro et al. 2012; Sparks et al. 2012). Based on the structure of the *Thermatoga maritima* RNase HII in complex with substrates containing a (5') RNA–DNA (3′) junction (Rychlik et al. 2010), the protein specifically interacts with the 2' OH of the ribonucleotide, explaining the specificity of the cleavage. A conserved Tyr residue contacts and distorts the substrate at the RNA–DNA junction enabling the substrate to coordinate with the active site metal ion. Four highly conserved residues in the active site, Asp18, Glu19, Asp107, and Asp124 are important for catalysis.

The primary function of RNase HII is most likely related to its ability to repair misincorporated ribonucleotides in DNA (Rydberg and Game 2002). Nevertheless, an *E. coli* mutant strain that lacks both RNase HI and RNase HII is viable at low temperatures, indicating that these enzymes are not essential (Itaya et al. 1999). The *rnhB* gene is located at 4.4 min on the *E. coli* genetic map and is in an operon that also contains the upstream *lpxA*, *lpxB*, *lpxD* and *fabZ* genes involved in lipid metabolism and the downstream *dnaE* gene encoding DNA polymerase III (Itaya 1990; Rudd 1998).



YbeY is the *E. coli* member of the highly conserved UPF0054 family of bacterial proteins (Gil et al. 2004). Early work revealed that YbeY is a heat shock protein that somehow affects translation (Rasouly et al. 2009). Its role in RNA metabolism was uncovered in an analysis of a strain in which the *ybeY* gene had been deleted (Davies et al. 2010). Loss of YbeY led to slightly slowed growth and to a pleiotropic phenotype that included defects in the maturation of rRNAs, particularly that of both termini of 16S rRNA. Subsequently, YbeY was shown to be a metal-dependent, single strand-specific endoribonuclease that could effectively degrade rRNA and mRNA in vitro (Jacob et al. 2013). Zn^{2+} is the most effective cation supporting the degradation of total RNA (Taviti and Deutscher, unpublished). Using a synthetic 30 nt substrate that mimics part of the 3′ end of unprocessed 16S rRNA, YbeY displays a preference for cleavage after U residues, and the 2' OH groups on the RNA substrate are essential for activity. Surprisingly for a metal-dependent nuclease (Zhan et al. 2005), cleavage by YbeY results in 3′ phosphoryl and 5' hydroxyl termini (Jacob et al. 2013).

Based on the sequence of the *E. coli ybeY* gene at 14.91 min, YbeY is a protein of 155 amino acids with a calculated mass of 17,526 Da. Although the structure of *E. coli* YbeY has been determined (Zhan et al. 2005), it is still not clear whether the protein functions as a monomer or a higher-order structure. YbeY, like other members of the UPF0054 family, contains a conserved H3XH5XH motif that coordinates a metal ion (Davies et al. 2010). Mutation of the conserved His residues revealed that the first one (His114) is required for recovery of the pleiotropic effects caused by the absence of YbeY, whereas the other two His residues (His118 and His124) have little effect. A conserved Arg residue, Arg59, is also required for complementation (Davies et al. 2010). Purification of mutant proteins containing either a H114A or an R59A mutation indicated that these residues are also essential for the RNase activity of YbeY (Jacob et al. 2013).

On the one hand, the physiological role of YbeY in *E. coli* has been somewhat controversial. It was originally suggested that the endoribonuclease activity of YbeY participates in the removal of the 33 nts present at the 3′ end of the precursor to 16S rRNA, and strains lacking this protein are defective in 3′ maturation (Davies et al. 2010; Jacob et al. 2013). On the other hand, other studies indicated that the exoribonucleases, RNase R, PNPase, RNase II, and RNase PH are required for removal of the 33 precursor residues (Sulthana and Deutscher 2013). The absence of the four exoribonucleases leads to accumulation *in vivo* of 16S rRNA precursors containing 33 extra residues (Sulthana and Deutscher 2013), and purified exoribonucleases can remove the extra residues from 30S precursor particles *in vitro* (Smith et al. 2018); purified YbeY, in contrast, cannot remove the extra residues (Smith et al. 2018). In addition, removal of the four exoribonucleases leads to inviability (Sulthana and Deutscher 2013), as might be expected if the cell is unable to mature 16S rRNA, whereas removal of YbeY only leads to slightly slowed growth (Davies et al. 2010). Nevertheless, there is no question that YbeY is required for efficient 3′ maturation of 16S rRNA in *E. coli* (Jacob et al. 2013). YbeY interacts with ribosomal protein S11 and the ribosome-associated GTPase, Era, suggesting that YbeY is recruited to the ribosome (Vercruysse et al. 2016). Moreover, at elevated temperature, cells lacking YbeY process 16S rRNA extremely poorly (Jacob et al. 2013). In recent studies, it was also found that overexpression of Era partially suppresses the growth phenotype of a strain lacking YbeY, and increases 16S rRNA maturation and ribosome assembly (Ghosal et al. 2018). Most interestingly, the suppression requires the functions of RNase II, RNase R, and RNase PH, linking Era, YbeY, and the exoribonucleases in the processing of 16S rRNA. However, the exact role of YbeY in the overall process remains a mystery.

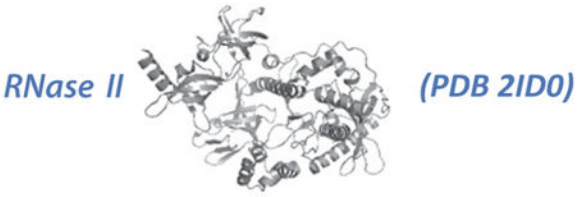
In addition to its role in rRNA maturation, YbeY has also been implicated in several other processes in *E. coli*. YbeY participates in ribosome quality control, a process that leads to the degradation of defective 70S ribosomes by a mechanism mediated by the 30S subunit, and that works in conjunction with the exoribonuclease, RNase R (Jacob et al. 2013). Details of the process are not completely understood, and so far, it has only been shown for ribosomes *in vitro*, but the process could serve to ensure cell survival under a variety of stress conditions. YbeY also plays a role in small RNA expression (Pandey et al. 2014). Strains lacking YbeY are markedly resistant to killing by hydroxyurea, and expression of 28 sRNAs was found to be YbeY-dependent upon hydroxyurea-induced stress in a process that also affects the target mRNAs (Pandey et al. 2014). YbeY also has an effect on colonization

by enterohemorrhagic *E. coli* due to alterations in the type III secretion system (McAteer et al. 2018). The effect appears to be due to a reduction in the number of initiating ribosomes in cells lacking YbeY. Another connection between YbeY and rRNAs is that it is required for proper rRNA transcription antitermination. In strains deleted for YbeY, transcription is inhibited in the presence of the "Nut-like" sequences required for antitermination (Grinwald and Ron 2013). Based on all of these studies, it is evident that YbeY is important for ribosome maturation and function, but what specific role might be played by its endoribonuclease activity is not at all clear.

General properties of the *E. coli* endoribonucleases are summarized in Table 1.

Escherichia coli *exoribonucleases*

Seven *E. coli* exoribonucleases are known and have been studied in detail. These include RNase II, RNase R, polynucleotide phosphorylase (PNPase), RNasePH, RNase D, RNase T, and oligoribonuclease (Orn) (Table 1). An eighth enzyme, RNase BN, has both endoribonuclease and exoribonuclease activity and has already been described with the endoribonucleases. All of the known *E. coli* exoribonucleases digest RNA in the 3′ to 5′ direction. Despite extensive searching and many years of study, no exoribonuclease with a 5′ to 3′ mode of action has been identified in *E. coli*, although such activities are known in Gram-positive organisms (see below). Of the eight known exoribonucleases (including RNase BN), six act hydrolytically, releasing nucleoside 5′ monophosphates, whereas two, PNPase and RNase PH, use inorganic phosphate to release nucleoside diphosphates. Inasmuch as nucleoside diphosphates are high-energy compounds, PNPase and RNase PH can act reversibly, utilizing the diphosphates to synthesize RNA molecules. Many of the exoribonucleases have overlapping substrate specificities *in vitro*, and this extends to their physiological roles as well. Nevertheless, all have been maintained in *E. coli*, suggesting that despite their overlap in some metabolic processes, each one also manifests unique properties that are important to the cell. Based on their structural and catalytic properties, several of the RNases are closely related. These include RNase II and RNase R (sometimes called the RNR family, 353); PNPase and RNase PH (PDX family); and RNases D, T, and Orn (DEDD family).



The two members of the RNR family, RNase II and RNase R, are large, single-chain proteins. Both contain multiple RNA-binding domains and a conserved central catalytic region with several highly conserved motifs. Nevertheless, the two subfamilies can be distinguished. While *E. coli* and many other organisms contain one member of each subfamily, some eubacteria contain only an RNase R homolog (Zuo and Deutscher 2001), and an example is known in which the only RNR family member is an RNase II homolog

(Matos et al. 2012). Both enzymes are processive, nonspecific exoribonucleases, but their catalytic properties differ significantly since RNase II is specific for single-stranded RNA molecules, whereas RNase R can efficiently digest double-stranded substrates as well (Cheng and Deutscher 2002).

RNase II is the most active exoribonuclease in extracts of *E. coli*, accounting for 95–98% of hydrolytic activity when poly (A) is used as the substrate (Cheng et al. 1998). Because it is such an active enzyme, RNase II was one of the first RNases to be identified and purified (Wade 1961). Based on the sequence of the *rnb* gene encoding RNase II, RNase II is a protein of 644 amino acids with a calculated molecular mass of 72.5 kDa (Coburn and Mackie 1996). It migrates on gel filtration with an apparent molecular mass of ~80 kDa (Deutscher et al. 1984), confirming that it is a single-chain protein, and suggesting that it is not associated with other macromolecular components in extracts. The crystal structure of *E. coli* RNase II has been determined both as the free protein (Frazao et al. 2006; Zuo et al. 2006) and in a complex with a fragment of RNA (Frazao et al. 2006). The protein contains three RNA-binding domains, two at the N-terminus and one at the C-terminus, which assemble into a clamp (or anchor) that leads to a narrow, basic channel. The clamp and the channel have dimensions that would only allow entry of a single-stranded RNA, explaining the enzyme's inability to act on structured substrates.

The catalytic center in the central catalytic domain is at the bottom of the channel that together with the clamp can interact with 9–10 nts of RNA, in good agreement with the number of nucleotide residues required for processivity (Cheng and Deutscher 2002). Fewer than 10 nts leads to distributive, rather than processive, hydrolysis, and much weaker binding of the substrate to RNase II (Zuo et al. 2006). The X-ray structure also suggests that duplex RNA can approach closer to the active center using an alternate path that avoids the clamp (Zuo et al. 2006). Such an alternative entry to the channel would explain how RNase II can leave a 3′ overhang with as few as 4 nts, such as in mature tRNA (Li and Deutscher 1996), and how digestion of a duplex RNA with a 3′ overhang of 17 nts can be shortened to fewer than 7 nt (Zuo et al. 2006). The catalytic center contains four conserved sequence motifs that include four essential Asp residues, Asp201, Asp207, Asp209, and Asp210, three of which coordinate a metal ion at the active site (Frazao et al. 2006; Zuo et al. 2006). The possibility of a second metal ion has also been suggested (Frazao et al. 2006). Other work has shown that Asp209 is essential for catalytic activity, but not for RNA binding (Amblar and Arraiano 2005). Interestingly, the widely used RNase II-negative strain, S296, contains a mutation that converts Asp209 to Asn (Amblar and Arraiano 2005).

Early work on the specificity of RNase II indicated that it hydrolyzes a variety of RNA substrates in a reaction that requires Mg^{2+}, and that is stimulated by monovalent cations (Shen and Schlessinger 1982). Its action on synthetic homopolymers greatly exceeds that on natural RNAs (Cheng and Deutscher 2002), most likely due to the extensive secondary structure present in the natural substrates, and the sensitivity of RNase II to structure. RNase II slows dramatically as it approaches within ~10 nt of a double-stranded region (Coburn and Mackie 1996), although the strength of the double-stranded region affects its stalling and dissociation. RNase II can act slowly on short, single-stranded RNAs (Cannistraro and Kennell 1999), and given sufficient enzyme, molecules as short as 3 to 5 nts in length

ultimately can be generated (Cheng and Deutscher 2002). *In vivo*, RNase II can generate mature 3′ termini on tRNAs (Li and Deutscher 1996) (Figure 3), indicating that it can digest to within 4 nts of the double-stranded aminoacyl stem. Studies with synthetic RNA substrates initially led to the suggestion that RNase II contains an anchoring site that binds the substrate 15 to 25 nts from the 3′ end in addition to the 3′ end bound at the catalytic site (Cannistraro and Kennell 1999), and this has been confirmed by the X-ray structure (Frazao et al. 2006). However, the anchoring site cannot be essential for RNase II action given that much shorter molecules can be substrates for the enzyme. RNase II also has the ability to digest a single-stranded DNA oligomer, dT_{17}, at a slow rate (Cheng and Deutscher 2002), in keeping with the observation that the interaction with the ribose 2'-OH groups only helps to orient the substrate (Frazao et al. 2006). RNase II does not require a free 3′-hydroxyl group to initiate degradation since the presence of a 3′-phosphoryl does not hinder its action (Cheng and Deutscher 2002).

RNase II is a relatively large protein for the apparently simple hydrolytic reaction that it catalyzes, and a number of studies have been carried out to obtain additional structure–function information about its multiple domains and about specific amino acid residues. Of its three RNA-binding domains (two at the N-terminus, one at the C-terminus), the C-terminal S1 domain is most important for RNA binding (Amblar et al. 2006). Truncation of RNase II to remove this S1 domain leads to a dramatic reduction in RNA binding and exoribonuclease activity. Substitution of S1 domains from either PNPase or RNase R can partially reverse the effect of S1 removal (Amblar et al. 2007). It was also found that the S1 domain does not determine whether either of these RNases can digest through the secondary structure, nor does it affect the size of the limit products of each enzyme (Amblar et al. 2007). Rather, it appears that Tyr253 plays a major role in setting the size of the end product of the RNase II action (Barbas et al. 2009). Two other residues important for RNase II activity are Arg500 and Glu542. Arg500 is present in the active site of RNase II and is essential for activity, but not for RNA binding. Its conversion to Ala essentially eliminates RNase II activity. Arg500 contacts the phosphate group 2 nt from the 3′ end keeping the residue fixed in position, and thereby increasing the efficiency of the cleavage step. Glu542 is most interesting in that its conversion to Ala increases the k_{cat}/K_m of RNase II close to 10^6 (Barbas et al. 2009). Since Glu542 contacts the nt product of the reaction, these data suggest that dissociation of the mononucleotide product may be rate limiting.

Due to its high activity, the primary role of RNase II in *E. coli* appears to be in mRNA metabolism (Figure 4), and when both RNase II and PNPase are absent, cells lose viability and fragments of mRNA accumulate (Donovan and Kushner 1986). In some instances, RNase II can also stabilize mRNA since it is very effective in removing the poly (A) tails that are needed for degradation (Coburn and Mackie 1996). In fact, analysis of the stabilizing effects of RNase II revealed that steady-state levels of 31% of *E. coli* mRNAs actually decrease in the absence of RNase II (Mohanty and Kushner 2003). As noted, the activity of RNase II often overlaps functionally with that of the other two processive exoribonucleases, PNPase and RNase R. As a consequence, RNase II is not essential for the normal growth of *E. coli*. However, recent work has shown that cells lacking RNase II lose viability during starvation or during extended periods of stationary phase (Sulthana et al. 2017). This phenotype can be suppressed if another exoribonuclease, RNase PH, is also

absent. RNase PH levels normally decrease dramatically during starvation, but this does not occur in an RNase II^{-} background leading to excessive ribosome degradation and ultimately inviability. The mechanism by which RNase II regulates RNase PH levels is not yet understood.

RNase II plays a major role in several other cellular processes as well. As discussed above, it is primarily responsible for the removal of poly (A) tails from RNAs. For example, it removes the poly (A) tail from *rpsO* mRNA leading to a more stable message (Marujo et al. 2000), and it also removes poly (A) from 23S rRNA (Mohanty and Kushner 2000b). In the absence of other tRNA processing enzymes, RNase II can maintain *E. coli* viability, indicating that it is able to mature the 3′ end of all essential tRNA molecules (Li and Deutscher 1994; Figure 3). It also participates in the maturation of the leuX precursor tRNA (Mohanty and Kushner 2010). Additionally, RNase II plays a role in mRNA breakdown following ribosome pausing, degrading sequences downstream of the A site (Garza-Sanchez et al. 2009).

RNase II is encoded by the monocistronic *rnb* gene located at 29.0 min on the *E. coli* genetic map (Zilhão et al. 1993). RNase II is expressed from two functional promoters (Zilhão et al. 1996), but the significance of having two *rnb* mRNAs is not understood. A number of factors are known to affect the amount of RNase II present in cells. For example, deletion of the *gmr* gene increases the amount of RNase II protein and RNase II activity ~3-fold due to stabilization of RNase II protein (Cairrao et al. 2001). The amount of RNase II changes somewhat in different growth media, and this regulation disappears in a *gmr* deletion strain. Since Gmr is a cyclic-di-GMP phosphodiesterase (Weber et al. 2006), these findings suggest that variations in the amount of cyclic-di-GMP can affect RNase II levels, but how these diverse observations are related is unclear. RNase II also is affected by PNP (Zilhão et al. 1996). In the absence of PNP, the amount of RNase II increases, and overproduction of PNP reduces the amount of RNase II. Again, the mechanism of this regulation has not been ascertained, but it appears to be reciprocal, as removal of RNase II elevates PNP. Perhaps, the clearest example of RNase II regulation comes from the observation that Lys501 is acetylated and that this modification reduces the catalytic activity of the enzyme (Song et al. 2016). Acetylation increases in stationary phase and other conditions that lead to slowed growth resulting in reduced RNase II activity under these conditions. Acetylation is a reversible process dependent on the acetyltransferase, Pka, and the deacetylase, CobB. These findings identify a direct connection between environmental conditions and RNase II activity.

Another feature of RNase II that has the potential to be extremely important is that the protein has been found to be associated with the cell membrane through an N-terminal amphipathic helix (Lu and Taghbalout 2013). This association appears to be physiologically relevant since under conditions in which RNase II is essential for growth (i.e. in the absence of PNP), viability is affected if the association with the membrane is disrupted due to alterations in the amphipathic helix (Taghbalout et al. 2014). Clearly, additional studies are needed to completely understand all the ramifications of these observations.

RNase R was originally identified based on the presence of residual RNase activity in *E. coli* cells lacking RNase II, and the observation that this activity was dependent on a gene distinct from that encoding RNase II (Kasai et al. 1977). It is the primary hydrolytic activity in RNase II-negative extracts able to degrade synthetic polynucleotides (Cheng et al. 1998), but its ability to also degrade rRNA led to its naming as RNase R (Deutscher et al. 1984). Subsequent studies revealed that RNase R is encoded by the *vacB* gene (now called *rnr*), required for *E. coli* virulence. Based on the sequence of this gene, RNase R is a protein of 813 amino acids with a calculated molecular mass of 92.1 kDa (Cheng et al. 1998), in close agreement with its size determined by gel filtration (Cheng and Deutscher 2002), and indicating that it is a monomer in solution. RNase R has a strong tendency to self-aggregate, particularly at salt concentrations below 300 mM (Cheng and Deutscher 2002), complicating studies to determine whether it associates with other proteins. Sequence analysis suggests that RNase R has a domain structure very similar to that of RNase II consisting of two RNA-binding domains in the N-terminal region and one in the C-terminal region, as well as a central catalytic core (Vincent and Deutscher 2006). However, RNase R also has two additional domains, one at each end of the protein (Vincent and Deutscher 2009a), that are known to participate in the regulation of the enzyme (see below).

