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RNA maturation/degradation in *Mycobacterium smegmatis*: a study of the role of an RNase J ortholog and RNase E on messenger and ribosomal RNA processing

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Index

SUMMARY……...pag. 6

INTRODUCTION……pag. 8

1. The RNA decay in *E. coli*……..pag. 9

1.1 The endoribonucleases……..pag. 9

1.1.A RNase E……pag. 9

1.1.B. RNase G……...pag. 14

1.1.C. RNase III…….pag. 14

1.1.D. RNase P, RNase Z and RNase LS……...pag. 15

1.2. The exoribonucleases………..pag. 16

1.2.A. Polynucleotide Phosphorylase (PNPase)…….pag. 16

1.2.B. RNase II……pag. 17

1.2.C. RNase R……...pag. 18

1.2.D. Oligoribonuclease……pag. 20

1.3 The degradosome……...pag. 21

2. The RNA decay in *B. subtilis*…….pag. 24

2.1. Endoribonucleases……pag. 24

2.1.A. RNases J1/J2……...pag. 24

2.1.B RNase Y……...pag. 29

2.1.C Bs-RNase III……pag. 31

2.1.C RNase M5……pag. 32

2.1.D RNase P and RNase Z……pag. 32

2.2 Exoribonucleases……...pag. 33

2.2.A PNPase……....pag. 33

2.2.B RNase R, RNase PH and YhaM…….pag. 34

2.2.C NanoRNase…….pag. 35

2.3 The RNA degradosome in *B. subtilis*……..pag. 35

3. The RNA decay in Mycobacteria……pag. 36

4. Maturation of ribosomal RNA in bacteria…….pag. 37

4.1 16S rRNA……..pag. 39

4.2 23S rRNA……..pag. 43

4.3 5S rRNA……....pag. 45

5. The *furA-katG* region in Mycobacteria……pag . 48

5.1 Expression of the *furA-katG* region in mycobacteria……..pag. 49

6. Aim of the project……...pag. 51

RESULTS……....pag. 53

1. Maturation of the *furA-katG* transcript………...pag. 54

1.1 *In vitro* processing of the *furA-katG* transcript……..pag. 54

1.2 Construction of the *rne* and *rnj* conditional mutants……....pag. 56

1.2.A Identification of mycobacterial endoribonucleases……pag. 56

1.2.B Construction of conditional expression mutants in *M. smegmatis* RNAse E and RNAse J orthologous genes……..pag. 58

1.2.C Growth analysis of *M. smegmatis rne* and *rnj* conditional mutant strains……..pag. 60

1.2.D Construction of an RNase J null mutant in *M. smegmatis*……...pag. 61

1.2.E Construction of a conditional mutant in *M. tuberculosis rne* gene……..pag. 61

1.3 Analysis of the *furA-katG* transcript maturation in the *rne* and *rnj* mutant strains……..pag. 63

2. *M. smegmatis* RNase J ortholog characterization……pag. 65

2.1 *M. smegmatis* encodes a protein with high similarity to *B. subtilis* RNases J1/J2..pag. 65

2.2 The MSMEG_2685 protein has endoribonucleolytic activity……pag. 68

2.3 The mycobacterial RNase J has 5'-3' exoribonucleolytic activity dependent on the 5' phosphorylation state of the substrate……pag. 70

3. Ribosomal RNA maturation……...pag. 70

3.1 *M. smegmatis* rRNAs operons……..pag. 70

3.2 Pre-16S rRNA maturation……pag. 71

3.3 Pre-23S rRNA maturation……pag. 75

3.4 Pre-5S subunit maturation……pag. 76

DISCUSSION.....pag. 78

4. Discussion.....pag. 79

4.1 In vitro M. smegmatis furA-katG transcript processing……pag. 79

4.2 Mycobacterial mutants in ribonuclease E and J…….pag. 80

4.3 In vitro M. smegmatis furA-katG transcript processing……pag. 80

4.4 In vitro activity of M. smegmatis RNAse J……....pag. 81

4.5 Maturation of the 5' end of rRNA in *M. smegmatis.*……..pag. 83

MATERIAL AND METHODS……pag. 87

5.1 Bacterial strains and growth conditions…….....pag. 88

5.2 Plasmids……..pag. 89

5.3 Preparation of crude extracts……pag. 90

5.4 Crude extract processing assay……pag. 90

5.5 In vitro transcription……..pag. 90

5.6 RNase J overexpression and purification…….....pag. 91

5.7 RNase J processing assays……....pag. 92

5.8 Site-direct mutagenesis…….....pag. 93

5.9 Total RNA isolation……...pag. 93

5.10 Primer extension…….pag. 93

5.11 Bioinformatic tools……..pag. 94

5.12 Oligos used in this work……..pag. 95

Bibliography……...pag. 96

Annexes……..pag. 107

Summary

Escherichia coli and *Bacillus subtilis* have very different sets of ribonucleases, in particular the presence of RNase E and RNase J, respectively, that have been used to explain significant differences in RNA metabolism between these the two model organisms. However, these studies might have somewhat polarized our view of RNA metabolism, while recent works outline models of RNA degradation that are more similar than has been thought. In fact, the recent characterization of RNase Y as the scaffold for the degradosome assembly in *B. subtilis* lead to the consideration that RNA degradation in *B. subtilis* might begin through an endonucleolitycal cleavage, followed by exonucleolytical degradation.