As is true for RNase II, RNase R is most active against synthetic polynucleotides, such as poly (A), but it also displays significant activity against structured RNAs, such as rRNA and tRNA (Cheng and Deutscher 2002). In fact, 16S and 23S rRNAs can be digested essentially to completion by the enzyme. Such data implied that RNase R might be able to digest through extensive secondary structure, and direct analysis revealed that in contrast to the other processive exoribonucleases, RNase II and PNPase, RNase R had the unique ability to completely digest a 17-mer double-stranded RNA as long as a single-stranded 3′ extension was present; a perfect double-stranded 17-mer oligoribonucleotide was not a substrate (Cheng and Deutscher 2005). More detailed analysis indicated that a 3′ single-stranded overhang of at least 10 nts was required for optimal substrate binding and catalysis, although those with overhangs as short as 4 nts retained partial activity (Vincent and Deutscher 2006). Molecules with a 5’ overhang were not substrates, and also bound extremely poorly, indicating that the substrate must thread into the enzyme’s catalytic channel with a 3′ to 5’ polarity. While the RNA-binding domains of RNase R are important for substrate binding and efficient catalysis, the catalytic domain, by itself, is sufficient for digestion of structured RNAs (Matos et al. 2009; Vincent and Deutscher 2009b). The catalytic domain of RNase R binds RNA more tightly than its counterpart in RNase II, and this property contributes to the ability of RNase R, but not RNase II, to degrade structured RNAs (Vincent and Deutscher 2009b, 2009a). The C-terminal RNA-binding domain also contributes to structured RNA degradation (Matos et al. 2011).

In addition to its nuclease activity, RNase R also contains an intrinsic RNA helicase activity that is independent of the nuclease activity (Awano et al. 2010; Hossain et al. 2015). The helicase activity requires ATP, but ATP hydrolysis is not required (Hossain et al. 2015). Two ATP-binding Walker A and Walker B motifs are present, one in the N-terminal region, and one in the C-terminal region, that are required for helicase activity. The motifs come together to generate a functional ATP-binding site only when double-stranded RNA is present and is required for efficient nuclease activity against double-stranded substrates particularly at low temperatures and with stable duplexes (Hossain et al, 2015). The helicase activity utilizes the same catalytic channel as the nuclease activity, and is an intrinsic component of the enzyme's ability to digest structured RNAs since mutations that interfere with ATP binding prevent digestion of structured RNA, but have no effect on the digestion of single-stranded RNA molecules (Hossain et al. 2016). *Escherichia coli* strains in which the helicase activity of RNase R has been eliminated by mutation of the Walker motifs exhibit growth defects at low temperatures (Hossain and Deutscher 2016). Moreover, cells also lacking PNPase and dependent on RNase R for growth, do not grow at 31 °C, and grow extremely poorly at 34, 37, and 42 °C. Such cells accumulate high levels of ribosomal RNA fragments and lose viability. Based on these findings, the intrinsic helicase activity of RNase R is essential for its proper functioning *in vivo*. The crystal structure of a truncated form of *E. coli* RNase R has been determined recently and reveals that the protein has two RNA-binding channels and that it contains a 'wedge' region that appears to induce RNA unwinding (Chu et al. 2017). How the 'wedge' relates to the intrinsic RNA helicase activity remains to be determined.

RNase R is encoded by the *rnr* (previously named *vacB*) gene located at 94.9 min on the *E. coli* genetic map (Cheng et al. 1998). Although very little work has been carried out on expression of the *rnr* gene (Cairrao et al. 2003; Cairrao and Arraiano 2006), it appears to be part of an operon that contains the *nsrR* gene (coding for a NO-dependent transcriptional repressor) upstream, and *rlmB* (encoding a 23S rRNA methyltransferase) and *yjfI* downstream. Mutant strains lacking RNase R grow essentially normally at temperatures between 31 and 42 °C, but display slowed growth at temperatures of 25 °C and below (Cairrao et al. 2003; Hossain and Deutscher 2016). In contrast, a mutant strain lacking both RNase R and PNPase does not grow (Cheng and Deutscher 2003), suggesting overlap in an essential cellular function. Analysis of a mutant strain lacking RNase R and containing a $PNPase^{ts}$ mutation revealed that fragments of 16S and 23S rRNA accumulate to high levels and that ribosome assembly is defective (Cheng and Deutscher 2003). These data indicate that RNase R and PNPase participate in a quality control process that normally removes the rRNA fragments as rapidly as they are generated, presumably due to errors during ribosome assembly or to premature transcription termination. Additionally, RNase R participates in rRNA degradation that occurs under conditions of glucose starvation (Basturea et al. 2011). RNase R also is important for mRNA decay (Cheng and Deutscher 2005; Andrade et al. 2006; Figure 4), particularly of regions in the mRNA with extensive secondary structure, such as REP elements (Cheng and Deutscher 2005). RNase R also plays an important role in removing mRNA during *trans*-translation (Richards et al. 2006; Liang and Deutscher 2013; Domingues et al. 2015), and in regulating the amount of translating ribosomes (Barria et al. 2019).

RNase R levels increase dramatically in response to a variety of stress conditions, such as starvation, stationary phase and cold shock (Cairrao et al. 2003; Chen and Deutscher 2005). RNase R is an extremely unstable protein, and its increased level during stress is primarily due to its stabilization under such conditions (Chen and Deutscher 2010), although an increase in *rnr* message also contributes to increased RNase R during cold shock (Cairrao et al. 2003; Chen and Deutscher 2010). The usual short half-life of RNase R in exponential phase cells is dependent on the binding of transfer-messenger RNA (tmRNA) and its associated protein, SmpB, to the C-terminal region of RNase R (Liang and Deutscher 2010). Binding is much tighter to exponential phase RNase R because the protein in this phase of growth is acetylated on Lys544, whereas the protein in stationary phase is not acetylated (Liang et al. 2011). Acetylation disrupts an interaction between the C-terminal region of RNase R and the Lys residue that facilitates binding of tmRNA-SmpB. Only exponential phase RNase R is acetylated because the acetylating enzyme, Pka, is not present in stationary phase cells (Liang and Deutscher 2012a). Binding of tmRNA-SmpB to RNase R promotes its proteolysis by Lon or HslUV proteases, each of which binds to the N-terminal region of RNase R and initiates proteolysis (Liang and Deutscher 2012b). These findings define a complicated regulatory process that leads to an increase in the amount of RNase R in stationary phase cells, and to cells under other stress conditions.

RNase R is also regulated due to its binding to ribosomes (Liang and Deutscher 2013; Malecki et al. 2014). Approximately 80% of RNase R in exponential phase cells is bound to ribosomes which stabilizes the protein and also enables its participation in *trans*-translation (Liang and Deutscher 2013). Binding to ribosomes requires tmRNA-SmpB and ribosomal protein S12. The remaining, unbound RNase R, which is very deleterious to cells, is extremely unstable with a half-life of only two minutes. Inhibition of RNase R binding to ribosomes leads to a major increase in RNA degradation and slower growth emphasizing the importance of keeping RNase R sequestered in growing cells. In contrast, RNase R is not bound to ribosomes in stationary phase cells (Liang and Deutscher 2013), and it is stable because it is not acetylated. In this phase of growth, RNase R is required for the degradation of rRNA, and likely other RNAs as well.

Two of the exoribonucleases present in *E. coli*, PNPase and RNase PH (PDX family) use P_i instead of H_2O as the nucleophile and generate nucleoside diphosphates rather than nucleoside monophosphates as the product. As a consequence, these enzymes can use the high-energy diphosphates to synthesize RNA in a reversal of the degradative reaction. In fact, it was the ability to synthesize RNA that originally led to the discovery of PNPase, and it was only after the discovery of DNA-dependent RNA polymerase that attention focused on PNPase's degradative properties as its primary function (see Littauer and Soreq (1982) for early references). Although studies of PNPase date back to the 1950s, the enzyme is still

the subject of active investigation, and recent studies of its structure, physiological role, and regulation continue to provide a wealth of fascinating information.

Escherichia coli PNPase is a large, polymeric protein. Its basic structure is an α_3-trimer, but it is also isolated as an $\alpha_3\beta_2$-structure (Portier 1975), in which the ß subunit is now known to be the RNA helicase, RhlB (Lin and Lin-Chao 2005). However, the function of this form of PNPase is not understood. Additional PNPase purifies with the degradosome, a multienzyme complex that contains in addition to PNPase, the endoribonuclease, RNase E, the RNA helicase, RhlB, and enolase (Carpousis et al. 1994; Miczak et al. 1996). Based on the sequence of the *pnp* gene, the PNPase monomer is a multidomain polypeptide of 711 amino acids with a calculated molecular mass of 77.1 kDa (Regnier et al. 1987). Each monomer contains two domains that are closely related to RNase PH, an N-terminal PH1 domain and a centrally located PH2 domain that are linked by an AAHD α-helical region (Symmons et al. 2000). KH and S1 RNA-binding domains occupy the C-terminal region of each monomer. The PH domains pack together in each of the three subunits forming a core hexameric ring structure containing a central channel (Shi et al. 2008; Nurmohamed et al. 2009). The KH and S1 domains, located on the upper surface of the core (Matus-Ortega et al. 2007) feed the RNA substrate into the central channel. The catalytic site located in the channel is largely in the PH2 domain, but mutations in the AAHD and PH1 domains also affect catalysis (Symmons et al. 2000). Overall, based on a detailed structural analysis of a PNPase homolog from *Caulobacter*, single-stranded RNA threads into the central channel after engagement with the RNA-binding domains, and is directed to the active site area within the central channel (Hardwick et al. 2012).

PNPase catalyzes three reactions *in vitro*: the synthesis of RNA from nucleoside diphosphates, the phosphorolytic degradation of RNA to form nucleoside diphosphates, and an exchange reaction between P_i and nucleoside diphosphates (Littauer and Soreq 1982). In its degradative mode, PNPase can rapidly and processively digest RNA chains (Littauer and Soreq 1982; McLaren et al. 1991); however, it is strongly inhibited by secondary structure. Stem-loop structures with as few as 7 bp stop the action of the enzyme (Spickler and Mackie 2000). PNPase requires a single-stranded region in order to bind and begin the digestion of an RNA substrate, and as it approaches within 6–9 residues of a double-stranded region, it stalls and ultimately dissociates (Spickler and Mackie 2000; Cheng and Deutscher 2005). This would explain the need for an association of PNPase with an RNA helicase *in vivo*, either as part of the degradosome (Miczak et al. 1996; Py et al. 1996) or in association with RhlB by itself (Lin and Lin-Chao 2005). As the length of an RNA substrate shortens, PNPase action becomes more distributive, and ultimately the enzyme dissociates. However, limit products of 2 to 3 nts can be obtained (Cheng and Deutscher 2005).

Although *E. coli* cells devoid of PNPase retain viability, their growth rate slows (McMurry and Levy 1987), and this becomes much more pronounced as the growth temperature decreases (Hossain and Deutscher 2016). PNPase is an important participant in many RNA metabolic reactions, and its absence affects multiple cellular processes (Briani et al. 2016). Together with RNases II and R, PNPase plays a major role in mRNA decay (Figure 4). Cells lacking both PNPase and RNase II are inviable, and they accumulate large fragments of mRNA (Donovan and Kushner 1986), indicating that both enzymes contribute to overall

mRNA degradation and overlap functionally. However, for many specific messages, PNPase and RNase II serve different functions (Mohanty and Kushner 2003). Cells lacking PNPase and RNase R also are inviable (Cheng et al. 1998), indicating functional overlap between these two enzymes as well. These cells accumulate structured mRNA fragments derived from REP sequences (Cheng and Deutscher 2005), and fragments derived from rRNAs, which also are highly structured molecules (Cheng and Deutscher 2003). Since PNPase is often associated with RhlB RNA helicase, and RNase R is able to digest structured RNAs by itself, it is not surprising that these two RNases would be the primary enzymes involved in the removal of structured RNA fragments. PNPase also participates in the removal of defective tRNA precursors (Li et al. 2002), and in the stability of sRNAs (Saramago et al. 2014; Cameron and De Lay 2016). In addition to its extensive involvement in degradative reactions, PNPase also has a role in maturation of various RNAs, including the 3′ end of 16S rRNA (Sulthana and Deutscher 2013; Figure 2), tRNAs (Reuven et al. 1997; Mohanty and Kushner 2010; Figure 3), and sRNAs (Piazza et al. 1996; Saramago et al. 2014). Counterintuitively, in some cases, PNPase also serves to protect sRNAs against degradation (Bandyra et al. 2013), and it can also act in the synthetic mode to add residues to RNA molecules *in vivo* (Reuven et al. 1997; Mohanty and Kushner 2000a).

Since PNPase plays a role in so many RNA metabolic processes, it is not surprising that its absence affects cells in many ways leading to a multiplicity of phenotypes. For example, bacterial cells lacking PNPase are affected in cell motility and exhibit increased biofilm formation (Carzaniga et al. 2012; Pobre and Arraiano 2015); they display increased sensitivity to antibiotics (McMurry and Levy 1987); they exhibit growth defects at low temperatures (Hossain and Deutscher 2016); they are unable to maintain control of cysteine homeostasis (Tseng et al. 2015); they are more sensitive to oxidative stress (Wu et al. 2009); and they are affected in DNA recombination and repair (Carzaniga et al. 2017).

PNPase is encoded by the *pnp* gene at 71.3 min on the *E. coli* genetic map (Rudd 1998) that lies downstream of the *rpsO* gene, encoding ribosomal protein S15. *pnp* is expressed from either of two promoters, one upstream of *rpsO* that generates a dimeric transcript for both *rpsO* and *pnp* and one just upstream of *pnp* that generates a monomeric transcript (Evans and Dennis 1985). PNPase expression is subject to a complex autoregulatory mechanism that controls the amount of PNPase at the posttranscriptional level. In the favored model for this autoregulation (Carzaniga et al. 2009), the endoribonuclease RNase III cleaves *pnp* in a stem-loop in the 5’ UTR that creates a 3′ end to which PNPase can bind and initiate degradation of the generated fragment (Jarrige et al. 2001). Once the complementary 37-nt fragment has been removed by PNPase, RNase E initiates degradation of the *pnp* message by acting on the single-stranded region that has become exposed. Mutations that eliminate PNPase activity or that remove the KH and S1 RNA-binding domains abolish autocontrol (Jarrige et al. 2002; Matus-Ortega et al. 2007; Wong et al. 2013). Mutations that inactivate RNase III also elevate PNPase expression (Portier et al. 1987). By this mechanism, PNPase can facilitate the degradation of its own message resulting in autoregulation. The small regulatory RNA, CsrA, has also been implicated in the regulation of PNPase by acting as a translational repressor of the RNase III-cleaved *pnp* message (Park et al. 2015). Recently, evidence has also been obtained for a second, RNase III-independent mechanism in which PNPase autoregulates by acting as a repressor of its own translation (Carzaniga et al. 2015).

The small RNA, SraG, located between *rpsO* and *pnp*, but encoded by the opposite strand, has been suggested to be an antisense regulator affecting the stability of the *pnp* transcript (Fontaine et al. 2016).

PNPase is induced upon cold shock (Jones et al. 1987), and this also is related to its autoregulation (Beran and Simons 2001; Mathy et al. 2001). At low temperatures, PNPase is less efficient at degrading its own message, perhaps because it is unable to remove the 37-nt RNase III cleavage product under these conditions, resulting in increased *pnp* expression (Mohanty and Kushner 2002). The *pnp* gene contains a number of intergenic rho-dependent transcription termination sites, and these are suppressed at low temperatures (Marchi et al. 2007), contributing to the elevation of PNPase under these conditions.

Interestingly, PNPase is also regulated by the Krebs cycle intermediate, citrate (Nurmohamed et al. 2011). *In vitro*, Mg-citrate binds to PNPase, and inhibits its activity, whereas free citrate binds at a distinct site and acts as an allosteric activator. Most importantly, a cell dependent on PNPase for viability grows poorly when citrate levels are increased. The citrate effect is evolutionarily conserved as bacterial, archaeal, and eukaryotic PNPases all are inhibited by citrate (Stone et al. 2017). The widespread occurrence of this apparent regulatory mechanism suggests an important cellular requirement to link RNA degradative pathways with the pathways of central metabolism. Further studies will be necessary to define the details of the linkage between these pathways.

Escherichia coli and many other bacteria contain a second P_i-dependent exoribonuclease, termed RNase PH. RNase PH was initially identified during studies of tRNA processing as a P_i-dependent activity that could mature the 3′ terminus of a tRNA precursor (Cudny and Deutscher 1988). Further examination revealed that the activity was distinct from PNPase, the only phosphorolytic exonuclease known at that time (Deutscher et al. 1988; Ost and Deutscher 1990). RNase PH is now known to be the founding member of a large family of widely distributed nucleases (Zuo and Deutscher 2001).

RNase PH degrades RNA only when P_i is present (Kelly and Deutscher 1992a). Like PNPase, RNase PH also has a polymerizing activity, with the ability to add a nucleoside diphosphate to the 3′ terminus of an RNA chain (Ost and Deutscher 1990). In its degradative mode *in vitro*, RNase PH can act on homopolymers and on tRNA-type substrates. On tRNAs, the preferred substrate is one containing a few residues following the −CCA sequence from which it removes nucleotides to generate tRNA-CCA. In contrast, tRNA-CCA and tRNA-CC are considerably poorer substrates. In fact, the K_m value for tRNA-CCA is ~10-fold higher than that for tRNA-CCA-$C_{2–3}$, suggesting that tRNA-CCA would tend to dissociate from the enzyme once it is generated. RNase PH requires a free 3′-hydroxyl group to act on tRNA-type substrates (Kelly and Deutscher 1992a).

Based on analysis of the *rph* gene encoding RNase PH, RNase PH is a polypeptide 238 amino acids in length with a molecular mass of 25.5 kDa (Poulsen et al. 1984; Ost and Deutscher 1991). However, the protein tends to aggregate. The smallest active form on gel filtration is the size of a dimer, but oligomeric forms as large as 200 kDa are also present (Poulsen et al. 1984). While a crystal structure for *E. coli* RNase PH has not been reported, those from *Aquifex aeolicus* (Ishii et al. 2003), *Pseudomonas aeruginosa* (Choi et al. 2004), and *B. subtilis* (Harlow et al. 2004) tend to form a hexameric ring structure as a trimer of dimers, very similar to that originally reported for the core of PNPase, suggesting that all of these enzymes may function as oligomeric rings (Symmons et al. 2002). These findings would explain why *E. coli* RNase PH tends to aggregate, and they raise the possibility that the putative active dimers observed by gel filtration may actually have oligomerized during the assay to the true active form (Poulsen et al. 1984). In fact, mutations in the *P. aeruginosa* enzyme that prevent its association into a hexameric structure lead to the formation of inactive dimers (Choi et al. 2004). The X-ray structures have identified the catalytic site of RNase PH, and residues important for binding P_i that are conserved among the bacterial enzymes (Ishii et al. 2003; Choi et al. 2004; Harlow et al. 2004). In addition, this P_i-binding site also superimposes well on the second core domain of PNPase, which was predicted to be the catalytic center of that closely related enzyme (Symmons et al. 2002). In RNase PH, the catalytic site is located at the bottom of a cleft, which would allow only the 3′-single-stranded region of a tRNA precursor to enter easily. This would explain the preferential activity on precursor tRNAs compared with that on mature forms. It has also been observed that RNase PH interacts with RNase E *in vitro* and *in vivo* through specific binding domains (Martinez et al. 2014), although the physiological significance of this interaction is unknown. Overall, however, structure–function studies of RNase PH have been limited, and additional work clearly is warranted.