In this work, we have identified a functional RNase J in *Mycobacterium smegmatis* and characterized its *in vitro* 5'-3' exo- and endonucleolytic activities. Furthermore, we constructed two mutants in *M. smegmatis rnj*: a conditional and a knock out mutant, thus demonstrating that in *M. smegmatis* the gene is not essential, contrary to the RNase J1 function in *B. subtilis*.

In *M. smegmatis* RNase J co-exists with RNase E, a configuration that enabled us to study how these two key nucleases collaborate. A conditional mutant in the *rne* gene was constructed, demonstrating that this function is essential for *M. smegmatis*, as it is in *E. coli*. Moreover, a conditional mutant in *Mycobacterium tuberculosis*, confirmed its essentiality also in this organism.

We studied the respective roles of the *M. smegmatis* RNase J and RNAse E ribonucleases in the 5' end maturation of the *katG* transcript, previously demonstrated to derive from an endoribonucleolytic processing. Here we find that RNase E is responsible of the specific cleavage of the 5' *katG* end.

Further, we show that RNase E and RNase J are involved in the 5' end processing of all three ribosomal RNAs. Thus the maturation pathways of rRNAs in *M. smegmatis* are quite different

from those observed in both *E. coli* and *B. subtilis*. Studying organisms containing different combinations of key ribonucleases can thus significantly broaden our view of the strategies directing RNA metabolism used by various organisms.

INTRODUCTION

The RNA decay plays an important role in the nucleotide turnover and in the regulation of the intracellular levels of RNA species in the different growth phases or conditions. Normally, the mRNAs are rapidly degraded to continuously adjust the message population to the needs of the cell for specific proteins, while the stable RNAs, as tRNA and rRNA, are degraded only under certain stress conditions or when an RNA molecule is defective. In considering the mRNA decay, we have to distinguish between the factors that determine the initiation of the degradative process and those implicated in the final degradation to lead to the free nucleotides. Finally, the RNA stability is strictly related to the level of maturation and degradation, consequently also the level of protein expression in the cells is controlled by this system of maturation/degradation (Deutscher, 2006). RNA decay is carried out by two general types of ribonucleases (RNases): endoriboncleases, which cleave at internal sites in a RNA molecule, and exoribonucleases, which degrade a RNA molecule one nucleotide at a time from the ends. Extensive studies on the bacterial RNases were carried out in *E. coli* and *B. subtilis*, belonging respectively to the gram-negative and gram-positive bacteria. A comparison of the genomes of the two species has shown that the pattern of RNases is quite different between the gram-negative and gram-positive organisms, suggesting that they have evolved different systems of RNA decay(Condon and Putzer, 2002).

1. The RNA decay in *E. coli*

In this part of the introduction we will discuss on the principal RNases involved in the RNA metabolism in *E. coli*.

1.1 The endoribonucleases

1.1.A RNase E

The RNase E has been discovered as the enzyme involved in the 9S rRNA processing to mature 5S rRNA (Apirion and Lassar, 1978). The RNase E is also implicated in the maturation of the

16S rRNA (li, 1999A). It is equally implicated in the degradation of the mRNAs of the bacteriophage T4 (Mudd et al., 1990; Mudd et al., 1988). All studies from the last two decades confirm that the degradation of mRNA in *E. coli* begins with an endonucleolytic cleavage by the RNase E(Kushner, 2002; Regnier and Arraiano, 2000). This endoribonuclease is essential for *E. coli* and no other gene product seems able to compensate its function in the cell (Jain et al., 2002). The RNase E, coded by the *rne* gene, is composed of 1061 amino acids devised in two functional domains: the N- and C-terminal domains. The N-terminal domain (amino acids 1-498) encompasses the endoribunucleolytic activity and it is sufficient for RNase E-mediated cleavage of a short single-stranded RNA(McDowall and Cohen, 1996). It shares a high degree of sequence similarity with its paralog, RNase G (about 50% similarity), and is well conserved among the many homologs identified in bacteria.

The C-terminal domain is much more divergent among the many homologs **(Fig.1) (**Marcaida et al., 2006), it bears an Arginin-rich region that is important to bind the RNA target, moreover it constitutes the scaffold to assemble the different components of the degradosome, a multyenzime complex that plays an important role in the *E. coli* RNA decay of which we will discuss later. Further, the C-terminal domain is necessary for binding the RNase E to the inner cytoplasmic membrane (Khemici et al., 2008).

Comparing the regions processed by the RNase E, it doesn't exist a specific consensus sequence recognized by this endoribonuclease. Normally, it is a single-strand-specific endoribonuclease with a preference for AU-rich sequences (Lin-Chao et al., 1994; McDowall et al., 1994). The efficiency of the cleavage is dependent on the 5'end state of the substrate: the RNase E processes the 5' monophosphorylated substrates more efficiently than those carring a hydroxyl or triphosphate group (Jiang and Belasco, 2004; Mackie, 1998).