Although RNase PH is not an essential enzyme in *E. coli*, it and RNase T are the major contributors to 3′ maturation of tRNA molecules (Figure 3), and in their absence cells grow poorly and large amounts of tRNA precursors accumulate (Li and Deutscher 1996; Mohanty et al. 2012). RNase T is unable to digest through adjacent C residues, and RNase PH is required for processing of those tRNA precursors in which such sequences are present (Zuo and Deutscher 2002c). Thus, even in the absence of RNase PH, by itself, certain tRNA precursors are incompletely processed, indicating that all the other RNases present are unable to efficiently carry out the function of RNase PH (Li and Deutscher 1996). After RNase T, RNase PH is most effective in supporting the growth of cells lacking multiple exoribonucleases (Kelly and Deutscher 1992b), indicating that RNase PH can take over the functions of the multiple missing RNases quite well. Additionally, RNase PH also participates in the 3′ maturation of a number of small, stable RNAs in *E. coli* (Li et al. 1998). Interestingly, truncated, catalytically inactive RNase PH, which is found in many laboratory strains of *E. coli* (see below), inhibits the RNase P-mediated 5’-end maturation of 5’ triphosphate-containing primary tRNAs with short leader sequences (Bowden et al. 2017). Presumably, the inactive RNase PH bound at the 3′ end interferes with 5’ end-maturation when a 5’ triphosphate is present.

RNase PH also plays a prominent role in rRNA metabolism (Figure 2). Ribosomes, which generally are stable in growing cells, become substrates for degradation under conditions of

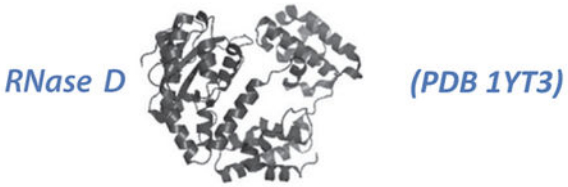
nutrient deprivation (Deutscher 2003) or as a consequence of an RNA quality control process that removes defective ribosomes (Cheng and Deutscher 2003). During starvation, degradation of 16S rRNA initiates with shortening of its 3′ end in a reaction dependent on RNase PH (Basturea et al. 2011), followed by endonucleolytic cleavage by RNase E (Sulthana et al. 2016). In the absence of RNase PH, degradation of 16S rRNA under starvation conditions is limited. In contrast, RNase PH is not required for the 16S rRNA decay that occurs as a consequence of quality control (Basturea et al. 2011). Based on *in vitro* studies, RNase PH removes ~20 nts from the 3′ end of 16S rRNA, although *in vivo* the shortening reaction extends to 120 nts (Sulthana et al. 2016). It is not yet known how the extended shortening occurs *in vivo*, or how the 3′ shortening of 16S rRNA ultimately leads to RNase E cleavage during starvation. rRNA degradation has also been found to occur in strains that lack the two known phosphorolytic RNases, PNPase and RNase PH (Zhou and Deutscher 1997). Such strains are very cold sensitive and are defective in 50S subunit assembly. Apparently, some essential process in ribosome metabolism can be carried out only by the phosphorolytic nucleases.

RNase PH also contributes to rRNA maturation (Figure 2). It is one of four exoribonucleases required for maturation of the 3′ end of 16S rRNA, which involves removal of 33 precursor-specific nts (Sulthana and Deutscher 2013). The presence of RNase PH by itself is sufficient to allow 3′ maturation and cell growth, although it is not as effective as the other exoribonucleases, RNase R, PNPase and RNase II. Moreover, *in vitro*, RNase PH can remove 33 nts from the 3′ end of 16S rRNA precursors present in small subunit assembly intermediates (Smith et al. 2018), confirming its direct role in the processing reaction. Cells lacking RNase PH display increased growth sensitivity to the aminoglycoside antibiotics neomycin and paromomycin, and they contain decreased amounts of ribosome subunits and increased amounts of 16S rRNA precursor (Frazier and Champney 2012). RNase PH also has been implicated in the 3′ end maturation of 23S rRNA (Gutgsell and Jain 2012) and in the degradation of structured RNAs (Jain 2012). In a mechanism that is not yet understood, RNase PH has also been found to stabilize certain Hfq-bound sRNAs against premature degradation (Cameron and De Lay 2016).

RNase PH is encoded by the *rph* gene at 82.2 min on the *E. coli* genetic map. *rph* exists in an operon together with the downstream *pyrE* gene, that encodes orotate phosphoribosyltransferase, an enzyme required for pyrimidine biosynthesis (Ost and Deutscher 1991). The two genes are coupled, since the translation of *rph* is necessary to transcribe past the *pyrE* attenuator (Poulsen et al. 1984). In addition, the *rph* message differs depending on pyrimidine metabolism (Andersen et al. 1992). When cellular UTP is low, a dicistronic *rph-pyrE* mRNA is produced; however, when UTP levels are high, a monocistronic *rph* mRNA is made due to attenuation of *pyrE* expression. In many widely used *E. coli* strains derived from W3110 and MG1655, a GC base pair is missing near the 3′ end of the *rph* gene (Jensen 1993). This results in a frameshift that reduces the size of RNase PH by 10 amino acids, and largely eliminates its activity. Also, as a consequence of the altered translation of *rph*, orotate phosphoribosyltransferase is reduced and the cells become dependent on pyrimidines for optimal growth in minimal medium. Of particular interest in this regard is the finding that during laboratory evolution of MG1655, the most common

adaptive mutation observed was the deletion of 82 nt in the *rph-pyrE* operon that apparently relieved the pyrimidine biosynthesis defect and led to faster growth (Conrad et al. 2009).

Very little is known about the regulation of RNase PH. However, a recent study revealed that it decreases close to 90% in starving cells due to the instability of RNase PH protein under these conditions (Sulthana et al. 2017). This reduction limits the amount of rRNA degradation that normally occurs as a consequence of nutrient limitation since, as discussed previously, RNase PH is required to initiate the rRNA degradative process. Of particular interest is the finding that reduction of RNase PH does not occur when cells lack RNase II, leading to extensive rRNA degradation and ultimately to cell death. How RNase II can regulate the stability of RNase PH is not yet understood, but a clarification of this unusual mechanism of regulation is sure to provide fascinating information.



Escherichia coli contains three members of the DEDD family: RNase D, RNase T, and oligoribonuclease (Orn). Although the overall structures of the three RNases differ, they share a common catalytic core that consists of four invariant acidic residues, as well as other conserved residues, distributed in three separate motifs (Zuo and Deutscher 2001). The nucleases of this superfamily, which also includes many DNA exonucleases, are thought to act by a common catalytic mechanism that involves two metal ions (Steitz and Steitz 1993). The DEDD nucleases fall into two subfamilies based on whether they contain a histidine or tyrosine residue in motif III (Zuo and Deutscher 2001). RNase D belongs to the DEDDy subfamily, whereas RNase T and Orn are in the DEDDh subfamily. The latter two enzymes also are more closely related structurally and catalytically (see below). RNase D family members are widespread in all organisms.

The discovery of *E. coli* RNase D led to the first indications that exoribonucleases could display a high degree of substrate specificity (Cudny et al. 1981a). The few exoribonucleases known at that time all displayed broad specificities, whereas RNase D was found to be specific for tRNA-like molecules, and was essentially inactive against homopolymers, such as poly (A). In fact, RNase D was originally identified because of its action on “denatured” tRNAs (Ghosh and Deutscher 1978). Subsequent work showed that it could generate mature tRNA from an artificial tRNA precursor, suggesting that it might participate in 3′ maturation of tRNA precursors (Cudny and Deutscher 1980), now known to be the case.

Based on its deduced amino acid sequence and gel filtration, RNase D is a single-chain protein of 375 amino acids with a molecular mass of 42.7 kDa (Cudny et al. 1981b; Zhang and Deutscher 1988a), and there is no evidence that RNase D associates with other proteins in cell extracts. The crystal structure of RNase D has been determined to 1.6 Å (Zuo et al. 2005). The protein contains three domains that come together to form a funneled ring structure. The catalytic center containing the DEDD residues is found in one of these

domains in a region rich in ß-sheet. The other two domains, which consist mainly of α-helical structure, form a region of positively charged residues on the side of the funnel away from the catalytic center. This region likely serves as the RNA substrate-binding site.

RNase D is most active on tRNA molecules that contain residues following the mature 3′ terminus and on molecules that lack all or part of the −CCA sequence. It is much less active on mature tRNA. The resistance of tRNA-CCA is not due to the −CCA sequence *per se* because a second −CCA sequence following the original one can be removed rapidly (Cudny et al. 1981a). Rather, it appears that RNase D is sensitive to tRNA structure, a conclusion supported by the increased activity of RNase D on denatured tRNA compared to native tRNA (Ghosh and Deutscher 1978). The structure of the 3′-terminal residue also affects RNase D activity. tRNA-C-Cp, containing a 3′-terminal phosphate residue, is inactive as a substrate, and tRNAs with a terminal dial-dehyde or dihydroxy structure are much less active than those with an intact 3′-terminal ribose (Cudny et al. 1981a). However, the identity of the 3′-terminal base does not appear to play a role. RNase D apparently acts distributively on tRNA, as it dissociates from the substrate after removing the terminal AMP from tRNA-CCA (Cudny et al. 1981a). The action of RNase D on other natural RNA substrates has not been examined.

The role of RNase D *in vivo* is much less clear. Removal of RNase D has little effect on growth or on the plating of bacteriophage T4 (Blouin et al. 1983). However, RNase D can artificially be made essential for viability if four other exoribonucleases, RNases II, BN, T, and PH, are eliminated by mutation (Kelly and Deutscher 1992b; Figure 3). Such cells grow poorly, indicating that while RNase D can support viability, it cannot completely take over the functions of the missing RNases. In wild-type cells, RNase D can degrade tRNA molecules when it is overexpressed (Zhang and Deutscher 1988b). Since it is able to support growth in the absence of four other exoribonucleases, it is able to process all essential tRNA precursors, but it does so relatively inefficiently (Reuven and Deutscher 1993; Li and Deutscher 1996). RNase D also participates in the maturation of several other small *E. coli* RNAs in the absence of other exoribonucleases (Li et al. 1998). Inasmuch as these conditions would never arise naturally, the primary function of RNase D that has led to its maintenance in *E. coli* has not yet been determined. Recently, it has been found that RNase D and its message are greatly reduced in stationary phase cells (Taylor, Dutta, and Deutscher, unpublished), suggesting that RNase D functions primarily in exponential phase. In these studies, it was also found that the regulatory protein CsrA and its message are elevated ~3-fold in stationary phase cells lacking RNase D compared to wild type, suggesting that this mRNA might be a substrate of RNase D. Further work is necessary to understand the significance of these observations. Interestingly, in contrast to *E. coli*, RNase D is a component of the degradosome in *Caulobacter crescentus* (Voss et al. 2014), but its role has not been ascertained. The site on *C. crescentus* RNase E with which RNase D interacts has not been conserved in *E. coli* RNase E, providing an explanation for the absence of RNase D from the *E. coli* assemblage.

The *rnd* gene encoding RNase D is located at 40.6 min on the *E. coli* genetic map (Zaniewski and Deutscher 1982). The *rnd* message is controlled by a single promoter with transcription initiating at a position located 70 nts upstream of an unusual UUG translation

initiation codon (Zaniewski and Deutscher 1982). Compared to an AUG initiation codon, RNase D expression is down-regulated ~20-fold by this UUG codon. Expression of RNase D is also affected by a stem-loop structure followed by eight U residues that are immediately downstream of the promoter. Surprisingly, removal of the stem-loop structure affected RNase D expression at the translational level and did not alter levels of *rnd* mRNA (Zhang and Deutscher 1989, 1992). The sequence of eight U residues following the stem-loop serves as a ribosome-binding site, and its mutation decreased RNase D protein and activity by as much as 95%. Although overall RNase D expression can be affected by the UUG codon, the stem-loop structure, the U-rich region, as well as by a Shine–Dalgarno sequence that is present (Zhang and Deutscher 1992), it is not clear how these unusual features actually modulate RNase D levels *in vivo*. Moreover, no work has been done on RNase D expression since these initial studies despite the presence of all these possible regulatory sequences.

RNase T was originally identified as the enzyme responsible for removal of the 3′ terminal AMP residue of the −CCA sequence of tRNA (Deutscher et al. 1984) as part of the end-turnover process in which this residue is removed and restored by tRNA nucleotidyltransferase (Deutscher 1990). In fact, RNase T is the only nuclease in *E. coli* that can efficiently carry out this reaction due to its ability to digest close to a double-stranded stem. Subsequent studies revealed that RNase T also participates in other RNA metabolic processes, such as tRNA maturation, and that it is required for normal growth. Given its importance for RNA metabolism, it is somewhat surprising that the distribution of this enzyme is restricted to only a small group of bacteria, the γ division of proteobacteria (Zuo and Deutscher 2001). However, a close structural homolog is present in Mycobacteria (Abendroth et al. 2014; Romano et al. 2014).

Based on the sequence of the *rnt* gene encoding RNase T and structural analysis, RNase T is an α_2 dimer with a molecular mass of ~50 kDa. Each monomer contains 215 amino acids with a molecular mass of 23.5 kDa (Deutscher and Marlor 1985; Huang and Deutscher 1992). The dimer form of RNase T is required for activity both *in vitro* and *in vivo* (Li et al. 1996b, 1996a). RNase T has not been found to associate with any other components in crude extracts (Deutscher et al. 1984). A detailed structural and functional model for RNase T originally was developed based on site-directed mutagenesis (Zuo and Deutscher 2002a, 2002b), and this was confirmed by X-ray crystallography (Zuo et al. 2007; Hsiao et al. 2011). In the dimeric structure adopted by RNase T, the DEDD catalytic center on one monomer comes in close contact with a large basic patch involved in substrate binding on the other monomer to generate the complete active site. This arrangement was confirmed experimentally by reconstituting active dimers from monomers derived from two inactive mutant proteins, one defective in catalysis and one in substrate binding (Zuo and Deutscher

2002a, 2002b), and it explains why RNase T must dimerize to function. Detailed analyses of multiple RNase T-DNA complexes revealed that the protein is designed to bind a duplex structure with a short 3′ overhang and to generate products with 1- or 2-nt overhangs (Hsiao et al. 2011), exactly the products produced *in vivo* (see below). The structural analyses also explain why the action of RNase T is blocked by C residues, and why it stops trimming DNA and RNA at the correct position (Hsiao et al. 2012; Duh et al. 2015).

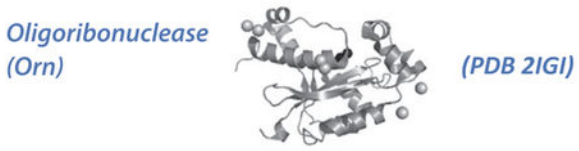
The preferred tRNA substrate for RNase T is intact tRNA-CCA, from which it removes primarily only the terminal AMP residue. tRNA-CA and tRNA-CCA-CC are weak substrates, whereas tRNA-CC and tRNA-CU are essentially inactive as substrates (Deutscher et al. 1984). Detailed analysis of RNase T base specificity using oligo-nucleotide substrates showed that the enzyme discriminates against C residues (Zuo and Deutscher 2002c). A single 3′-terminal C residue reduces RNase T action by >100-fold, and two consecutive terminal C residues essentially stop the enzyme. These unusual properties explain why only the terminal AMP residue is removed from tRNA-CCA, and why certain tRNA precursors are substrates for RNase T *in vivo* and others are not (Li and Deutscher 1995). RNase T can digest short oligonucleotides, but those shorter than six residues are poor substrates. RNase T acts distributively, releasing from a substrate after each catalytic cycle. The enzyme requires a free 3′-hydroxyl group to initiate degradation. RNase T works actively on single-stranded substrates, and it can digest right up to a double-stranded region (Zuo and Deutscher 2002c). It also can effectively digest single-stranded DNA, with a K_m value much lower than that for RNA (Viswanathan et al. 1998; Zuo and Deutscher 1999). This property, coupled with its ability to digest up to a double strand, makes RNase T a useful reagent for generating blunt-end DNAs (Zuo and Deutscher 1999).

RNase T plays a very important role in RNA metabolism in *E. coli*. Its absence leads to a small but significant decrease in doubling time (from 25 to 30 min) in otherwise wild-type cells, and to a delay in the recovery of cells from starvation (Padmanabha and Deutscher 1991). These data indicate that there is no other exoribonuclease in *E. coli* that can completely take over the functions of RNase T. Moreover, from among the five exoribonucleases, RNase II, D, BN, PH, and T, re-introduction of RNase T is most effective in restoring growth to a strain that lacks all of these RNases (Kelly and Deutscher 1992b), suggesting that it can efficiently substitute for the other missing enzymes. These findings are explained by the fact that only RNase T can complete the 3′ processing of 5S rRNA (Li and Deutscher 1995; Figure 2) and 23S rRNA (Li et al. 1999a) (Figure 2) because it is the only exoribonuclease able to remove precursor nucleotides close to double-stranded regions, as are present in these RNAs. In the absence of RNase T, neither RNA is fully matured, although both RNAs can still be assembled into large ribosomal subunits as slightly 3′-elongated precursors. Cells survive under these conditions, but the presence of the mutant ribosomes is likely responsible for the decreased growth rates observed in RNase T^- strains. RNase T also has a major role in the 3′ maturation of tRNA (Li and Deutscher 1996) (Figure 3) and other small, stable RNAs (Li et al. 1998). However, in these processes, other RNases can substitute if RNase T is absent or if the presence of adjacent C residues precludes RNase T action. In tRNA precursors, the residues to be removed are >4 nts from the aminoacyl stem, enabling other nucleases to participate. In contrast, RNase T is the only

enzyme that can remove the 3′ terminal AMP during the end-turnover process because that residue is too close to the aminoacyl stem.

There is evidence that RNase T also participates in DNA metabolism in *E. coli*. As noted, RNase T can effectively digest single-stranded DNA (Viswanathan et al. 1998; Zuo and Deutscher 1999), and, in high copy, it can suppress the UV repair defects of a RecJ, Exo I, Exo VII mutant (Viswanathan et al. 1999). It trims the 3′ ends of bulged, bubbled or Y-structured DNA, and it can coordinate with Endo V in DNA repair pathways (Hsiao et al. 2014).