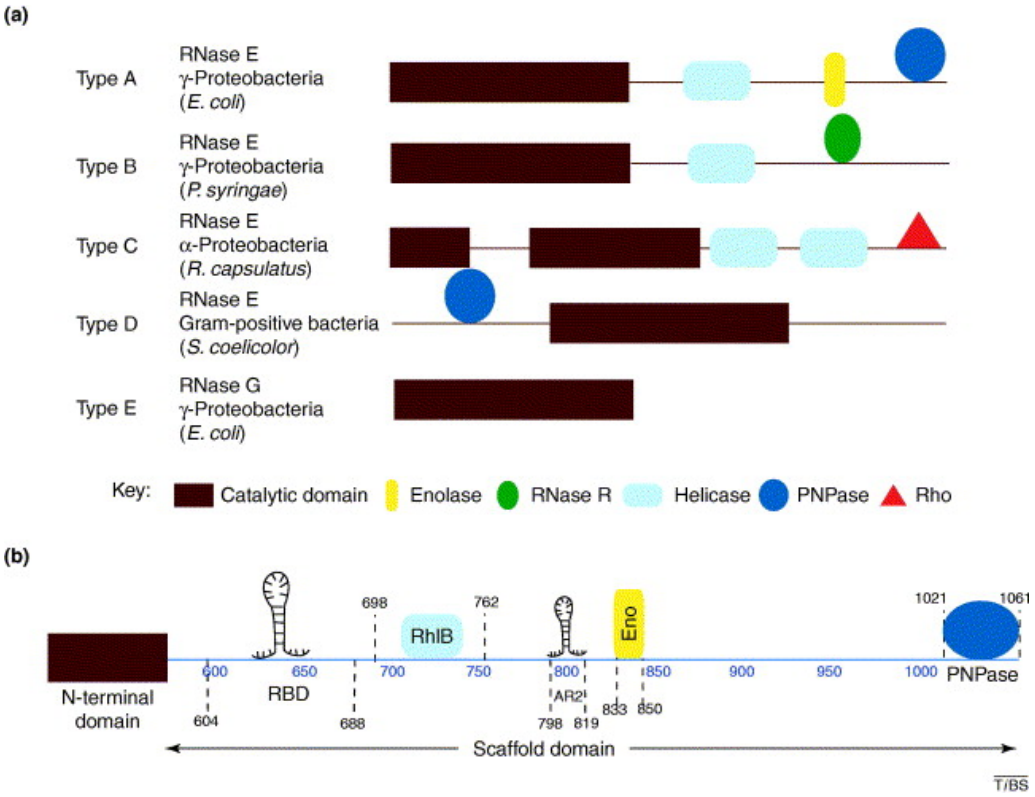


Figure 1. RNase E, the *E. coli* RNA degradosome and putative bacterial degradosomes. (a) Primary structure of RNase E (type A) and its shorter paralogue RNase G (type E). The C-terminal half of *E. coli* RNase E forms the scaffold for the degradosome and has binding regions for RNA, helicase, enolase and PNPase. RNase E homologues from other bacteria, which we classify as groups A–D according to the extra peptide segments attached to the RNase G-like catalytic core, are also indicated. **(b) Summary of the protein–protein and protein–RNA recognition microdomains of *E. coli* RNase E**. Near the recognition sites for helicase (Rhlb) and enolase (Eno) are two arginine-rich regions that might be independently involved in RNA binding: the RNA-binding domain (RBD) and arginine-rich region 2 (AR2). We define 'microdomains' as the short segments on the scaffold domain of RNase E that are required for protein–protein and protein–RNA interaction (Marcaida et al., 2006).

Recently, a pyrophosphorylase, RppH, was identified in *E. coli* belonging the NUDIX hydrolase family. This enzyme removes the pyrophosphate group from the 5' end of the substrate leaving a

monophosphorylated 5'end that becomes, consequently, a good substrate for the RNase E-mediated degradation **(Fig.2)** (Celesnik et al., 2007; Deana et al., 2008**)**. Further, the presence of stable secondary structures near the 5' end reduces the efficiency with which Rpph converts a triphosphate to a monophosphate group and sterically blocks the interaction with the 5' monophosphate binding pocket of RNase E (Arnold et al., 1998; Bouvet and Belasco, 1992; Chen et al., 1991).

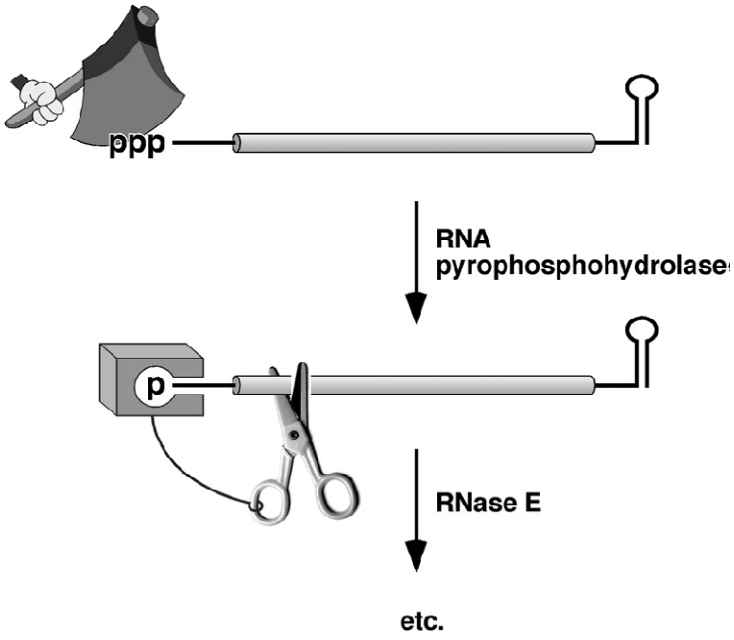


Figure 2. Mechanism of the 5' end-dependent pathway for RNA degradation in *E. coli.* An RNA pyrophosphohydrolase (hatchet) removes the γ and β phosphates at the 5' terminus of a triphosphorylated primary transcript, either simultaneously (as shown) or sequentially. The resulting monophosphorylated decay intermediate is then rapidly cleaved by RNase E (scissors linked to a 5'-sensor domain), an endonuclease with a marked preference for RNA substrates bearing a single phosphate group at the 5' end (Celesnik et al., 2007).

The RNA molecule is bound in a pocket within the RNase E catalytic domain and its 5' monophosphate group is engaged in a semicircular ring of hydrogen bonding interactions. This explains why a 5'-OH group could not form this interaction network. Further, a triphosphate group or harpin near the 5' end may be sterically occluded from the site.

The crystallographic resolution of RNase E structure gave us more insights on the influence of a 5' monophosphate substrate on the activity of the enzyme (Koslover et al., 2008). The hypothesis is that the RNase E is in a free open conformation without the RNA substrate (**Fig. 3**). When a 5' monophosphate substrate bind RNase E, the enzyme switches in a closed conformation that holds the RNA and carries it near the active site for the cleavage (Carpousis et al., 2009).

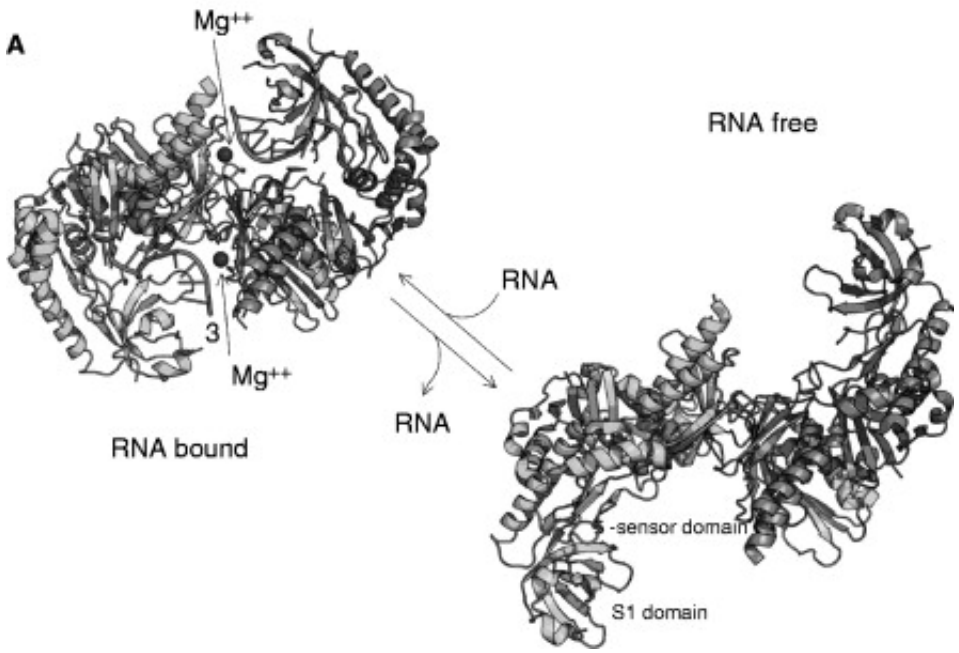


Fig. 3. Structural changes associated with RNA binding. A view of the RNA bound form along the twofold axis of the principle dimer in the upper left panel, and the same view of the apo-form in the lower right panel (Carpousis et al., 2009).

1.1.B RNase G

The RNase G, coded by the *rng* gene, shares with the RNase E the 5'end maturation of the 16S rRNA (Li et al., 1999b). This endoribonuclease has a high homology with the N-terminal domain of the RNase E corresponding to the active site of the protein. Contrary to the RNase E, the RNase G is not essential, although an overproduction of the N-terminal tagged protein can restore viability to cells defective in RNase E (Lee et al., 2002; Ow et al., 2003). The RNase G shares with the RNase E many characteristics important to recognize and process the RNAs as: single stranded, AU rich regions and RNAs with a monophosphate at its 5'end (Jiang et al., 2000; Tock et al., 2000). Moreover, the RNase G is much more sensitive to the 5'end monophosphorylated substrates than the RNase E (Jiang and Belasco, 2004; Tock et al., 2000) and completely unable to process the 5' triphosphorylated substrates. Thus, it has been suggested that RNase G acts primarly on intermediates generated by other endoribonucleases (Ow et al., 2003).

1.1.C RNase III

The *E. coli* endoribonuclease III, coded by the *rnc* gene, represents a family of double-stranded RNA (dsRNA) endonucleases. This enzime was found in all studied procariotic and eucariotic organisms. The ribonuclease III plays a primary role in the maturation of 16S and 23S rRNA from the 30S primary transcripts (Hofmann and Miller, 1977), however in strains *rnc*$^-$, the rRNA is still matured indicating that there is an RNase III-indipendent mean of processing 30S RNA, although it is less efficient.

The bacterial RNase III enzime contains an endonuclease domain (endoND) and a dsRNA binding domain (dsRBD) (Blaszczyk et al., 2004); its activity is not dependent by a specific sequence, it cleaves double-stranded RNAs. The RNase III cleavage generates upstream and downstream products with 5' monophosphates and 3' hydroxyl groups respectively so it has the

potential to stimulate cleavage by RNase E and G and to provide a new end for 3' exonucleolytic attack.

Further, the RNase III regulates its own production by cleaving its *rnc* message within the 5' UTR (Bardwell et al., 1989).

1.1.D RNase P, RNase Z and RNase LS

RNase P and RNase Z were prior identified as implicated in tRNA maturation (Pellegrini et al., 2003; Schedl et al., 1976), further studies have also demonstrated a direct role of these two endoribonucleases in mRNA degradation in *E. coli*.