RNase T is encoded by *rnt* gene located at 37.2 min on the *E. coli* genetic map (Case et al. 1989). It is transcribed from a σ^{70} promoter upstream of the *rnt* coding region (Huang and Deutscher 1992), and is cotranscribed with a large open reading frame, termed *lhr*, which encodes a putative helicase (Reuven et al. 1995) that is a member of a widespread protein family (Ordonez and Shuman 2013; Ejaz and Shuman 2018). However, it is not known whether there is any functional relationship between RNase T and Lhr nor is there anything known about the expression of *rnt*.



Orn, together with RNase D and RNase T, are the three members of the DEDD exoribonuclease family in *E. coli*. Orn was initially identified on the basis of its ability to preferentially digest short oligoribonucleotides (Niyogi and Datta 1975). Its unusual substrate specificity distinguished Orn from all other *E. coli* exoribonucleases (Datta and Niyogi 1975; Yu and Deutscher 1995), and strongly suggested that it was a distinct enzyme, and not a minor activity of another protein. Subsequent genetic and biochemical studies confirmed this conclusion (Zhang et al. 1998). Purified Orn is a homodimer containing subunits 180 amino acids in length with a molecular mass of 20.7 kDa. Based on the crystal structure of Orn (Fiedler et al. 2004) and Protein Data Bank 2IGI), the two subunits are arranged very similarly to those in RNase T in which the catalytic site is on one subunit, and a small basic patch involved in substrate binding is on the other subunit. However, since the substrate for Orn is so short, the catalytic DEDD cavity itself plays a role in substrate binding.

Although Orn works best at pH 8–9 in the presence of Mn^{2+}, Mg^{2+} also supports activity (Niyogi and Datta 1975). Oligonucleotides 2–5 nt in length are the preferred substrates, and the smaller the oligoribonucleo-tide, the more rapid is the rate of hydrolysis. Only single-stranded chains are substrates, and a free 3′-hydroxyl group is preferred. As with RNase T, DNA is also a substrate (Mechold et al. 2006). Orn also utilizes 5’-p-nitrophenyl thymidylate as a substrate enabling the use of a spectrophotometric assay to measure its activity (Young Park et al. 2008). pAp, an intermediate in sulfur metabolism, binds strongly to Orn and inhibits its activity (Mechold et al. 2006).

Orn is the only exoribonuclease essential for *E. coli* viability (Ghosh and Deutscher 1999). This is probably due to the fact that at their normal cellular levels none of the other exoribonucleases can effectively remove oligoribonucleotides (Figure 4). RNase T does have activity against oligonucleotides (Young Park et al. 2008), but it is relatively low. Short oligonucleotides are continually generated in cells because the processive exoribonucleases, RNase II, RNase R and PNPase, are unable to digest RNAs to completion. In the absence of Orn, these oligonucleotides, 2–5 residues in length, accumulate to high levels (Ghosh and Deutscher 1999), and likely are deleterious. However, the mechanism by which the accumulation of oligonucleotides leads to loss of viability remains unexplained. In many bacterial species, Orn also is needed to remove pGpG, a breakdown product of the second messenger, cyclic di-GMP (Cohen et al. 2015; Orr et al. 2015). In the absence of Orn, cyclic di-GMP accumulates, disrupting normal signaling by this second messenger and interfering with many cellular processes including biofilm formation, pathogenicity, and tolerance to oxidative stress.

Orn is encoded by the *orn* gene located at 94.6 min on the *E. coli* genetic map (Zhang et al. 1998). Orn appears to be monocistronic, but the *orn* promoter has not been identified, and, currently, nothing is known about *orn* expression.

General properties of the aforementioned *E. coli* exoribonucleases are summarized in Table 1.

Other RNases

RNase LS is an *E. coli* endoribonuclease that degrades mRNAs encoded by phage T4, as well as those of the host (Otsuka and Yonesaki 2005). The *rnlA* gene is essential for RNase LS activity and appears to encode the active protein. However, RNase LS activity is associated with a large complex containing 10 or more proteins, complicating its identification (Otsuka and Yonesaki 2005). Further analysis indicated that RnlA is part of a TA system that also contains the unstable antitoxin RnlB that neutralizes RNase LS activity. Upon T4 infection, RnlB is degraded, increasing RNase LS activity, and thereby antagonizing the phage infection (Koga et al. 2011). Much remains to be learned about the functional role of RNase LS in uninfected cells.

RNase AM is a recently discovered enzyme that can remove 5’ mononucleotides from 5’-phosphorylated RNA or DNA oligonucleotides (Ghodge and Raushel 2015). 5’-unphosphorylated molecules are not hydrolyzed. The enzyme removes mononucleotides in the 5′-to-3′ direction, making this the first exoribonuclease with this specificity to be identified in *E. coli*. At present, it is not known how long a substrate can be hydrolyzed, although DNA oligonucleotides 14 nts in length can be completely digested. This protein, whose activity was previously unknown, had been called TrpH or YciV. If this enzyme can also work on long RNA molecules, it has the potential to carry out RNA metabolic reactions for which no enzyme has yet been identified.

Escherichia coli also contains a number of other RNases whose description is beyond the scope of this review, but are mentioned here for completeness. Chief among these are the

bacterial toxins. Many of these are RNases that normally are not active in growing cells, but are activated under stress conditions as the antitoxins that normally keep them under control are destroyed. These TA systems have been extensively reviewed, but some recent relevant ones are in references (Hall et al. 2017; Orr et al. 2018; Nikolic 2019). In *E. coli*, the most well studied RNase toxins are RelE, MazF, and HicA, all of which are endoribonucleases that cleave mRNAs with varying degrees of specificity. RelE cleaves mRNAs in the A site of the ribosome. The cleavage shows some codon specificity, and generally occurs between the second and third nucleotides of the codon in the A site. Purified MazF cleaves mRNA, specifically between the A and C residues in an ACA sequence. In contrast to RelE, purified MazF cleaves free RNA. HicA also cleaves free RNA, but with little specificity.

Bacillus subtilis *endoribonucleases*

As can be seen in Figure 1, the overlap of RNases between *E. coli* and *B. subtilis* is only partial. The two major mRNA decay enzymes of *E. coli*, endonuclease RNase E and 3′ exonuclease RNase II, have no sequence homolog in *B. subtilis*. This is also the case for Orn, the essential oligoribonuclease of *E. coli* that is required to degrade small oligonucleotide products that are not substrates for other 3′ exonucleases. Similarly, in the case of RNases acting primarily on stable RNAs, *B. subtilis* has no homolog of *E. coli* RNase G, the endonuclease required for 16S rRNA maturation, RNase T, the 3′ exonuclease required for tRNA, 23S and 5S rRNA maturation, and RNase D, a tRNA processing enzyme. In some cases, the RNase functions observed in *E. coli* are accomplished by *B. subtilis* RNases that, although they are non-homologous, have similar activities. In other cases, RNase functions in *B. subtilis* are accomplished by RNases with quite different activities, as detailed below.

RNase Y

The major decay-initiating endonuclease in *B. subtilis* and many Firmicutes is RNase Y, a 59-kDa member of the HD superfamily of metal-dependent phosphohydrolases. RNase Y protein contains five recognizable domains: (1) an N-terminal, 25 amino-acid membrane-spanning domain. Association with the cell membrane is essential for RNase Y function; a mutant that is deleted for the N-terminal domain is inviable (Lehnik-Habrink, Newman, et al. 2011). This membrane localization is similar to that of *E. coli* RNase E, which is also membrane-bound via an internal 21 amino-acid sequence, although this does not constitute a membrane-spanning domain (Khemici et al. 2008). (2) an N-proximal coiled-coil/disordered domain. This domain may be involved in interactions with other proteins, similar to how the C-terminal disordered region of RNase E is the scaffold for proteins in the *E. coli* degradosome. (3) a KH RNA-binding domain. (4) an HD catalytic domain. (5) a C-terminal domain to which no function has been assigned, but which is conserved in all RNase Y coding sequences. A truncated version of RNase Y that is missing only the C-terminal domain cannot support normal growth of *B. subtilis* (Lehnik-Habrink, Newman, et al. 2011).

No crystal structure has been reported for RNase Y, likely due to the disordered regions making it difficult to obtain crystals. Nevertheless, there is good evidence for RNase Y self-

interaction from bacterial-2-hybrid experiments (Commichau et al. 2009), and biophysical techniques suggest that RNase Y exists as a dimer (Lehnik-Habrink et al. 2011).

RNase Y is encoded by the *rny* gene, formerly known as *ymdA*, located at 1.77 Mb on the *B. subtilis* genetic map. (The location of *B. subtilis* ribonuclease genes are from the Subtiwiki site; Mader et al. 2012.) The *rny* transcript appears to have a long 5′ UTR of 174 nts, the function of which is unknown. Immediately downstream of the *rny* gene is the *ymdB* gene, and the two genes appear to be in a single transcription unit. The *ymdB* gene encodes a phosphodiesterase that cleaves cyclic 2′3′ or 3′5′ cyclic nucleotides (Diethmaier et al. 2014). Strains with a catalytically inactive YmdB are significantly affected in biofilm formation, motility, and sporulation. A microarray analysis showed that >800 genes were up- or down-regulated in the *ymdB* mutant strain, most likely indirectly due to changes in expression of regulatory genes. The set of affected genes in the *ymdB* mutant strain did not overlap with genes affected in an *rny* mutant strain (Diethmaier et al. 2014); thus, the significance of these two genes being in an operon is, as yet, unknown.

RNase Y was initially thought to be essential in *B. subtilis*, but this was later shown not to be correct (Figaro et al. 2013). An RNase Y-knockout strain can be isolated, although it grows quite slowly (doubling time more than twice as long as wild type), has altered cell morphology, and is defective in competence and sporulation. Importantly, the half-life of bulk mRNA increases two-fold in a strain that is depleted for RNase Y (Shahbabian et al. 2009), suggesting that RNase Y is the major mRNA decay-initiating endoribonuclease, similar to RNase E. Transcriptome-wide analyses have identified hundreds of RNAs whose level is affected by depleting RNase Y (Durand et al. 2012; Laalami et al. 2013). Many of these mRNAs increase in abundance, as would be expected by the absence of a decay-initiating activity. On the contrary, many showed decreased abundance, which is likely due to indirect effects of RNase Y on regulatory RNAs or mRNAs that encode regulatory proteins. These studies were done before it was found that RNase Y is not essential for survival in laboratory conditions, and, thus, RNase Y was depleted rather than eliminated. Such conditions complicate the analysis, as there may be sufficient RNase Y present for normal activity on high-affinity substrates. Performing a transcriptome analysis in an RNase Y deletion strain would likely not be informative, as the extremely perturbed phenotype of such a strain would complicate the interpretation of effects on RNA half-life and processing.

Another characteristic that RNase Y may share with RNase E is a preference for a 5′-monophosphorylated end. This has been shown so far for only one substrate *in vitro*, the *yitJ* riboswitch RNA, which is one of several S-adenosylmethionine-dependent riboswitch RNAs that are targeted by RNase Y (Shahbabian et al. 2009). The lack of *in vitro* studies on RNase Y stands in sharp contrast to numerous such studies with RNase E, and is due to the difficulty of obtaining purified, active enzyme. A recent publication that provides detailed purification protocols for various forms of RNase Y may begin to help in this regard (Mora et al. 2018). However, even in this description, the enzyme assay contained a 100-fold enzyme:substrate molar ratio, which suggests the *in vitro* system is missing other factors or protein partners crucial for RNase Y activity. Recently, a complex of three *B. subtilis* proteins – YlbF, YmcA, and YaaT – was reported to regulate RNase Y cleavage specificity

(DeLoughery et al. 2018), indicating perhaps that this "Y-complex" may be required for optimal RNase Y activity.

Until recently, identification of RNase Y cleavage sites had been reported for relatively few *in vivo* substrates. One endonuclease cleavage in *B. subtilis* that affected mRNA half-life was first discovered in studies on the expression of the *gapA* operon, which encodes enzymes in the glycolytic pathway. The first of six metabolic genes in the operon, *gapA*, is preceded by *cggR*, a regulatory gene that encodes a transcriptional repressor. There is a 100-fold differential expression between the regulatory protein CggR and the enzyme GapA, and it was shown that this is achieved by endonuclease cleavage at a site near the end of the *cggR* CDS, resulting in an unstable upstream *cggR* RNA fragment and a stable downstream operon RNA (Meinken et al. 2003). Several years later, it was discovered that this cleavage is specified by RNase Y (Commichau et al. 2009). At about the same time, the cleavage site of *yitJ* riboswitch RNA was mapped (Shahbabian et al. 2009). Maturation of RNase P RNA and small cytoplasmic RNA have also been shown to involve cleavage by RNase Y (Gilet et al. 2015). The recent publication of Deloughery *et al.*, mentioned above, mapped 20 additional RNase Y processing sites, but one could not identify from these a consensus sequence or conserved structural motif that is targeted by RNase Y (DeLoughery et al. 2018).

Whether RNase Y engages other enzymes in a "degradosome" complex is controversial. On the one hand, bacterial-2-hybrid and crosslinking experiments have indicated a *B. subtilis* degradosome-like complex, containing PNPase, RNases J1 and J2 (5′ exoribonucleases; see below), DEAD-box helicase CshA, and glycolytic enzymes enolase and phosphofructokinase (Commichau et al. 2009). These components would be similar to the major ones of the *E. coli* degradosome: RNase E, PNPase, DEAD-box helicase RhlB, and enolase. On the other hand, such a complex cannot be isolated from *B. subtilis* by pull-down experiments, as can be done with the *E. coli* degradosome, which suggests that interactions of these proteins in *B. subtilis* may be transient rather than forming a bona fide complex. Surface plasmon resonance analysis of interactions among mRNA decay proteins showed a relatively strong PNPase/RNase Y interaction with a Kd of 5 nM (Newman et al. 2012). Pull-down studies also provided evidence for *in vivo* interactions of CshA with RNase Y (Lehnik-Habrink et al. 2010). Other interactions measured by SPR were not as convincing, and these remain controversial. Indeed, the interaction of RNase Y with RNase J1 using the bacterial-2-hybrid assay (Commichau et al. 2009) could not be detected by the same assay in another study (Mathy et al. 2010).

RNase Y in other organisms

Several years before RNase Y was discovered in *B. subtilis*, the gene encoding this enzyme in *Staphylococcus aureus* was known as *cvfA*, for conserved virulence factor A (Kaito et al. 2005). Surprisingly, even though RNase Y plays an important role in *S. aureus* virulence, the molecular basis of which has since been described, deletion of the *rny* gene has little or no effect on the growth of wild-type *S. aureus* strains (Kang et al. 2010; Marincola et al. 2012). This is unlike the case in *B. subtilis* (above), and unlike the case in *C. perfringens*, where even depletion (not a total loss) of RNase Y results in extremely slow growth (Obana et al.

2017). Thus, RNase Y activity appears to be restricted in *S. aureus*, rather than being involved in bulk mRNA decay. Indeed, transcriptome analysis in the *S. aureus* Δ*rny* strain showed that the level of less than 10% of RNAs was strongly affected by the absence of RNase Y, and many of these showed decreased levels, indicating indirect effects (Khemici et al. 2015). Furthermore, it was shown that deletion of the N-terminal transmembrane domain (amino-acids 2–24) had a much more severe effect on growth than the deletion of the entire gene! The conclusion was that, in *S. aureus*, the activity of RNase Y is highly restricted by target specificity and by membrane localization. The presence of RNase Y free in the cytosol is apparently too much of a good thing. This may be true also of *B. subtilis* RNase Y, since deletion of RNase Y amino-acid residues, 5–24, which is the membrane-spanning domain, results in a loss of viability (Lehnik-Habrink et al. 2011). Ongoing studies are aimed at understanding the requirement for RNase membrane localization; see (Hadjeras et al. 2019) for a most recent study.

Streptococcus pyogenes also contains an RNase Y-encoding gene. Similar to *B. subtilis*, but unlike the case in *S. aureus*, an *S. pyogenes* strain that is missing RNase Y shows a 2-fold increase in bulk mRNA half-life. Despite this, the Δ*rny S. pyogenes* strain shows only a slightly slower growth rate than wild type (Chen et al. 2013). It is interesting that the importance of an RNase to overall growth cannot be estimated by its effect on global mRNA half-life. Rather, the processing and/or turnover of specific messages is likely to determine the overall relevance of any RNase to cellular health.

While relatively few RNase Y cleavage sites have been mapped in *B. subtilis*, the target site for almost 100 *in vivo* substrates for *S. aureus* RNase Y are known (Khemici et al. 2015). This mapping was done by ligating an oligonucleotide to monophosphorylated 5′ ends in wild-type and Δ*rny* strains, followed by RNA-seq to identify 5′ ends that were generated by RNase Y cleavage. The results suggested some preference for A/U-rich sequences, some specificity for cleavage after a guanosine residue, and perhaps a requirement for unstructured RNA around the cleavage site. It is remarkable that an endonuclease with such loosely conserved target site parameters should cleave long mRNAs, and even operon RNAs, at only one or a few sites. Other factors, such as ribosome flow and other RNA-binding proteins, will likely influence susceptibility to RNase Y cleavage.

Among the *S. aureus* RNase Y targets are sites in the coding sequence for RNase J2 and in the 5′ UTR of the bicistronic transcript that encodes RpoY and RNase J1. These cleavages suggest a regulatory network in which the cellular level of RNase Y is coordinated with the level of RNases J1 and J2. An RNase Y cleavage site was also mapped in the 5′ UTR of the transcript encoding RNase Y itself, suggesting autoregulation of RNase Y levels by cleavage of its own transcript, followed by message decay via 5′ exonuclease activity (Khemici et al. 2015).

RNase P

As in *E. coli*, RNase P of *B. subtilis* is responsible for 5′-end maturation of tRNA. However, both the protein and RNA components of *B. subtilis* RNase P are relatively (and curiously) dissimilar to the *E. coli* RNase P components. The proteins share 30% identity and 48% similarity, while the two RNAs are less than 50% similar. The pathway of RNase P RNA

maturation in *B. subtilis* differs substantially from that of *E. coli*. There was early *in vitro* evidence that *B. subtilis* RNase P RNA holoenzyme can undergo an autocatalytic reaction to produce the mature 5′ end and a 3′ end that is 4 nucleotides shorter than the mapped 3′ end (Loria and Pan 2000). However, more recently, RNase P RNA 3′ maturation was found to be due to RNase Y cleavage at the mapped 3′ end, and the 5′ end is generated primarily by transcription initiation at this site (Gilet et al. 2015). *Bacillus subtilis* RNase P can form dimers that associate with 30S ribosomal subunits with a relatively strong binding affinity (Barrera et al. 2002; Barrera and Pan 2004). It has been suggested that this association allows RNase P to act on mRNAs that require prior cleavage in a regulatory region to induce translation. A major difference between *E. coli* and *B. subtilis* RNase P is the differential binding affinity for precursor vs. mature tRNA: *B. subtilis* RNase P binds to precursor tRNA with a ~1600-fold greater preference than to mature tRNA. This differential binding is much smaller (~3-fold) for *E. coli* RNase P (Buck et al. 2005). Despite these dissimilarities, subunits of the *E. coli* and *B. subtilis* RNase P can function interchangeably *in vitro* (Guerrier-Takada et al. 1983) and *in vivo* (Wegscheid et al. 2006).