The RNase P matures the 5' end of the tRNA and it is also involved in the maturation of the 4.5S RNA (Bothwell et al., 1976). These endoribonuclease cut within intergenic regions of several polycistronic RNAs as *tna*, *secG*, *rbs* and *his* (Li and Altman, 2003) and there is evidence that cleavage by RNase E may precede cleavage by RNase P. Further, it has been shown that RNase P cleavage depends on the presence of ribosomes downstream of the cleavage site probably because they promote the formation of structures sensitive to the cleavage by the RNase P (Alifano et al., 1994).

The RNase Z was previously characterized as a phosphodiesterase zinc-dependent and its gene, not essential, named *elaC* (Vogel et al., 2002). This protein belongs to the ELAC1/2 family, present either in the eukaryotic organisms than in the Archeabacteria. It was shown that the ElaC protein of *E. coli* processes the tRNA precursors lacking a CCA determinant, both *in vivo* and *in vitro*, so the enzyme was renamed RNase Z (Perwez and Kushner, 2006). In *E. coli* all the tRNA precursors have the CCA determinant on the 3' side (Blattner et al., 1997) so the activity of RNase Z is not necessary for tRNA precursors maturation. Finally, the role of the RNase Z in *E. coli* is principally bound to the mRNA degradation, in fact, in strains *rnz*$^{-}$, there is at least a two-fold stabilization of more than 150 mRNA (Perwez and Kushner, 2006).

The RNase LS is the most recent endoribonuclease found to be implicated in mRNA degradation. This enzyme was initially described as the activity that induce *L*ate-gene *S*ilencing in bacteriophage T4, hence its LS designation (Kai et al., 1996). The gene coding for the RNase LS is still not known, but it was demonstrated that the gene *rnlA* is necessary for its activity (Otsuka et al., 2007).

1.2. The exoribonucleases

1.2.A Polynucleotide Phosphorylase (PNPase)

The PNPase protein belongs to the PDX family; it is widely conserved from bacteria to plants and metazoans (Zuo and Deutscher, 2001), but it is absent in the yeast and the Archea (Sarkar and Fisher, 2006). PNPase, involved in global mRNA decay in *E. coli*, is encoded by the *pnp* gene, located downstream the *rpsO* gene, its expression is regulated from two promoters: one upstream of the *pnp* gene and the other upstream the *rpsO* gene (Portier and Regnier, 1984). The *pnp* expression is negatively auto-regulated at the posttranscriptional level by the action of both PNPase and RNase III (Robert-Le Meur and Portier, 1992) with the RNase III cleaving at the 5' end in presence of a stable secondary structure. In an RNase III deficient strain, there is an increase of *pnp* message amount, thus the absence of RNase III cleavage impedes the autogenous control of the *pnp* mRNA (Portier et al., 1987; Robert-Le Meur and Portier, 1992).

PNPase is a cold-shock protein essential for growth at low temperature (Zangrossi et al., 2000).

PNPase is a processive phosphorolytic exoribonuclease that degrades the RNA in 3'-5' direction releasing nucleoside diphosphates from the 3' end (Sarkar and Fisher, 2006). It has been shown that, at low concentration of inorganic phosphate, the PNPase can catalyze the reverse reaction that is the polymerization of an RNA single-stranded tail from nucleoside diphosphates (Godefroy, 1970; Mohanty and Kushner, 2000). Both activities are involved in the RNA

degradation since the poly(A) or heteropolymeric tails are signals stimulating the RNA degradation.

Recent work demonstrated that ATP binds to PNPase and allosterically inhibits both its phosphorolytic and polymerization activities, suggesting that PNPase-dependent RNA tailing and degradation occur mainly at low ATP concentrations (Del Favero et al., 2008).

The PNPase in the cells forms a homotrimer of 78 kDa subunits (Portier, 1975). Each subunit comprises five domains: two homologous RNase PH domains associated to form the structural core, an α-helical domain that connects the two core domains, and two C-terminal RNA-binding domains, KH and S1. The role of the last two domains in the degradosome is to bind and stabilize single-stranded RNA released by RhlB (Matus-Ortega et al., 2007; Regonesi et al., 2004; Stickney et al., 2005). The three subunits associate via trimerization interfaces of the core domains, forming a central channel where is located the catalytic activity. The PNPase can operate alone or assembled into multienzyme complexes such as the degradosome where it interacts with the RNase E, the major component of the degradosome that provides the scaffold for the entire structure (Carpousis et al., 1994; Miczak et al., 1996).

1.2.B RNase II

E. coli RNase II belongs to the widespread RNase II family (Zuo and Deutscher, 2001). This enzyme, coded by the *rnb* gene, is not essential for viability (Piedade et al., 1995; Zilhao et al., 1995a). RNase II expression is regulated at posttranscriptional level by the PNPase (Zilhao et al., 1996) and RNase E and indirectly by RNase III (Zilhao et al., 1995b), and is the only exoribonuclease to be regulated at posttranslational level by the action of the Gmr protein (Cairrao et al., 2001).

RNase II is a 3'-exoribonuclease that cleaves hydrolytically single-stranded RNAs with a poly(A) tail. It participates in the terminal stage of mRNA degradation and is responsible for

90% of the degradation of poly(A) RNA *in E. coli* crude extracts (Deutscher and Reuven, 1991). RNase II degrades processively in direction 3'-5' the longer RNAs releasing 5'-nucleoside monophosphates. When the substrate is shorter than about 12 nucleotides the exoribonucleolytic activity becomes distributive (Cannistraro and Kennell, 1994). This exoribonuclease degrades preferentially the homopolymer poly(A) that normally serves as signal for the degradative action of other nucleases, thus the RNase II can act as a protector of some RNAs from degradation by impairing the access of other exoribonucleases (Coburn and Mackie, 1996; Folichon et al., 2005; Hajnsdorf et al., 1994; Marujo et al., 2000; Mohanty and Kushner, 2002; Pepe et al., 1994).