The *rnpA* gene, encoding the protein component of RNase P, is located at 4.21 Mb on the *B. subtilis* genetic map, placing it in the replication origin, similar to *E coli rnpA* (Ogasawara et al. 1985). As is the case in most bacterial species, *rnpA* is in a transcription unit with, and downstream of, the *rpmH* gene, encoding ribosomal protein L34. A Rho-independent transcription terminator immediately downstream of the *rpmH* coding sequence causes transcriptional polarity and is likely the major factor in the ~90-fold greater expression of the *rpmH* gene (Gossringer et al. 2006). Another factor is poor translational signals that direct expression of the *rnpA* coding sequence. Interestingly, a long, 2.3 kb non-coding RNA, S1579, is transcribed on the opposite strand in this region of the genome. Thus, antisense regulation may also play a role in *rnpA* expression. The *rnpB* gene, encoding the RNase P RNA component, is in a completely different location at 2.33 Mb on the *B. subtilis* genetic map, and appears to be transcribed by itself.

While almost all bacteria have an RNA-based RNase P enzyme, an exception was recently discovered. Like other bacteria, *Aquifex aeolicus*, a hyperthermophilic bacterium, has tRNA genes located within ribosomal RNA operons and these are processed by RNase P to give mature 5′ ends. However, there are no homologs in the *A. aeolicus* genome of either classic RNase P protein or RNase P RNA. The existence of RNA-free RNase P activities in higher species (Lechner et al. 2015) prompted a biochemical analysis of fractionated *A. aeolicus* protein for tRNA cleavage activity. This resulted in the identification of an RNase P enzyme that functioned in the absence of any RNA (Nickel et al. 2017). *Aquifex aeolicus* RNase P is the smallest known RNase P protein, consisting of a 23 kDa metallonuclease domain. It has limited sequence homology to RNA-free RNase P's of higher organisms, but does have three conserved aspartate residues in the catalytic center that are essential for activity. *Aquifex aeolicus* RNase P was able to complement *E. coli* and even yeast RNase P mutants. Many archaeal species and a few bacterial species encode a similar protein in their genome; some of these encode an RNA-based RNase P in addition. It remains to be determined whether both forms of RNase P are active in such cases.

In *E. coli*, all tRNAs genes have an encoded 3′ —CCA motif, whereas in *B. subtilis* only two-thirds of the tRNA genes encode a —CCA end. The third of *B. subtilis* tRNAs that are transcribed without the —CCA sequence is matured at their 3′ end by RNase Z, which cleaves such tRNA precursors immediately downstream of the discriminator base; the —CCA sequence is then added by a nucleotidyltransferase. RNase Z is, therefore, an essential RNase in *B. subtilis*. Those tRNAs that have an encoded —CCA sequence are not substrates for RNaseZ. RNase Z is also involved in the maturation of tmRNA, the RNA component of the ribosome rescue system (Gilet et al. 2015). The *rnz* gene, encoding RNase Z, is located at 2.48 Mb on the *B. subtilis* genetic map, and appears to be monocistronic.

Bacillus subtilis RNase Z is a 34 kDa protein that is a member of the β-lactamase superfamily of zinc-dependent metallo-hydrolases. As such, it is related to the RNase J enzymes. *B. subtilis* RNase Z and *E. coli* RNase BN share 48% identity and 65% similarity. The dimeric structure of *B. subtilis* RNase Z has been determined (Li de la Sierra-Gallay et al. 2005), as well its structure when bound to tRNA (Li de la Sierra-Gallay et al. 2006). The structure is a dimer of metallo-β-lactamase domains, with one subunit in a conformation that coordinates two zinc ions, via the conserved HxHxDH motif, and the other subunit that does not coordinate zinc and is apparently inactive for catalysis but is required for substrate recognition. As could be expected from the many different tRNA sequences that are substrates for RNase Z, the protein recognizes primarily the phosphate-sugar backbone. For the $tRNA^{Thr}$ that was used in the crystallization study, guanosine residues 1 and 19 made specific hydrogen bonds; these residues are highly conserved. It was suggested that when the discriminator nucleotide (U73 in the $tRNA^{Thr}$ used, which, in the free tRNA structure, stacks on the acceptor stem) is in the tRNA–RNase Z complex, it separates from the stem to enter the catalytic site of the enzyme (Li de la Sierra-Gallay et al. 2006). In tRNAs that contain an encoded —CCA sequence, the two C residues adjacent to the discriminator nucleotide, as well as a particular conserved Arg residue in the channel leading to the catalytic site, block movement of the tRNA into the active site (Dutta et al. 2013). Details of RNase Z structure/function have been extensively reviewed (Redko et al. 2007).

RNase M5

Unlike the multi-step maturation of 5S rRNA in *E. coli*, *B. subtilis* 5S rRNA is processed by a single activity, RNase M5, which cleaves on both sides of a double-stranded structure to give the final mature product (Figure 2; Stahl et al. 1984). RNase M5 catalyzes this reaction in conjunction with the binding of ribosomal protein L18, which may act as a chaperone to hold the 5S rRNA precursor in the optimal conformation for RNase M5 cleavage (Pace et al. 1984). The gene encoding RNase M5, *rnmV*, located at 0.05 Mb on the *B. subtilis* genetic map, is highly conserved among the low G + C Gram-positive bacteria. The *rnmV* gene is immediately upstream of the *ksgA* gene, encoding an rRNA methyltransferase. Intriguingly,

the *ksgA* coding sequence begins in the *rnmV* coding sequence, five nucleotides from the end. Whether this case of translational coupling, for genes involved in ribosome maturation, has any regulatory consequences has not been investigated.

An RNase M5 deletion strain shows no growth phenotype in laboratory conditions, indicating that precursor 5S RNA functions as well as the mature form (Condon et al. 2001). 5S rRNA is likely the only cellular substrate for RNase M5 (Condon et al. 2002).

RNase M5 is a 20.5 kDa protein (186 amino-acids) that is a member of the Toprim domain family ("To" for topoisomerase I and "prim" for DNA G primase; Aravind et al. 1998). The Toprim domain consists of a core of four hydrophobic β strands surrounded on three sides by α-helices, in a βαβαβαβ fold, with conserved residues at the edge of the three α helices. The RNase M5 Toprim domain comprises the N-terminal half of the protein (residues 6–81), which contains the key residues for catalytic activity. There are several highly conserved residues in this domain, including an Asp in between β1 and α1, a Gly immediately after β2, and a DxDxxG motif spanning β3–α3. It was shown that mutation of these conserved residues completely abolishes RNase M5 activity *in vivo* and results in either a complete loss of activity or a sharp reduction in activity *in vitro* (Allemand et al. 2005). Since the mutation of residues that affect topoisomerase cleavage of double-stranded DNA also affect RNase M5 cleavage of double-stranded RNA, it is likely that a similar enzyme mechanism occurs for both. The function of the RNase M5 C-terminal half of the protein is less clear, but may be required for RNA binding.

RNase III

The *B. subtilis* version of RNase III was first purified in the early 1980′s, and its activity as a double-strand-specific endoribonuclease was demonstrated on *B. subtilis* rRNA and on RNAs encoded by the *B. subtilis* bacteriophage SP82 (Panganiban and Whiteley 1983a, 1983b). A later study showed that there were significant differences in the substrate specificity of the *E. coli* and *B. subtilis* RNase III enzymes (Mitra and Bechhofer 1994). Subsequently, the *B. subtilis rnc* gene, encoding RNase III, was identified and its coding sequence revealed a 28 kDa protein that shared 36% amino-acid identity with *E. coli* RNase III, including the highly conserved NERLEFLGD sequence at the catalytic site (Wang and Bechhofer 1997). The *B. subtilis rnc* gene was able to complement the rRNA processing defect in an *E. coli rnc* deletion mutant. The domain organization of *B. subtilis* RNase III is similar to that of *E. coli* RNase III, with an N-terminal catalytic domain and a C-terminal double-stranded RNA binding domain. The *B. subtilis* enzyme presumably binds to RNA target sites as a dimer, cleaving either one or both strands of a hairpin sequence containing two duplexed regions.

The *rnc* gene is located at 1.67 Mb on the *B. subtilis* genetic map, in a four-gene cluster: *acp* (acyl carrier protein)-*rnc*-*smc* (chromosome condensation)-*ftsY* (signal recognition particle protein). There is a predicted Rho-independent transcription terminator immediately downstream of the *rnc* coding sequence, and, in a wild-type strain, only a ~1 kb transcript covering the *acp* and *rnc* genes can be detected. However, in a strain with an inactivating *rnc* point mutation (E138A), a prominent ~6 kb operon RNA can easily be detected. *rnc* mRNA showed a 4-fold stabilization in the point mutant, and, indeed, two RNase III cleavage sites

were mapped to the *rnc* coding sequence (DiChiara et al. 2016). Thus, *B. subtilis* RNase III autoregulates its own expression, as is the case for *E. coli* RNase III, where cleavage occurs in a 5′ leader region (Bardwell et al. 1989).

Unlike the case in *E. coli*, an *rnc* null mutant of *B. subtilis* could not be easily obtained, suggesting it was essential (Herskovitz and Bechhofer 2000). Rare *rnc* deletion mutants, that presumably had second-site mutations allowing them to survive (see below), showed an accumulation of unprocessed 30S rRNA, as well as precursor small cytoplasmic RNA (scRNA), an RNA component of the signal recognition particle that is cleaved by RNase III (Oguro et al. 1998). Alternative pathways for processing 30S rRNA are active to the extent that very little full-length 30S rRNA is detectable in a *B. subtilis rnc* mutant. In fact, this RNA species is detectable only by Northern blotting, unlike the case of an *E. coli rnc* strain where the 30S rRNA species is clearly observed in ethidium bromide-stained gels (Herskovitz and Bechhofer 2000). More recently, it was clarified that RNase III is essential only in *B. subtilis* strains carrying one or more prophage genomes that specify toxin genes whose mRNA transcript is kept under control by RNase III-mediated decay. Specifically, the Skin prophage carries the *txpA* gene, which encodes a toxic peptide. Expression of this peptide is down-regulated by binding of an antisense RNA, RatA (Silvaggi et al. 2005), forming a double-stranded RNA target that is cleaved by RNase III. A similar situation exists for the *yonT* gene carried on prophage SPβ. RNase III is not essential in strains lacking these prophages or their toxin genes (Durand et al. 2012).

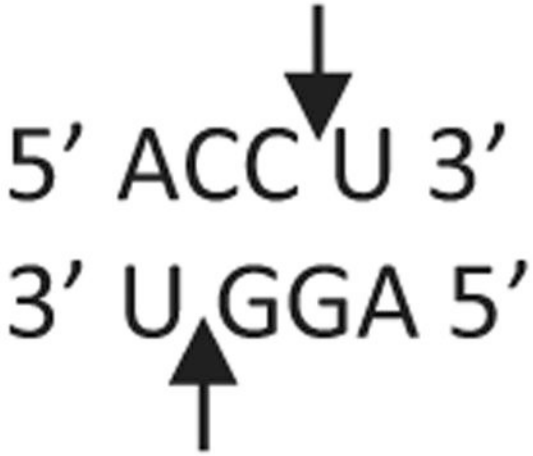
Since RNase III is not essential in the absence of the prophage toxin genes, it was possible to gather global information on RNase III targets, in addition to the stable RNAs mentioned above, by mapping 5′ ends that were present in the transcriptomes of an *rnc* wild-type strain but absent in an *rnc* deletion strain (DiChiara et al. 2016). This was done in strains that were deleted for the *rnjA* gene, encoding the 5′-to-3′ exonuclease RNase J1, to preserve the 5′ ends generated by RNase III cleavage. Using a conservative threshold to validate direct endonuclease targets, RNase III cleavage sites in 53 mRNAs and 5 intergenic RNAs were mapped. In *S. aureus* and *S. coelicolor* as well, RNase III was found to target many mRNAs (Lasa et al. 2011; Gatewood et al. 2012; Lioliou et al. 2012). In the *S. aureus* studies, RNase III cleavage was observed in 75% of sense RNAs, and this was most often associated with an antisense RNA pairing. The suggestion was made that, in *S. aureus*, the pairing of antisense transcripts to sense transcripts and cleavage by RNase III activity are key elements in regulating expression levels of sense RNAs and preventing pervasive transcription. However, this process was not observed widely in the case of *B. subtilis* (DiChiara et al. 2016). An example of *B. subtilis* RNase III cleavage that is required for regulation by a *trans*-acting RNA is the RoxS RNA, an sRNA that regulates genes involved in the response to oxidative stress (Durand et al. 2015).

Just as maturation at the 16S rRNA 5′ end and maturation of 5S rRNA in *B. subtilis* use RNases not found in *E. coli* (RNase J1, RNase M5), maturation of *B. subtilis* 23S rRNA also occurs by a different enzyme. Whereas in *E. coli*, 23S rRNA 3′ end maturation is accomplished by the 3′ exonuclease RNase T and 5′ end maturation occurs by an as-yet unidentified activity, in *B. subtilis*, maturation of 23S rRNA occurs in one step via a double-stranded RNA cleavage catalyzed by a novel enzyme, “Mini-III” (Figure 2) (Redko et al. 2008). This mechanism is similar to the double stranded RNA cleavage by RNase M5 that yields fully mature 5S rRNA.

The name “Mini-III” reflects the fact that this enzyme contains a similar N-terminal catalytic domain to RNase III, but is missing the C-terminal dsRNA-binding domain of RNase III. Mini-III is encoded by the *mrnC* gene, which specifies a 16 kDA protein. An alignment of the Mini-III sequence and the RNase III catalytic domains shows 25% identical and 41% similar amino-acids. Among bacterial species, Mini-III genes are limited to Firmicutes and Cyanobacteria. Mini-III can be found also in lower eukaryotes, such as *Chlamydomonas*, and even in plants, such as *Arabidopsis*, where its function in rRNA maturation and intron biology have been demonstrated (Hotto et al. 2015). Somewhat surprisingly, a *B. subtilis mrnC* deletion strain shows no major growth phenotype; 23S rRNA maturation occurs in this strain by an alternative pathway involving RNase J1 exonucleolytic processing at the 5′ end and RNase PH and YhaM processing at the 3′ end (Redko and Condon 2010).

The *mrnC* gene is located at 0.11 Mb on the *B. subtilis* genetic map, the third gene in an operon containing *gltX* (glutamyl-tRNA synthetase), *cysE* (serine acetyltransferase), *cysS* (cysteinyl-tRNA synthetase), *mrnC*, *yacO* (a putative rRNA methyltransferase), and *yacP*. The latter gene has recently been renamed *rae1*, for ribosome-associated endoribonuclease (Leroy et al. 2017). Rae1 is a translation-dependent endonuclease that cleaves in the ribosomal A site. It has been pointed out that both *rnmV* and *mrnC*, genes encoding rRNA maturation enzymes, are associated with genes encoding rRNA methyltransferases (*ksgA* and *yacO*) (Redko et al. 2008).

Presumably, Mini-III binds RNA as a dimer, as does RNase III, and models predict that the catalytic mechanism for RNase III and Mini-III is conserved despite a major difference in the mechanism of RNA recognition (Redko et al. 2008). Mini-III cleavage to produce mature 23S rRNA does not depend on prior RNase III cleavage of the processing stalk. However, Mini-III activity on precursor 23S rRNA in 50S ribosomal subunits is much more efficient than on naked RNA. It was shown that the L3 ribosomal protein, which lies close to the paired 5′ and 3′ ends of 23S rRNA, is the 50S subunit factor that stimulates Mini-III activity, likely by changing the substrate conformation (Redko and Condon 2009). Recently, the surprising finding was reported that *B. subtilis* Mini-III cleaves dsRNA at a preferred nucleotide sequence (Głów et al. 2015):



This is the first example of a dsRNA endonuclease that exhibits a degree of sequence specificity, which is quite different from RNase III, whose cleavage preference relates to structure much more than it does to sequence (Nicholson 2014). The basis for Mini-III specificity includes a particular alpha helix in the catalytic domain that is not present in RNase III (Głów et al. 2016).

YqfG

A recent study demonstrated that, in *B. subtilis*, processing of the 3′ end of 16S rRNA involved cleavage two nts downstream of the mature 3′ end by an unknown endonuclease (DiChiara et al. 2016). A 65-nt fragment that extended from this cleavage site down to the RNase III cleavage site in the processing stalk was detected by Northern blotting. A subsequent study suggested strongly that the endonuclease responsible for this cleavage is the product of the *yqfG* gene, located at 2.61 Mb on the *B. subtilis* genetic map (Baumgardt et al. 2018). The *yqfG* gene is in a three-gene operon and is located between *pgpH*, encoding a cyclic-di-AMP phosphodiesterase, and *dgkA*, encoding a diacylglycerol kinase, a membrane protein required for efficient sporulation. Strangely, these three genes are likely translationally coupled, as the *yqfG* coding sequence begins immediately after the *pgpH* stop codon, and the *dgkA* coding sequence begins 18 nt upstream of the yqfG stop codon.

Bacillus subtilis YqfG is a 17.6 kDa protein that is a homolog of the *E. coli* YbeY protein, with 23% amino-acid identity and 72% similarity. Although *in vitro* studies with YqfG have not yet been published, the results of *in vivo* end-mapping indicate that endonucleolytic cleavage by YqfG leaves a downstream fragment with a 5′ phosphate end (DiChiara et al. 2016). This is different from the observed 5′ hydroxyl end that is generated by YbeY (Jacob et al. 2013). While *E. coli* YbeY seems to play a role in the maturation of all three rRNAs, *B. subtilis* YqfG is involved only in the 3′-end maturation of 16S rRNA.

In a strain that has a low-level expression of the *yqfG* gene, there is a major loss of 70S ribosome particles, which is likely the reason why *yqfG* is essential in *B. subtilis* (Kobayashi et al. 2003; Baumgardt et al. 2018). The YbeY homolog in *Vibrio cholerae* is also essential (Vercruysse et al. 2014). This is different from *E. coli*, where *ybeY* mutants are viable

(Davies et al. 2010). Strikingly, YqfG is no longer essential in a strain that is deleted also for RNase R (Baumgardt et al. 2018). The explanation for this observation is that the 65-nt 3′ extension of precursor 16S rRNA, present in the absence of YqfG, is a substrate for processive RNase R exonucleolytic activity. Activation of RNase R rRNA quality control results in a shortage of 70S ribosomes. When RNase R is absent, the extended 16S rRNA can function, and so the *yqfG rnr* double mutant is viable.

Properties of the *B. subtilis* endoribonucleases described above are summarized in Table 1.

Bacillus subtilis *exoribonucleases*

Decades of work with *E. coli* revealed only exoribonucleases that operate in the 3′-to-5′ direction. Thus, the existence of a 5′-to-3′ exoribonuclease in bacteria was doubted. This was in contrast to the situation in eukaryotes, where the well-established XRN family of 5′-to-3′ exoribonucleases participates in many aspects of RNA decay and processing (Nagarajan et al. 2013). The lack of a bacterial 5′-to-3′ exoribonuclease was the accepted dogma, even for years after bacterial genome sequences began to appear in the mid-1990s. A significant milestone in the field of bacterial RNases was the discovery in 2007 of just such an activity in *B. subtilis*, specified by RNase J1 (Mathy et al. 2007). In fact, RNase J1 and another *B. subtilis* enzyme, RNase J2, had been identified 2 years earlier, but were thought to be exclusively endonucleases (Even et al. 2005). Nevertheless, the activity of RNase J1 on 16S rRNA was shown clearly to be exonucleolytic in the 5′-to-3′ direction. Thus, RNase J1 is a rare dual-acting enzyme, capable of exonucleolytic and endonucleolytic activities. The RNase J family of enzymes was found to be widely conserved in many bacterial phyla, especially in the Gram-positive Firmicutes and Actinobacteria (Even et al. 2005).

RNase J1 (encoded by the *rnjA* gene) and RNase J2 (encoded by the *rnjB* gene) are 61 kDa and 57 kDa proteins, respectively, with 49% amino-acid identity. The *rnjA* gene is located at 1.52 Mb on the *B. subtilis* genetic map, downstream of, and apparently transcribed with, the *rpoY* gene encoding the epsilon subunit of RNase polymerase. The significance of this arrangement is not known. The *rnjB* gene is located at 1.75 Mb and appears to be monocistronic. *A B. subtilis* Δ*rnjA* strain grows poorly and has several characteristics that are similar to a Δ*rny* strain (Figaro et al. 2013). This is rather surprising, as the presence of two similar RNase J′s in *B. subtilis* might suggest functional redundancy. However, it was found that the *in vitro* 5′ exonuclease activity of RNase J2 is at least 100-fold weaker than that of RNase J1, although they have similarly efficient endonuclease activities (Mathy et al. 2010). The poor 5′ exonuclease activity of RNase J2 explains why it cannot compensate for the loss of RNase J1 *in vivo*. In fact, a *B. subtilis* Δ*rnjB* strain grows identically to a wild-type strain in laboratory conditions. On the other hand, biological and biophysical experiments reveal that RNases J1 and J2 are present in a complex that could be a

heterotetramer (Mathy et al. 2010), but more likely a heterodimer (Newman et al. 2011). It has been argued that the *in vivo* significance of RNase J1 endonuclease activity is much more limited than its 5′ exonuclease activity (Condon 2010), and it has been proposed that *B. subtilis* RNase J2 functions *in vivo* solely as an endonuclease, providing substrates for the 5′ exonuclease activity of RNase J1 (Newman et al. 2011).

The RNase J domain structure consists of an N-terminal, catalytic β-lactamase domain, a central β-CASP domain (named after other members in this subfamily: metallo-β-lactamase CPSF, Artemis, SnmI, and Pso2), and a C-terminal domain that is also required for activity, perhaps to allow dimer formation. The first RNase J crystal structure was of *Thermos thermophiles* RNase J1, by itself, and bound to UMP (Li de la Sierra-Gallay et al. 2008). It was shown that a binding site for the nucleotide was immediately adjacent to the catalytic site at which a pair of cofactor Zn^{2+} ions is bound. Another metal divalent cation (Mg^{2+} or Mn^{2+}) is also required for activity (Pei et al. 2015). The nucleotide binding site was predicted not to be able to accommodate a 5′ nucleoside triphosphate, which clarified why RNase J1 exonuclease activity is inhibited by a terminal 5′ tri-phosphate (Deikus et al. 2008; Li de la Sierra-Gallay et al. 2008). While this initial structure readily explained a mechanism for the 5′ exonucleolytic activity of RNase J1 and its specificity for a 5′-monophosphate end, it was difficult to reconcile with the observed endonucleolytic activity. Subsequent structure studies provided a rationale for RNase J endonuclease activity, with an apparent channel in which longer RNAs could be threaded, delivering the cleavage target to the enzyme active site (Dorleans et al. 2011; Newman et al. 2011; Pei et al. 2015). It should be noted that mutagenesis of residues required for exonuclease activity also abolish endonuclease activity, indicating a single catalytic site for both activities (Li de la Sierra-Gallay et al. 2008). Several *in vivo* RNase J1 endonuclease cleavage sites have been mapped and show a preference for A/U-rich sequences (Bechhofer 2009), much like RNase E.

The exonuclease activity of RNase J1 removes RNA one nucleotide at a time in a processive manner, and becomes distributive when the substrate is less than 5 nts long (Dorleans et al. 2011). RNase J1 is somewhat inhibited by strong RNA secondary structure, but is able to digest through such structure in the 5′-to-3′ direction (Deikus et al. 2008). Although the same substrates have not been directly compared, the processivity of RNase J1 on RNA secondary structure appears to be much greater than that of PNPase in the 3′-to-5′ direction (Deikus and Bechhofer 2007). A transcriptome analysis of RNA 5′ ends in a Δ*rnjA* background supports the view that turnover of 3′-terminal RNA fragments, which includes Rho-independent transcription terminator structures, is accomplished primarily by RNase J1 (DiChiara et al. 2016).

As mentioned, the 5′ exonuclease activity of RNase J1 was first discovered in its role in 16S rRNA maturation (Mathy et al. 2007). Differences between *E. coli* and *B. subtilis* rRNA maturation are shown in Figure 2. In *E. coli*, 16S rRNA 5′ maturation occurs by endonuclease cleavages catalyzed by RNase E and RNase G. *B. subtilis* has neither of these enzymes. Instead, an unknown endonuclease cleaves downstream of the RNase III cleavage site, providing entry for RNase J1 5′ exonuclease processing up to the mature 5′ end. Processing of precursor 16S rRNA by RNase J1 occurs on assembled 70S ribosomes.

The involvement of RNase J1 5′ exonuclease activity in mRNA decay may occur in two different pathways (Figure 5). (1) RNase J1 may act at a 5′ end that is created by prior endonucleolytic cleavage, primarily by RNase Y. RNase Y cleavage yields an upstream fragment with a 3′ hydroxyl end susceptible to 3′-to-5′ exonucleolytic decay, and a downstream fragment with a 5′-monophosphate end susceptible to RNase J1 5′-to-3′ exonucleolytic decay. This pathway has been demonstrated for *rpsO* and *hbs* mRNA decay (Daou-Chabo et al. 2009; Yao and Bechhofer 2010; Braun et al. 2017). The presence of a processive 5′-to-3′ exonuclease would obviate the need for additional endonucleolytic cleavages by RNase Y. (2) Direct binding of RNase J1 at the 5′ end of a native transcript is inhibited by the presence of the 5′ triphosphate group. To allow RNase J1 binding, an activity specified by an RNA pyrophosphohydrolase (RppH), two of which are known to exist in *B. subtilis* (Hsieh et al. 2013; Piton et al. 2013), can remove the γ and β phosphates. (Note that the RppH activity that acts on mRNA 5′ ends was first discovered in the Belasco laboratory in *E. coli* (Celesnik et al. 2007)). The 5′ monophosphate end is then bound by RNase J1, followed by the initiation of decay of the message in the 5′-to-3′ direction. Such a mechanism has been suggested by experiments with model mRNAs that are known RppH substrates, where the half-life of the full-length mRNA increased several-fold when RNase J1 levels were depleted (Yao et al. 2009; Richards et al. 2011). The half-lives of these same mRNAs were unaffected by depletion of RNase Y.

The endonuclease activity of RNase J1 has also been shown to initiate decay of a regulatory RNA. Degradation of the small *trp* leader RNA, which is bound by TRAP and regulates read-through transcription into the *trp* operon (Gollnick et al. 2005), is apparently initiated by an RNase J1 cleavage that has been demonstrated *in vivo* and *in vitro* (Deikus et al. 2008; Deikus and Bechhofer 2011). Cleavage of *trp* leader RNA by RNase J1 was independent of the 5′ end, i.e. cleavage was observed even when a strong stem-loop structure was provided at the 5′ end. More examples of the involvement of RNase J1 endonuclease activity in *B. subtilis* RNA turnover will be needed to make a stronger case for the importance of this activity.

There is no evidence for cross-regulation at the transcriptional level between RNases J1, Y, and III (Durand et al. 2012). Similarly, there were no changes in the level of *rnjA* transcript in various 3′ exonuclease mutant strains (Liu et al. 2014). In general, little is known about the regulation of most RNase activities in *B. subtilis*.

RNase J in other organisms

Although many organisms contain RNase J1 and RNase J2 genes, others contain only a single RNase J gene, and still others three or four copies (Britton et al. 2007). *Streptomyces coelicolor* contains only one gene coding for an RNase J enzyme; nevertheless, this gene is not essential (Bralley et al. 2014). Contrast this with *S. pyogenes*, where both RNase J1 and RNase J2 are essential for growth (Bugrysheva and Scott 2010). In *S. aureus*, on the other hand, neither of the two RNase J genes is essential, but deletion of either one results in restricted growth at high and low temperatures and on certain media (Linder et al. 2014). The effect of the *S. aureus rnjB* deletion was clarified somewhat by the finding that an active site mutant of RNase J1 had the same effect as a deletion of the *rnjA* gene, whereas an active

site mutant of RNase J2 had no effect on growth, unlike the deletion of the *rnjB* gene. This suggested that the role of RNase J2 in *S. aureus* is not as an independent activity, but to allow the correct function of RNase J1 with which it forms a complex.

The first gene identified of the four known 3′ exoribonucleases of *B. subtilis* was the *rph* gene, encoding RNase PH, the phosphorolytic enzyme responsible for maturation of tRNAs that have an encoded —CCA sequence (Craven et al. 1992). The *rph* gene is located at 2.90 (Megabases (Mb)) on the *B. subtilis* genetic map and appears to be the first gene in an operon containing also *ysnA*, an inosine triphosphatase, and *ysnB*, a putative metallophosphoesterase. See the section above on *E. coli* RNase PH for structural details, which are likely shared by *B. subtilis* RNase PH that has 56% identical amino-acid residues to the *E. coli* protein. For the two-thirds of *B. subtilis* tRNAs that have an encoded —CCA motif, the precursor tRNA is trimmed back to the —CCA sequence primarily by RNase PH (Wen et al. 2005). This trimming function can be accomplished in *E. coli* by a number of redundant activities specified by RNases T, BN, D, II, and PNPase (Kelly and Deutscher 1992b). RNases T, BN, D and II have no homologs in *B. subtilis*. Experiments with multiply mutant *B. subtilis* strains defective in the other three known 3′ exonucleases (PNPase, YhaM, and RNase R) demonstrated that there is some redundancy in tRNA maturation function in *B. subtilis* as well. Interestingly, even a mutant that was missing all four of the known 3′ exonucleases had significant levels of mature tRNA, suggesting the existence of another 3′ exonuclease capable of trimming back from the 3′ end of precursor tRNAs. The identity of this enzyme is not yet known. A Δ*pnpA* Δ*rph* double mutant, missing both phosphorolytic 3′ exonucleases, is viable but grows slower than the Δ*pnpA* strain.

It is probable that RNase PH is not involved in mRNA decay since the enzyme is likely to resemble *E. coli* RNase PH in that it is distributive, rather than processive, on non-tRNA substrates (M.P. Deutscher, unpublished results). On the other hand, RNase PH appears to be involved, along with YhaM, in the maturation of the 3′ end of 23S rRNA in strains that do not contain Mini-III, the endonuclease that normally generates the mature 5′ and 3′ ends of 23S rRNA (Redko and Condon 2010).

The distribution of RNase PH in other organisms is variable. For example, among the proteobacteria, organisms in the α, β, and γ subdivisions have the enzyme, but organisms in the δ and ε subdivisions do not (Condon and Putzer 2002).

PNPase

The second gene identified of the four known 3′ exoribonucleases of *B. subtilis* was the *pnpA* gene, encoding PNPase, also a phosphorolytic enzyme. Biochemical analysis of strains with *pnpA* knockout constructs was used to demonstrate that PNPase specifies the

major RNA decay activity of *B. subtilis* (Wang W and Bechhofer 1996), confirming an earlier study that showed *B. subtilis* RNA decay is primarily phosphorolytic, unlike *E. coli* in which RNA decay is primarily hydrolytic (Deutscher and Reuven 1991). A possible rationale for this difference was proposed to be the nutrient-poor environment of *B. subtilis* (a soil-dwelling organism), which may necessitate conservation of phosphate energy in the form of nucleoside diphosphates that are the product of PNPase digestion.

The pnpA gene is located at 1.74 Mb on the *B. subtilis* genetic map. This is downstream of the *rpsO* gene, as it is in *E. coli*. Although it has been assumed that there is an *rpsO-pnpA* transcript (Mitra et al. 1996), there is no direct evidence for this. PNPase is a 77 kDa protein that functions as a trimer and is 52% identical with *E. coli* PNPase. Structural studies on *B. subtilis* PNPase have not been done, but homology modeling based on the *E. coli* crystal structure (Nurmohamed et al. 2009) was able to predict residues that were involved in catalysis and that interacted with RNase Y (Salvo et al. 2016). The enzyme has a central channel that narrows to the active site and that can accommodate only single-stranded RNA, providing a rationale for the observation that purified *B. subtilis* PNPase is inhibited by RNA secondary structure (Deikus and Bechhofer 2007). It is, therefore, likely that efficient degradation of long mRNA sequences, which may contain secondary structure, will require coupling of PNPase to a helicase activity. In fact, an interaction between PNPase and CshA, the major *B. subtilis* RNA helicase (Lehnik-Habrink et al. 2013) has been detected by both *in vivo* crosslinking and bacterial-2-hybrid experiments (Lehnik-Habrink et al. 2010). In experiments with purified PNPase, it was also determined that the enzyme could bind the 3′ end of an RNA efficiently (apparent dissociation constant of 1.0 nM) when provided with a single-stranded tail greater than 6 nucleotides. While Rho-independent transcription terminators should provide a tail of sufficient length for PNPase binding (de Hoon et al. 2005), the strength of the terminator stem-loop structure would likely prohibit PNPase processivity. It has been shown for the abundant *rpsO* mRNA that mRNA decay, which is PNPase-dependent, is not initiated from its 3′ end (Yao and Bechhofer 2010). Thus, the processive degradative activity of PNPase on *B. subtilis* mRNA likely initiates from an endonuclease cleavage site, as is the case in *E. coli*.

In *E. coli*, a strain that is deficient for PNPase and RNase II activity is not viable (Donovan and Kushner 1986). Although *B. subtilis* does not have an RNase II homolog, the *B. subtilis* Δ*pnpA* strain is surprisingly healthy, growing only slightly slower than wild type. This, despite the fact that substantial accumulation of mRNA decay intermediates occurs in this strain (Oussenko et al. 2005). Apparently, other 3′ exonucleases can substitute for PNPase in the recognition of critical mRNA targets that need to be degraded. Nevertheless, a global analysis of accumulation of 5′-proximal sequences in wild type versus Δ*pnpA* strains showed that ~10% of mRNAs depend exclusively on PNPase for degradation, i.e. the other 3′ exoribonucleases cannot compensate for the loss of PNPase, suggesting some specificity for 3′ exonucleolytic recognition and/or processivity.

The Δ*pnpA* strain shows several phenotypes. As mentioned in the Introduction, the *pnpA* gene was originally identified in a transposon-insertion screen for competence-deficient mutants (Luttinger et al. 1996). The basis for this deficiency has not been explained. Also not yet explained is why a PNPase deficiency makes the cells hypersensitive to tetracycline

(Bechhofer and Stasinopoulos 1998). A Δ*pnpA* strain is also cold sensitive, which is not unexpected since PNPase is a cold-shock protein that is required for survival at low temperature (Awano et al. 2008). Finally, the Δ*pnpA* strain grows in long chains, and, in a strain that is otherwise capable of swarming, the loss of PNPase results in a severe swarming deficiency (Liu et al. 2014). The interesting molecular basis for these latter phenotypes, in which PNPase-mediated mRNA decay controls the level of a negative regulator of gene expression, has been described (Liu et al. 2016). The general conclusion from this study is that precise control of mRNA half-life by the 3′ exonucleolytic activity of PNPase is an important element in the regulation of gene expression.

In a list of over 600 annotated bacterial genomes, ~95% contained a gene encoding PNPase, with the exceptions clustered in the order *Lactobacillales* and the Tenericutes class (Hui et al. 2014). PNPase has been found to be required for virulence, biofilm formation, and stress responses in many organisms (reviewed in Rosenzweig and Chopra 2013; Briani et al. 2016), which is not surprising since alterations in PNPase-mediated decay of specific mRNAs and regulatory RNAs will likely affect any adaptive process that involves multiple players. Most often, the precise target for PNPase whose accumulation causes defects in such processes has not been identified.

RNase R

In extracts of a *B. subtilis* Δ*pnpA* strain, there is a major hydrolytic ribonuclease activity that can degrade rRNA *in vitro* and that can degrade past RNA secondary structure when a 3′ "tail" is present downstream of the structure (Oussenko and Bechhofer 2000). Identification of the *rnr* gene encoding this activity was facilitated by the identification of the RNase R gene of *E. coli* (Cheng et al. 1998). The *B. subtilis* homolog has 37% identical amino acids and is a similar size (89 kDa). Interestingly, the *rnr* gene is located at 3.45 Mb on the *B. subtilis* genetic map, in a five-gene cluster, *secG-yvaK-rnr-smpB-ssrA*, which has a complex transcriptional pattern (Shin and Price 2007). The *smpB* and *ssrA* genes that follow *rnr* encode protein and RNA (tmRNA) components, respectively, of the ribosome rescue system. In *E. coli*, RNase R is the ribonuclease that digests nonstop mRNA fragments that are acted upon by the ribosome rescue system (Richards et al. 2006); the genetic arrangement of the secG cluster in *B. subtilis* suggests that this is the case in this organism as well.

A Δ*rnr* strain grows as well as wild type, but a Δ*pnpA* Δ*rnr* grows slower than the Δ*pnpA* strain. Again, the viability of such a strain is somewhat of a surprise, given the inviability of an *E. coli* strain that is deficient for PNPase and RNase R (Cheng et al. 1998). RNase R of *E. coli* is involved in the quality control of *E. coli* rRNA (Deutscher 2009), and the same is assumed to be the case for *B. subtilis*. Experiments to support this assumption come from a recent study involving strains deleted for *yqfG*, the endonuclease required for 16S rRNA maturation. As explained above in the *yqfG* section, while deletion of *yqfG* is lethal, this gene could be deleted in a strain that was also deleted for *rnr* (Baumgardt et al. 2018). The results suggest that the defect in 16S 3′ end maturation caused by the absence of YqfG triggers degradation of 16S rRNA by RNase R.

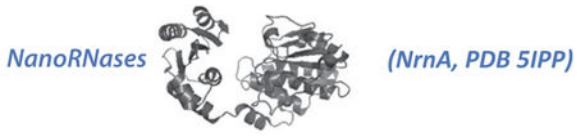
Unpublished RNA-Seq data (Liu and Bechhofer) shows that the absence of RNase R caused an accumulation of 5′-proximal reads in less than 0.5% of genes, compared to ~10% in the

strain missing PNPase. The absence of both RNase R and PNPase resulted in an accumulation of 5′-proximal reads in ~11% of genes, not much different from the strain lacking PNPase alone. Thus, the pool of mRNAs is unlikely to be a common target for RNase R activity. The primary function of RNase R in *B. subtilis*, then, would be in ribosome quality control.