The N-terminal region of the RNase II has a sequence similar to the cold shock domain (CSD). This seems to be implicated in the binding to single-stranded nucleic acids (Graumann and Marahiel, 1998). The C-terminal region contains an S1-domain important in the binding to RNAs (Bycroft et al., 1997). The S1 domain is more important than the CSD domain to determine the binding to the RNA (Amblar et al., 2006). The central domain of RNase II (RNB) contains the catalityc activity of RNase II implicated in the degradation of the RNA (Amblar et al., 2006).

1.2.C RNase R

RNase R was initially identified as an enzyme merely responsible for residual hydrolytic activity in a mutant for RNase II (Kasai et al., 1977). Only later the protein product of the gene previously called *vac*B, was identified as exoribonuclease R, a 92 kDa protein, and its gene named *rnr* (Cairrao et al., 2003; Cheng et al., 1998). RNase R belongs to the RNase II family of exoribonucleases (Grossman and van Hoof, 2006). The *rnr* gene is second in an operon together with *nsr*R (a transcriptional regulator), *rlm*B (rRNA methyltransferase), and *yjf*I (unknown function).

Like PNPase, RNase R is upregulated in cold shock and this was shown to be the consequence of a significant stabilization of the *rnr* transcripts at low temperatures (Cairrao et al., 2003). Further, RNase R is regulated at the posttranscriptional level by RNase E, although RNase G may also participate to the *rnr* transcript processing (Cairrao and Arraiano, 2006).

RNase R and RNase R RNase II double mutants are viable but a RNase R PNPase double mutant is lethal (Cairrao et al., 2003; Cheng et al., 1998). *E. coli* RNase R was shown to be important for growth at low temperatures and RNase R-deficient colonies are smaller especially in the cold (Cairrao et al., 2003).

RNase R is a processive, sequence nonspecific, 3′–5′ exoribonuclease that acts through a hydrolytic mechanism releasing nucleoside monophosphates. RNase R is able to degrade a duplex RNA provided there is a single-stranded 3′ overhang of more than 7-nucleotides in length (Cheng and Deutscher, 2002; Vincent and Deutscher, 2006). Unlike RNase II or PNPase, RNase R tightly binds the RNA and does not detach when approaching a double-stranded RNA region, proceeding very efficiently through even extensive secondary structures (Cheng and Deutscher, 2005; Vincent and Deutscher, 2006).

RNase R is growth-phase regulated and its levels increase in the stationary phase of growth (Andrade et al., 2006). It was demonstrated that RNase R can be a modulator of gene expression in stationary-phase cells(Andrade et al., 2006). Moreover, *E. coli* RNase R seems to be a general stress-induced protein whose levels are not only upregulated in stationary phase (2-fold), but also in response to heat shock (2-fold) and cold shock (7–8-fold) (Andrade et al., 2006; Andrade et al., 2009; Cairrao et al., 2003). RNase R seems thus to have a central role in the cellular adaptation to new stressful conditions. Finally, RNase R has been implicated in the establishment of virulence in a growing number of pathogens including *Shigella flexneri*, enteroinvasive *E. coli*, and *Aeromonas hydrophila* (Erova et al., 2008; Tobe et al., 1992). In fact, RNase R-deficient bacteria have been shown to be less virulent than the wild-type parental strains.

1.2.D Oligoribonuclease

The end products resulting from the degradation of PNPase, RNase II, and RNase R constitute a serious problem for cell viability, since these enzymes release RNA fragments 2–5 nucleotides in length whose accumulation may be deleterious (Ghosh and Deutscher, 1999).

Oligoribonuclease was described for the first time, as an enzyme able to hydrolyze short oligoribonucleotide chains (Niyogi and Datta, 1975; Stevens and Niyogi, 1967). The oligoribonuclease coding gene, *orn*, is essential in *E. coli* (Ghosh and Deutscher, 1999). It represents the only exoribonuclease required for cell viability in *E. coli* (Ghosh and Deutscher, 1999). It has been reported to copurify with PNPase (Yu and Deutscher, 1995).

Oligoribonuclease belongs to the DEDD family of exoribonucleases and contains a well-conserved ExoIII domain (Zuo and Deutscher, 2001). This enzyme processively hydrolyzes, in the 3′–5′ direction, short oligoribonucleotides to produce mononucleotides (Niyogi and Datta, 1975). Oligoribonuclease has a higher affinity for a 5-mer oligoribonucleotides than smaller substrates. However, the hydrolysis reaction rate also decreases with the increasing chain length (Datta and Niyogi, 1975).

Oligoribonuclease has close homologues in other organisms (Zhang et al., 1998) such as the human protein Sfn, which exhibits a 3′–5′ exoribonuclease activity on small single-stranded RNA and DNA oligomers (Nguyen et al., 2000), and the XC847 protein from the plant pathogen *Xanthomonas campestris* (Wu et al., 2005). Oligoribonucleases are inhibited by the nucleotide 3′-phosphoadenosine-5′-phosphate (pAp) that is generated in both prokaryotes and eukaryotes during the process of sulfur assimilation (Mechold et al., 2006).