YhaM

A hydrolytic ribonuclease activity in extracts of a *B. subtilis* Δ*pnpA* Δ*rnr* strain was attributed to YhaM, a member of a family of 3′ exoribonucleases with a unique domain structure (Oussenko et al. 2002). YhaM has an N-terminal OB domain (for oligonucleotide/oligosaccharide binding domain) and a C-terminal HD domain (found in metal-dependent phosphohydrolases). This combination is unique and is found only in Gram-positive organisms. YhaM enzyme activity is optimal in the presence of Mn^{2+}, and this can be substituted with Co^{2+}, but YhaM shows no activity *in vitro* in the presence of Mg^{2+} – another unusual property. The *yhaM* gene is located at 1.07 Mb of the *B. subtilis* genetic map and appears to be monocistronic.

The true function of YhaM is not yet clear. As mentioned above, the 5′ and 3′ ends of 23S rRNA are normally generated by Mini-III endonuclease cleavage, but in the absence of Mini-III, the 3′ end of 23S rRNA appears to be produced by 3′ exonucleolytic trimming activities of RNase PH and YhaM (Redko and Condon 2010). Several observations suggest that YhaM is involved in DNA replication: (1) YhaM can degrade single-stranded DNA (Oussenko et al. 2002). (2) A close homolog of YhaM in *S. aureus*, CBF1, binds to a plasmid replication enhancer (CBF = *cmp*-binding factor). CBF1 was shown to also have 3′ exoribonuclease activity. (3) YhaM is a member of the *B. subtilis* replisome (Noirot-Gros et al. 2002). Most recently, the Charpentier group published a transcriptome-wide analysis of targets of 3′ exoribonucleases in *S. pyogenes* (Lecrivain et al. 2018). They found that YhaM acts on the majority of transcripts and trims a few nucleotides from either the native 3′ end or from sites of endonuclease cleavage. The purpose of such trimming has not been clarified.



NanoRNAs are short oligonucleotides (≤5 nts) that are the limit digestion product of most exoribonucleases. Oligoribonuclease of *E. coli*, encoded by the *orn* gene, degrades nanoRNAs to mononucleotides (see above). This is a critical function that allows a complete turnover of mRNAs to nucleotide building blocks, and, indeed, the *orn* gene is essential in *E. coli*. As mentioned, Orn homologs are present in diverse species, from prokaryotes to eukaryotes and even humans. It was therefore surprising to find no *orn* homolog in the *B. subtilis* genome. Instead, the product of the *B. subtilis yqtI* gene (later named *nrnA*, for nanoRNase A) was shown to have oligoribonuclease activity *in vitro* (Mechold et al. 2007). The *nrnA* gene is located at2.99 Mb of the *B. subtilis* genetic map and appears to be monocistronic. Most bacterial species contain either an Orn homolog (e.g. β- and γ-

Proteobacteria, Actinobacteria) or an NrnA homolog (Firmicutes, Bacteroidetes, δ-Proteobacteria), but not both. *E. coli* Orn and *B. subtilis* NrnA differ in their substrate specificities: the preferred substrates for Orn are 5-mers, while NrnA degrades 5-mers at a much slower rate than Orn, and prefers 3-mers. Nevertheless, NrnA could functionally complement an *orn* deletion strain of *E. coli*. NrnA was poorly active on a 24-mer oligonucleotide that was tested (Mechold et al. 2007).

NrnA is a member of the DHH family of phosphoesterases (Aravind and Koonin 1998). The N-terminal catalytic domain consists of four motifs that each contains at least one Asp residue, with the conserved DHH sequence occurring in motif III. The DHH motif and other aspartates coordinate two divalent metal cations, with Mn^{2+} being the preferred metal ion for the NrnA reaction *in vitro* (Mechold et al. 2007). NrnA contains a C-terminal conserved domain, found also in other DHH proteins and referred to as the DHHA1 domain (DHH-associated domain 1), which is thought to function in substrate binding. The crystal structure of *B. subtilis* NrnA was determined recently (Schmier et al. 2017) and was found to be similar to the structure of RecJ, a 5′-to-3′ exoribonuclease specific for DNA. Indeed, biochemical assays showed that, while NrnA is most active as a 3′-to-5′ exoribonuclease on 3-mers, it was able to attack a longer oligo (12-mer) from the 5′ end. It appeared that NrnA digested the 12-mer in the 5′-to-3′ direction down to a 5-mer, after which the 3′-to-5′ exoribonuclease activity took over to degrade the 5-mer to mono-nucleotides (Schmier et al. 2017). Thus, NrnA is a bidirectional exonuclease, the only enzyme known to act this way on RNA. Other bidirectional exonucleases, such as exonuclease VII (Chase and Richardson 1974) and RecBCD (Wang et al. 2000), act on DNA.

The *nrnA* gene is not essential in *B. subtilis*, suggesting the existence of another oligoribonuclease gene. This was discovered to be the *yngD* or *nrnB* gene (Fang et al. 2009). NrnB *in vitro* activity resembles that of Orn more than it does NrnA, with similar activity to Orn on 5-mers. The *nrnB* gene is located at 1.95 Mb of the *B. subtilis* genetic map and appears to be transcribed with a relatively long (72 nt) 5′ untranslated region, the significance of which is not known. *nrnB* orthologues are present also in ε-Proteobacteria and in some archaea. Somewhat unexpectedly, a double deletion of *nrnA* and *nrnB* is viable, with no observable effect on growth rate. In the same study, it was found that YhaM also has oligoribonuclease activity, but only high-level expression of YhaM could complement an *E. coli orn* mutant. The preferred substrate for YhaM was actually a short DNA oligo, indicating that it likely does not function well as an oligoribonuclease *in vivo*. Furthermore, even a triple *nrnA nrnB yhaM* mutant was viable, with only a small effect on growth rate. This suggests the existence of another activity capable of degrading nanoRNA. While RNase J1 can degrade longer RNAs to mononucleotides *in vitro*, without accumulation of limit products (i.e. 2–5-mers) (Mathy et al. 2007; Deikus et al. 2008), it has not yet been determined whether RNase J1 would have appreciable activity on nanoRNAs (Fang et al. 2009).

RNase H

As described above, *E. coli* has two RNase H enzymes, RNase HI and RNase HII, whose substrates are RNA bound to complementary DNA or ribonucleotides embedded in double-

stranded DNA. The *B. subtilis* genome contains a gene, *ypdQ*, which is a sequence homolog of *E. coli* RNase HI (30% amino-acid identity; 46% amino-acid similarity). However, YpdQ is missing key residues required for catalytic activity and does not show RNase HI activity *in vitro* (Ohtani et al. 1999; Randall et al. 2018). *Bacillus subtilis* is therefore not considered to contain an RNase HI-like enzyme. The product of the *B. subtilis rnhB* gene, located at 1.68 Mb on the *B. subtilis* genetic map, is very similar to *E. coli* RNase HII (48% amino-acid identity, 65% amino-acid similarity), and is designated RNase HII. Another *B. subtilis* gene, *rnhC*, located at 2.93 Mb on the *B. subtilis* genetic map, encodes a product with 25% identity and 40% similarity to *E. coli* RNase HII. This enzyme, designated RNase HIII, represents a third class of RNase H enzymes (Itaya et al. 1999; Ohtani et al. 1999). *Bacillus subtilis* RNases HII and HIII have low similarity to each other (20%), but they share several well-conserved regions. The conserved DEDD (RNase HII) or DEDE (RNase HIII) motif in the C-terminal catalytic domain is responsible for metal ion coordination. In general, bacterial organisms will have either an RNase HI/HII pair or an RNase HII/HIII pair, but not an RNase HI/HIII pair, suggesting that these two enzymes have redundant functions (Kochiwa et al. 2007).

A recent in-depth biochemical analysis was reported for *B. subtilis* RNases HII and HIII, using three substrates: (1) a complementary 20-mer RNA:DNA hybrid; (2) a 20-mer double-stranded DNA molecule with four RNA nucleotides embedded in one DNA strand; (3) a 20-mer double-stranded DNA molecule with a single RNA nucleotide embedded in one DNA strand (Randall et al. 2018). Assays were done in the presence of Mn^{2+} or Mg^{2+} at physiological concentrations. The data showed that RNase HII and HIII were similarly active on the 20-mer RNA:DNA in the presence of Mg^{2+} (1 mM), but RNase HIII was much more active than RNase HII in the presence of Mn^{2+} (10 μM). Both RNase HII and RNase HIII were similarly active on the substrate with four embedded ribonucleotides, in the presence of either metal at physiological concentrations, but RNase HII was much more active than RNase HIII on the substrate with one embedded ribonucleotide under the same conditions (Randall et al. 2018).

Many of the Bacilli synthesize RNases that are either secreted or anchored to the cell wall. The most famous of these is barnase, expressed in *Bacillus amyloliquefaciens*; see Ulyanova *et al.* for a review and references (Ulyanova et al. 2011). Barnase is a small, 12.4 kDa protein (110 amino acids) that cleaves RNA primarily at guanine residues, forming 2′,3′ phosphodiesters that resolve to give a 3′ phosphate end. Barnase and the well-characterized RNase T1 from *Aspergillus oryzae* are representative members of the microbial N1/T1 family of RNases. Expression of similar low-molecular-weight, guanyl-specific ribonucleases of bacilli (but not barnase) is induced by conditions of low phosphate, suggesting that their function is to degrade RNA to provide a source of phosphate. Intracellular barnase is maintained in an inactive state by the binding of barstar, an 89

amino-acid protein that binds barnase tightly. Barnase and the barnase/barstar complex have been intensively studied as models for protein folding and protein-protein interactions. *Bacillus subtilis* does not contain a barnase gene.

Another secreted RNase, found in *B. subtilis*, is RNase Bsn, encoded by the *yurI* gene (Nakamura et al. 1992), which is located at 3.34 Mb on the *B. subtilis* genetic map. RNase Bsn is a nonspecific RNase that is larger than barnase, with a molecular weight of 32 kDa. Cleavage by RNase Bsn yields 5′-monophosphate ends. Similar to the low-molecular-weight, guanyl-specific RNases mentioned above, expression of RNase Bsn is induced by conditions of phosphate starvation (Allenby et al. 2005).

Assays of RNase activity in a protein extract from a *B. subtilis* strain that was missing PNPase, RNase R and YhaM revealed the presence of an additional RNase encoded by the *yhcR* gene, located at 0.99 Mb on the *B. subtilis* genetic map (Oussenko et al. 2004). YhcR is a very large protein of 132 kDa, one of only a few proteins with such high molecular weight in *B. subtilis*. Following a signal peptide, it has five recognizable domains, including a novel NYD domain (for new YhcR domain), two OB-folds, an SNase (*Staphylococcal* nuclease) domain, a metallophosphatase domain, and a 5′-nucleotidase domain. Near the C-terminus is an LPXTS sequence, which is a recognition sequence for sortase enzymes that anchor proteins to the cell wall of Gram-positive organisms (Marraffini et al. 2006). Interestingly, while *B. subtilis* has two sortase-like proteins, one of which is YhcS, encoded in an operon with YhcR, there appears to be only one sortase substrate – YhcR (Pallen et al. 2001). One would assume that the function of YhcR on the cell wall is to degrade nucleic acids to serve as a metabolic source for phosphate and nucleotides. However, it was found that a *yhcR* deletion strain grew as well as wild type in a minimal medium that contained RNA as the only source of phosphate (Oussenko et al. 2004).

YhcR is a Ca^{2+}-dependent enzyme that is inactive in the presence of Mg^{2+} and partially active in the presence of Mn^{2+}. In the presence of Ca^{2+}, YhcR cleaves endonucleolytically to generate 3′-monophosphate nucleotides, which is similar to *S. aureus* micrococcal nuclease activity. Other than YhcR, two other Ca^{2+}-dependent nucleases, YncB and YokF, are observed in zymogram analysis of total *B. subtilis* protein, and these are low-molecular-weight enzymes that are similar to micrococcal nuclease (Sakamoto et al. 2001).

RNA interferases

We mention briefly two *B. subtilis* RNases that target mRNAs endonucleolytically. The first is EndoA, encoded by the *ndoA* gene (Pellegrini et al. 2005). EndoA is a 12.8 kDa protein that is homologous (25% identity) to *E. coli* MazF. The structures of these two RNases are very similar, and they share a target sequence specificity of UAC, with cleavage occurring after the U residue, leaving a 3′ phosphate and a 5′ hydroxyl. Like MazF, EndoA is a member of a type II toxin-antitoxin pair, and is inhibited by the product of the *ndoAI* (EndoA inhibitor) gene (Pellegrini et al. 2005). EndoA cleavage occurs independently of the ribosome. The second mRNA cleavage enzyme is the recently-identified Rae1, a 19.5 kDa protein that contains an N-terminal catalytic domain and a C-terminal helical domain that is similar to those found in rRNA binding proteins (Leroy et al. 2017). Unlike EndoA, Rae1 is an A-site endonuclease that cleaves mRNA only in the context of translation on the

ribosome. For the single substrate that was tested, Rae1 cleaved upstream of a lysine codon (AAA or AAG). Rae1 does not appear to be associated with a specific inhibitor.

Properties of the *B. subtilis* exoribonucleases described above are summarized in Table 1.

Conclusions and future directions

As is obvious from the length of this review, there has been a massive increase in information about bacterial RNases in recent years. This has included the discovery and study of new enzymes, elucidation of the structure of many of these proteins and their mechanism of action, determination of the physiological roles of the majority of RNases and initial insights into the regulation of this diverse group of enzymes. Most importantly, we now have an appreciation for how crucial RNases are to every aspect of RNA metabolism and that many RNases are required for RNA metabolism to proceed efficiently and accurately. From initial studies on the *E. coli* enzymes, it is now clear that a large number of RNases originally identified in this organism extends to other bacteria as well. In addition, the extensive studies of *B. subtilis* RNases have shown that while some of the enzymes may be homologous to those originally found in *E. coli*, many are not, revealing a spectrum of new activities with different structures and mechanisms of action, but nevertheless, ultimately satisfying very similar RNA metabolic requirements.

What might we expect from future studies of bacterial RNases? It appears that the catalog of RNases in the model organisms, *E. coli* and *B. subtilis*, is nearing completion. Studies in these organisms will, therefore, focus more on filling in the physiological roles of respective RNases, and determining which RNase(s) is responsible for each step in RNA metabolism. In addition, much attention will probably focus on how the levels of the RNases are regulated, and how they respond to changing environmental conditions. The studies that have already been undertaken have identified a number of different mechanisms for regulating the RNases, and other interesting mechanisms might be expected. Finally, it is likely that the study of RNases will expand to many other bacterial species. Some studies in other organisms have already been undertaken, and have revealed differences from the model organisms. It would not be surprising if further work opens up a panoply of bacterial RNases for future study.

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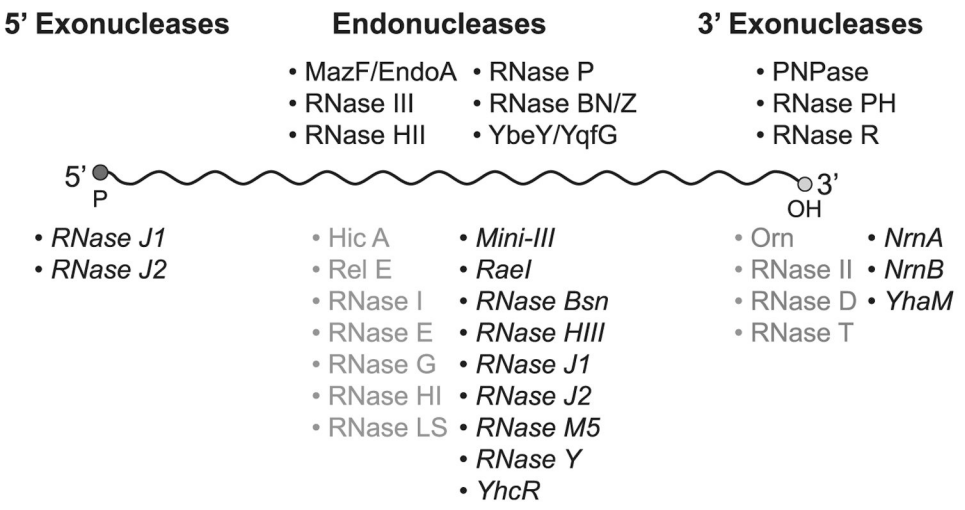

Figure 1.

Escherichia coli and *Bacillus subtilis* RNases. RNases present in both *E. coli* and *B. subtilis* are shown above the RNA schematic. RNases unique to one or the other organism are shown below the RNA schematic; *E. coli* enzymes in gray, *B. subtilis* enzymes in italics. Not shown are several other *E. coli* toxin RNases; only those discussed in the text are listed (MazF, HicA, and RelE).

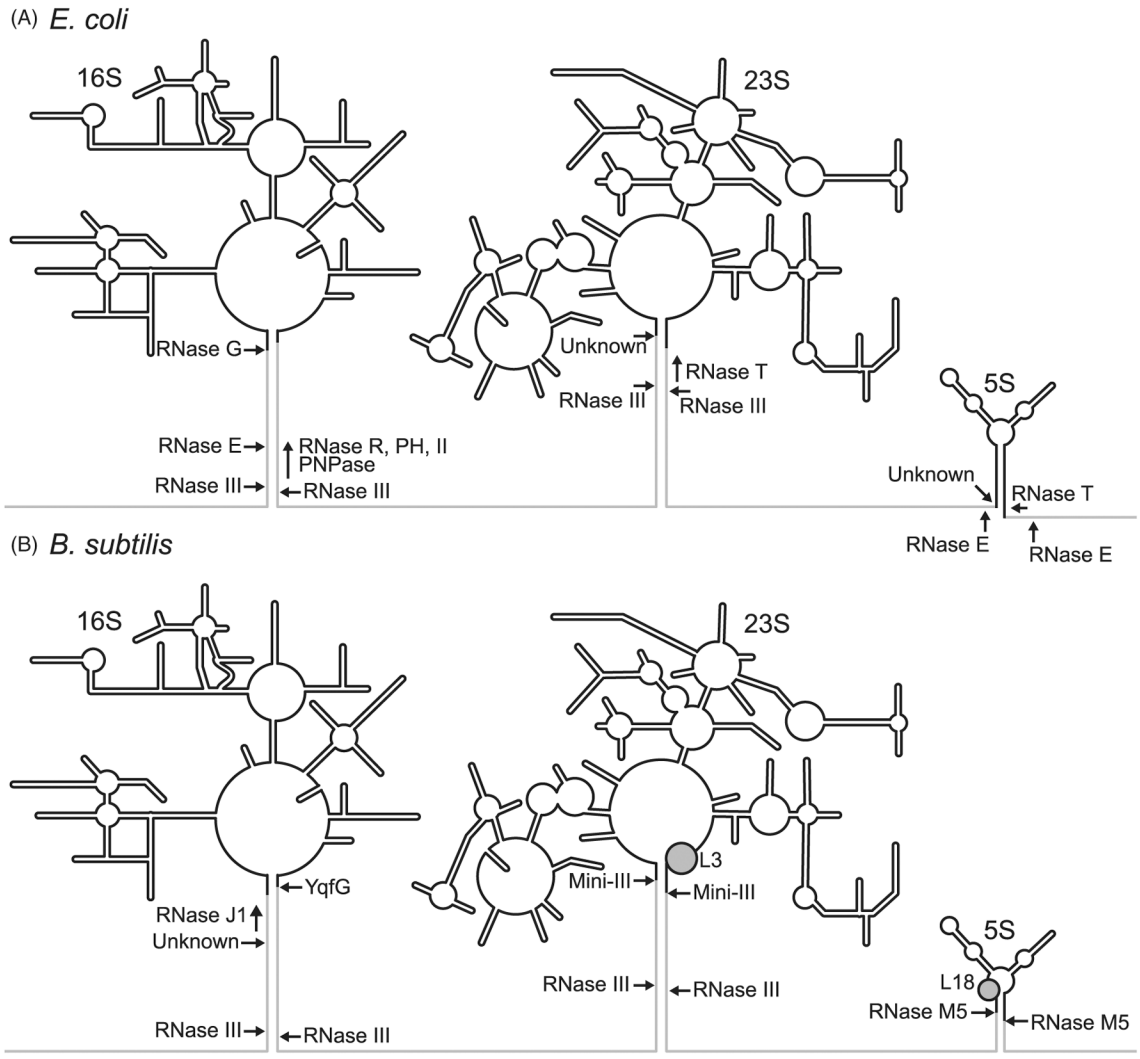

Figure 2.

rRNA processing. Processing of 16S, 23S, and 5S rRNA in (A) *E. coli* and (B) *B. subtilis*. Mature rRNA, in black, is depicted as a schematic of its secondary structure. Precursor rRNA not present in the mature rRNA is in gray. Location of endonucleolytic cleavages and direction of 5′ or 3′ exonucleolytic processing are indicated by arrows. In *B. subtilis*, ribosomal proteins L3 and L18 are required for efficient 16S and 5S rRNA processing, respectively.