1.3 The degradosome

The RNA degradosome is a large multiprotein complex discovered during the purification of *E. coli* RNase E (Carpousis et al., 2001; Carpousis et al., 1994; Ehretsmann et al., 1992; Py et al., 1994). The major components of the RNA degradosome include RNase E, PNPase and the DEAD-box RNA helicase B (RhlB) (Miczak et al., 1996; Py et al., 1996).

The DEAD-box RNA helicases are a ubi-quitous family of enzymes (Linder, 2006; Tanner and Linder, 2001). In vitro, these proteins often have RNA-dependent ATPase activity and ATPase-dependent RNA unwinding activity. However, these helicases generally unwind only a few base pairs of duplex RNA. The presence of RhlB bound to the degradosome facilitates the degradation of highly structured RNA from its 3' end by PNPase (Py et al., 1996). Moreover, RhlB can bind directly PNPase to form a "mini-degradosome" (Liou et al., 2002).

The complex also contains enolase, a glycolytic enzyme, as an integral component. Enolase is an abundant protein in *E. coli* and only a small proportion is bound to RNase E as a component of the RNA degradosome (Py et al., 1996). Enolase and RNase E are involved in the response to phosphosugar stress (Morita et al., 2004), so it might serve as a sensor linking the energetic state of the cell to mRNA degradation.

Associated proteins, present in substoichiometric amounts, include polyphosphate kinase (PPK), DnaK and GroEL. Interactions with other enzymes, such as *E. coli* poly(A) polymerase and the ribosomal protein S1, have also been described (Feng et al., 2001; Raynal and Carpousis, 1999). The role of enolase, PPK and other associated proteins in the degradation of mRNA remains to be clarified.

A 'minimal' degradosome containing RNase E, RhlB and PNPase can be reconstituted *in vitro* from purified components (Coburn et al., 1999; Mackie et al., 2001).

Three noncatalytic regions extend from the tetrameric catalytic core of RNase E (**Fig.4**) (Marcaida et al., 2006). Each noncatalytic region associates with a monomer of RhlB, a dimer of enolase, and a trimer of PNPase. The catalytic site of RNase E appears to be at the center of the RNA degradosome.

There is evidence that the RNA degradosome is modified during cold shock (Prud'homme-Genereux et al., 2004). The DEAD-box RNA helicase, CsdA, which is a cold shock protein, associates with the RNA degradosome at low temperature. CsdA reconstituted into the RNA degradosome can replace the function of RhlB in vitro.

Strains of *E. coli* expressing truncated forms of RNase E, in which part or all of the noncatalytic region have been deleted, are viable (Kido et al., 1996; Vanzo et al., 1998). Strains in which the genes encoding RhlB, enolase, and PNPase have been disrupted are also viable, although growth of a strain lacking enolase requires special feeding conditions. In an *rne131* strain, lacking the noncatalytic region, mRNA degradation is slower (Lopez et al., 1999). In fact, the effect of *rne131* is comparable to the effect of a temperature-sensitive *rne* allele at the nonpermissive temperature. Recent work suggests, however that the assembly of the RNA degradosome is necessary for normal mRNA decay (Bernstein et al., 2002; Bernstein et al., 2004). That is, in a strain with the *rne131* allele as well as strains in which the genes encoding RhlB, enolase, or PNPase have been inactivated, the levels of many mRNAs are significantly perturbed.

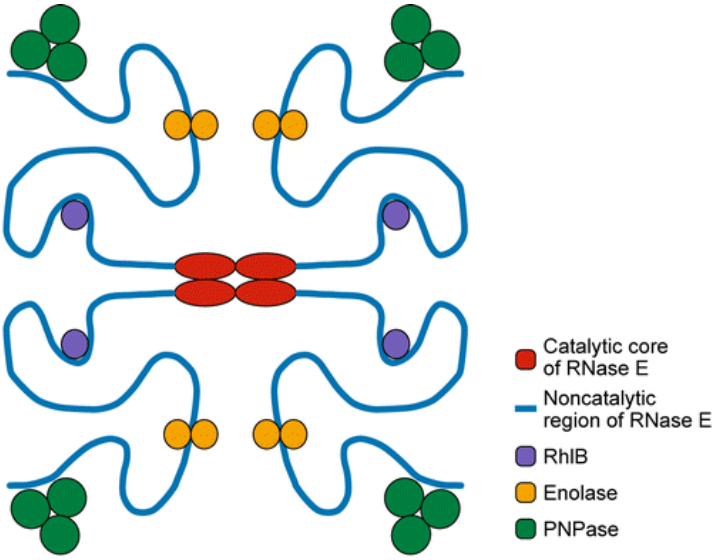


Fig.4 A model of the structure of the RNA degradosome. The components of the RNA degradosome are color coded. The noncatalytic region extending from the catalytic core of RNase E has been drawn symmetrically. However, the natively unstructured protein in each noncatalytic extension is likely to form a dynamic, random coil–like structure, and each extension is expected to act independently of the other noncatalytic extensions (Carpousis, 2007).

2. The RNA decay in *B. subtilis*

2.1 Endoribonucleases

2.1.A RNases J1/J2

Identification. RNase J is widespread among Bacteria and the Archaea. Although it is found in the Proteobacteria (particularly the α-, δ- and ε-subdivisions) and in the Cyanobacteria, it is most highly concentrated in the Gram-positive, Firmicutes and Actinobacteria (Even et al., 2005). Indeed multiple paralogues are often found in the Firmicutes, with four paralagous genes present in *Bacillus thuringiensis*.