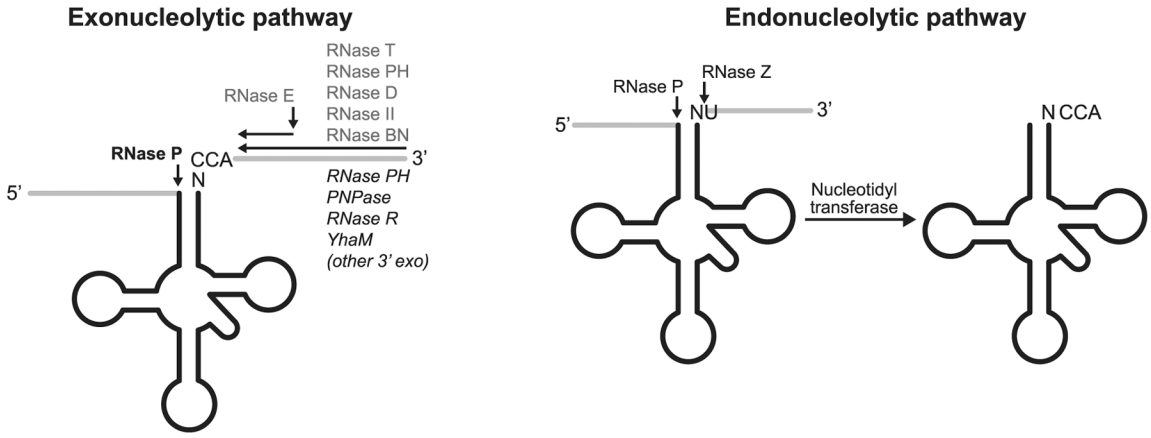

Figure 3.

tRNA processing. Two pathways for tRNA processing are shown, for tRNAs that do (left) or do not (right) have an encoded −CCA sequence in the genome. In *E. coli*, all tRNAs have an encoded −CCA sequence; in *B. subtilis*, only a third of tRNAs have an encoded −CCA sequence. For all tRNAs, RNase P cleaves endonucleolytically to give the mature 5′ end. For 3′ maturation, tRNAs with an encoded −CCA sequence follow the exonucleolytic pathway. Primarily RNase PH removes precursor nucleotides starting from either the native 3′ end or, in *E. coli*, from a site generated by RNase E cleavage. Other *E. coli* RNases (T, D, and II; shown above the tRNA 3′ extension) can participate in 3′ processing in the absence of RNase PH. *B. subtilis* does not have these three RNases, but other 3′ exonucleases (PNPase, RNase R, YhaM, and an as yet unidentified enzyme; shown in italics below the tRNA 3′ extension) can produce the mature 3′ end. For tRNAs without a −CCA sequence (in *B. subtilis*), the endonucleolytic pathway is followed in which RNase Z cleaves adjacent to the discriminator nucleotide (N). The −CCA sequence is added by nucleotidyl transferase.

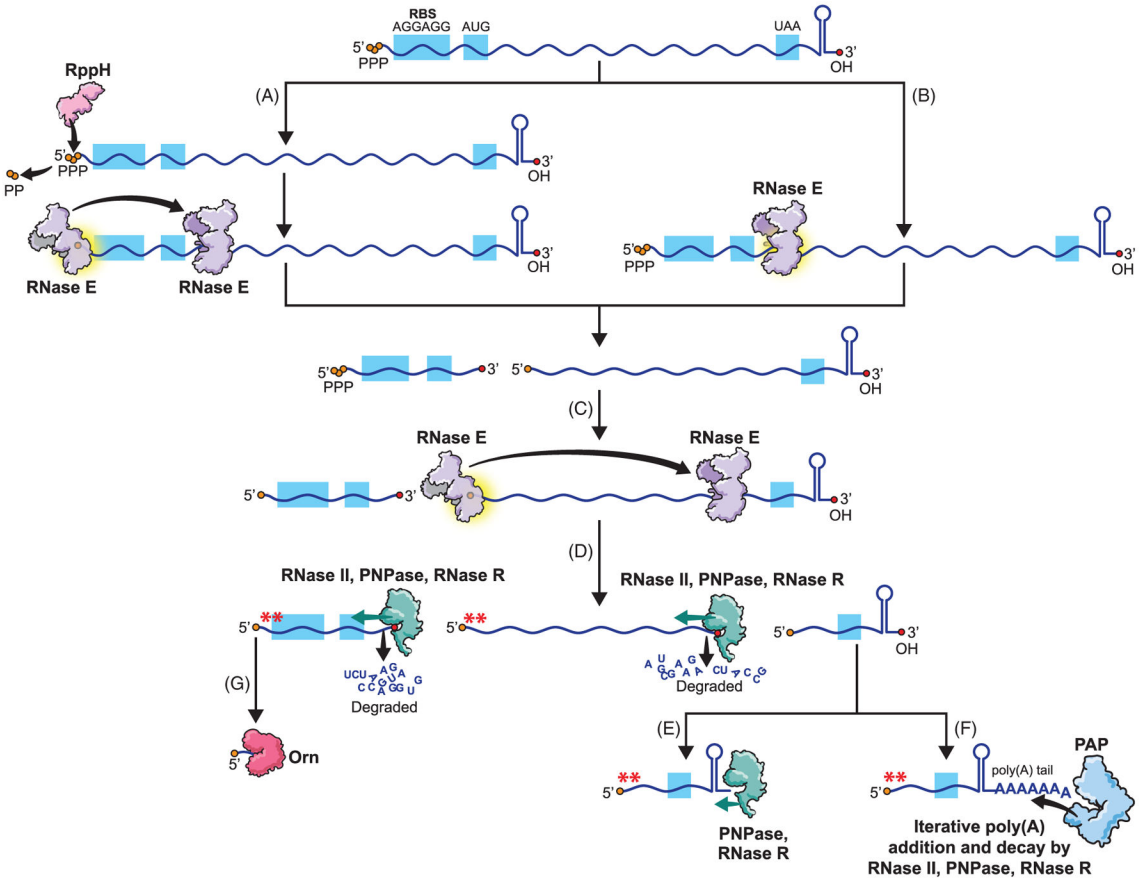

Figure 4.

Mechanism of *E. coli* mRNA decay. An mRNA is depicted as a wavy line, showing 5′ triphosphate and 3′ hydroxyl ends, ribosome binding site (RBS), start and stop codons, and the 3′-proximal transcription termination structure. Enzymes acting on the mRNA are stylized traces of structures from the Protein Data Bank. Initiation of mRNA decay in *E. coli* is primarily via endonuclease cleavage by RNase E. (A, B) RNase E can access its internal target either by first binding to a monophosphorylated 5′ end and then scanning in the 3′ direction (A) or directly (B). When RNase E activity occurs by the 5′-end-dependent pathway, efficient binding of RNase E to the 5′ end requires conversion of the 5′ nucleoside triphosphate to a nucleoside monophosphate by the action of RppH. RNase E cleavage in the body of the message generates an upstream fragment with a free 3′ hydroxyl end, and a downstream fragment with a 5′ monophosphate end. (C) Subsequent RNase E cleavage, enhanced by the monophosphate 5′ end of the downstream fragment, generates additional mRNA decay intermediates. (D) The upstream products of RNase E cleavage are susceptible to 3′-to-5′ exonucleolytic decay by RNase II, PNPase, or RNase R. (E) 3′-terminal fragments that contain the Rho-independent transcription terminator structure are degraded either by PNPase, likely in conjunction with an RNA helicase, or by RNase R, which has intrinsic helicase activity. (F) Alternatively, the 3′-terminal fragment can be made vulnerable to decay by the iterative action of poly(A) polymerase and 3′ exonucleolytic attack. (G) In all cases of 3′ exonuclease degradation, a limit oligonucleotide product of ≤5

nts remains. These are degraded by the oligoribonuclease, Orn. Nucleotides affected by Orn activity are indicated by the double asterisks.

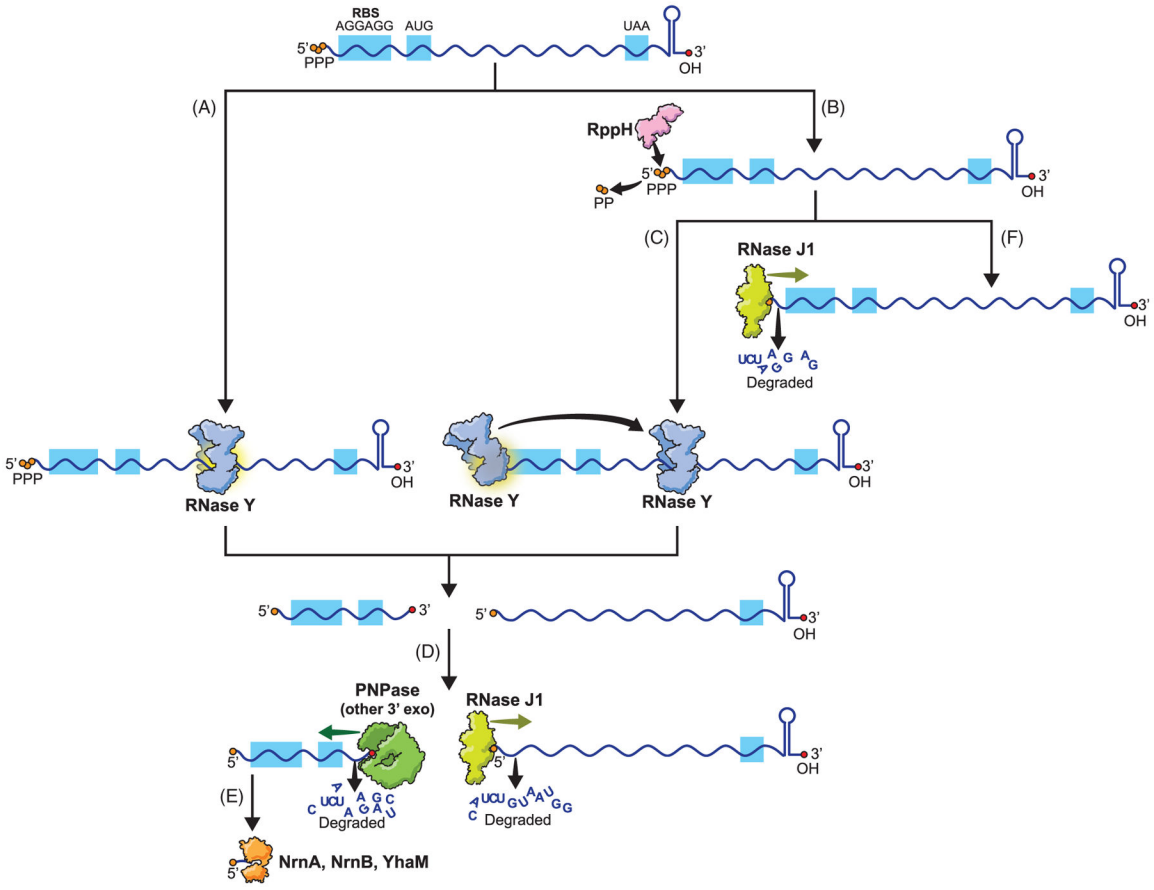

Figure 5.

Mechanism of *B. subtilis* mRNA decay. (A) *B. subtilis* endonuclease RNase Y is likely the functional homolog of *E. coli* RNase E. An internal cleavage in the body of an mRNA by RNase Y initiates decay in the same way as described for RNase E. It is thought that RNase Y can bind directly to an internal cleavage site. (B, C) In an alternative decay initiation pathway, the 5′ triphosphate end is converted to a monophosphate nucleotide by the action of RppH. RNase Y binds to the 5′ end and accesses an internal site. (D) A single RNase Y cleavage in the body of an mRNA can result in complete turnover of the mRNA via 3′ exonucleolytic decay of the upstream fragment (primarily by PNPase) and 5′ exonucleolytic decay of the downstream fragment by RNase J1. There is likely also another (yet unidentified) 3′ exonuclease that can degrade the upstream fragment. (E) The 3′ exonucleases leave an oligonucleotide limit product (≤5 nts), which is degraded to mononucleotides primarily by the oligoribonucleases, NrnA and NrnB. YhaM may also act as an oligoribonuclease. (F) RNase Y-independent pathway in which, subsequent to generation of a 5′ monophosphate end by RppH, RNase J1 degrades the entire mRNA in the 5′-to-3′ direction.

Table 1.

Properties of RNases discussed in the text.

E. coli RNases	Homolog in *B. subtilis*[a]	Gene name	Molecular mass (kDa)[b]	Subunit structure	Activity	Major function(s)	Regulation
RNase I	No	*rna*	27.2	Monomer	Endonuclease		periplasmic
RNase III	Yes (RNase III)	*rnc*	51.2	Homodimer	Double-strand specific endonuclease	rRNA maturation	autoregulation, phosphorylation, trans-acting protein
RNase P	Yes (RNase P)	*rnpA/rnpB*	13.8/377 nt	Protein/RNA dimer	Endonuclease	5′ tRNA maturation	
RNase BN	Yes (RNase Z)	*rbn*	65.8	Homodimer	Endonuclease/ exonuclease	sRNA decay	mRNA stabilization
RNase E	No	*rne*	472	Homotetramer	Endonuclease	mRNA decay, rRNA and tRNA maturation	autoregulation, phosphorylation, trans-acting protein
RNase G	No	*rng*	53.8	Dimer	Endonuclease	rRNA maturation	
RNase HI	No	*rnhA*	17.6	Monomer	Endonuclease on RNA primers and R-loops in DNA	DNA replication	
RNase HII	Yes (RNases HII and HIII)	*rnhB*	21.5	Monomer	Endonuclease on single RNA nt in DNA	DNA repication	
YbeY	Yes (YqfG)	*ybeY*	17.5		endonuclease	rRNA maturation and quality control	
RNase II	No	*rnb*	72.5	Monomer	3′ to 5′ exonuclease	mRNA decay, rRNA and tRNA maturation	acetylation
RNase R	Yes (RNase R)	*rnr*	92.1	Monomer	3′ to 5′ exonuclease	mRNA decay, rRNA and tRNA maturation	acetylation
PNPase	Yes (PNPase)	*pnp*	77.1	Trimer	3′ to 5′ exonuclease, phosphate-dependent	mRNA decay, rRNA and tRNA maturation	autoregulation, trans-acting protein and sRNA
RNase PH	Yes (RNase PH)	*rph*	51.0 for dimer	Dimer, oligomerizes	3′ to 5′ exonuclease, phosphate-dependent	tRNA and rRNA maturation, rRNA degradation	proteolysis
RNase D	No	*rnd*	42.7	Monomer	3′ to 5′ exonuclease	tRNA maturation	
RNase T	No	*rnt*	47	Dimer	3′ to 5′ exonuclease	tRNA and rRNA maturation	
Orn	No	*orn*	41.4	Dimer	3′ to 5′ exonuclease	removal of short oligos	
RNase Y	No	*rny*	117.5	Homodimer	endonuclease	mRNA decay	
RNase J1	No	*rnjA*	61.34	Heterodimer (with RNase J2)	5′-to-3′ exonuclease/ endonuclease	16S rRNA 5′ maturation, mRNA decay	
RNase J2	No	*rnjB*	56.67	Heterodimer (with RNase J1)	5′-to-3′ exonuclease (weak)/ endonudease		
RNase P	Yes (RNase P)	*rnpA/rnpB*	13.66/401 nts		Endonuclease	tRNA 5′ maturation	

E. coli RNases	Homolog in *B. subtilis*[a]	Gene name	Molecular mass (kDa)[b]	Subunit structure	Activity	Major function(s)	Regulation
RNase Z	Yes (RNase BN)	*rnz*	67.7	Homodimer	Endonuclease	tRNA 3′ maturation	
RNase M5	No	*rnmV*	20.53	Monomer	Endonuclease	5S rRNA maturation	
RNase III	Yes (RNase III)	*rnc*	56.6	Homodimer	Double-strand specific endonuclease	rRNA processing	autoregulation, mRNA stability
Mini-RNase III	No	*mrnC*	32.2	Homodimer	Endonuclease	23S rRNA maturation	
YqfG	Yes (YbeY)	*yqfG*	17.61		Endonuclease	16S rRNA 3′ maturation	
RNase PH	Yes (RNase PH)	*rph*	26.53	Homohexamer	3′-to-5′ exonuclease, phosphate-dependent	tRNA 3′ maturation	
PNPase	Yes (PNPase)	*pnpA*	154.6	Homotrimer	3′-to-5′ exonuclease, phosphate-dependent	mRNA decay	
RNase R	Yes (RNase R)	*rnr*	88.56	Monomer	3′-to-5′ exonuclease	rRNA quality control	
YhaM	No	*yhaM*	35.53		3′-to-5′ exonuclease	mRNA decay	
nanoRNase A	No	*nrnA*	34.93	Monomer	Exonuclease	nanoRNA decay	
nanoRNase B	No	*nrnB*	46.28		Exonuclease	nanoRNA decay	
RNase HII	Yes (RNase HII)	*rnhB*	28.20	Monomer	Endonuclease on RNA embedded in or hybridized to DNA	removal of ribonucleotides in DNA	
RNase HIII	Yes (RNase HIII)	*rnhC*	33.92	Monomer	Endonuclease on RNA embedded in or hybridized to DNA	removal of ribonucleotides in DNA	
RNase Bsn	No	*yurI*	31.94		Endonuclease	nonspecific	induced by phosphate starvation
YhcR	No	*yhcR*	132.47		Secreted endonuclease		
EndoA	Yes (MazF)	*ndoA*	12.48	Monomer	Endonuclease, RNA interferase	mRNA inactivation	
Rae1	No	*yncP*	19.53		A-site pndonudpase	mRNA inactivation	

[a] Homolog name in parentheses.

[b] Molecular mass of the monomer is given for RNases with no direct structural information.

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