RNases J1/J2 were characterized for the first time as two enzymes involved in the maturation of the leader region of *B. subtilis thrS* mRNA (encoding threonyl tRNA synthetase). RNase J1/J2, encoded respectively by the *rnjA* (formerly *ykqC)*and the *rnjB* (formerly *ymfA*) genes, copurified from a ribosome-associated fraction (Even et al., 2005). Both enzymes are around 61 kDa and are 49% identical. RNase J1 is essential, while RNase J2 is not. *In vitro* results obtained with purified RNase J1 have, for the most part, been obtained also with purified RNase J2 confirming an overlapping activity. A strain in which RNase J1 is expressed conditionally (IPTG controlled) and RNase J2 is deleted, showed an increase in global mRNA half-life (from 2.6 to 3.6 min) when RNase J1 expression levels are decreased by removing IPTG (Even et al., 2005). Deletion of the RNase J2 gene alone had no effect on mRNA half-life. While the difference in global mRNA half-life under full vs. limited RNase J1 expression was relatively small.

RNase J1 endonuclease cleavage at site 2 in the *thrS* mRNA leader resulted in a downstream RNA product that is stabilized, relative to the full-length *thrS* mRNA (Condon et al., 1996). As the processing site is just upstream of a predicted stem-loop structure (**Fig. 5**), the downstream RNA product of RNase J1 cleavage is likely to be protected from further decay by virtue of this structure. RNase J1 would then function in gene regulation not only negatively as an initiator of

mRNA decay, but also positively as an mRNA processing enzyme that creates a stable, translatable product.

Fig. 5 Schematic of *thrS* mRNA, showing leader region and coding sequence. Sites 1 and 2 are the mapped 5′ ends resulting from RNase J1 processing. Site 1 is now known to be the result of exonucleolytic decay from the initial 5′ end, while site 2 is the result of endonucleolytic cleavage (Li de la Sierra-Gallay et al., 2008).

It is evident that the site 1 "A-G box" cleavage (**Fig. 5**) was not an endonuclease cleavage but the result of a block to 5′ exonuclease processivity (Li de la Sierra-Gallay et al., 2008). In fact, it has been demonstrated that this "cleavage" is sensitive to the phosphorylation state of the *thrS* mRNA 5′ end. Processing at the A-G box was much more efficient when the 5′ end of the substrate was monophosphorylated than when it was triphosphorylated.

The effect of the 5′ phosphorylation state on RNase J1 activity is thought to mirror the effect on *E. coli* RNase E, which has a strong preference for a monophosphorylated 5′ end over a triphosphorylated 5′ end (Jiang and Belasco, 2004; Mackie, 1998), but the consequences are different: the effect of the 5′ phosphorylation state is on the *endonuclease* activity of RNase E but on the *exonuclease* activity of RNase J1.

It is found that the initial rate of RNase J1 exonuclease activity is three times faster on a substrate bearing a 5′ monophosphate end, or a 5′ hydroxyl end, than on the same substrate with a 5′ triphosphate end (Mathy et al., 2007). Moreover, RNase J1 requires a single-stranded 5′ end to allow 5′-to-3′ exonuclease activity. This observation comes from an experiment in which a complementary oligonucleotide was hybridized to the 5′ end of a uniformly labeled substrate (Mathy et al., 2007). However, these experiments could greatly underestimate the effect of a 5′-triphosphate end on exonuclease activity, since release of labeled monophosphate nucleoside by exonuclease activity could occur following endonuclease cleavage of the RNA substrate, which would not reflect degradation starting at the 5′ terminus. Finally, RNase J1 can cleave a circular RNA that contains a known cleavage site, suggesting that access to internal cleavage sites on mRNA is not 5′-end-dependent.

Sites of *in vivo* RNase J1 endonuclease cleavage have been mapped in five cases, and these are found in mRNA leader regions and in a stable RNA. They include: *infC* leader RNA (Choonee et al., 2007), *thrS* and *thrZ* leader RNAs (Even et al., 2005), *trp* leader RNA (Deikus et al., 2008), and small cytoplasmic RNA or scRNA (Yao et al., 2007). The cleavages occur in single-stranded AU-rich regions that are next to a downstream secondary structure.

Structure of RNase J. The sequence of RNase J1 reveals that it is a member of the β-CASP subfamily of zinc-dependent metallo-β-lactamases ("β-CASP" is named after other members of this subfamily: metallo-β-lactamase CPSF, Artemis, SnmI, Pso2). Structural information comes from work on the single RNase J enzyme of *Thermus thermophilus*, which is 61% similar to RNase J1 of *B. subtilis* and which cleaves *thrS* mRNA at the same site as RNase J1 (Li de la Sierra-Gallay et al., 2008). RNase J consists of three domains (**Fig. 6A**): an N-terminal β-lactamase domain, a central β-CASP domain, and a C-terminal domain that is required for activity, possibly through dimerization. Deletion of the C-terminal domain severely affects activity, but it is not known whether this is due to lack of dimerization or due to an intrinsic role

of the C-terminal domain in catalysis. Structural determination of RNase J in the presence of UMP revealed a binding site for the nucleotide that is immediately adjacent to the predicted catalytic site (at which a pair of Zn^{2+} ions are coordinated), providing an explanation for the 5′-to-3′ exonuclease activity (**Fig. 6B**). Furthermore, the architecture at this site does not seem to accommodate a 5′ triphosphate nucleoside, immediately suggesting an explanation for the ability of RNase J1 to discriminate between 5′ monophosphate and 5′ triphosphate ends. The monomer conformation showed a remarkably similar overall shape and charge distribution to that of *E. coli* RNase E (**Fig. 6C**), which may be related to their similar endonuclease activities.

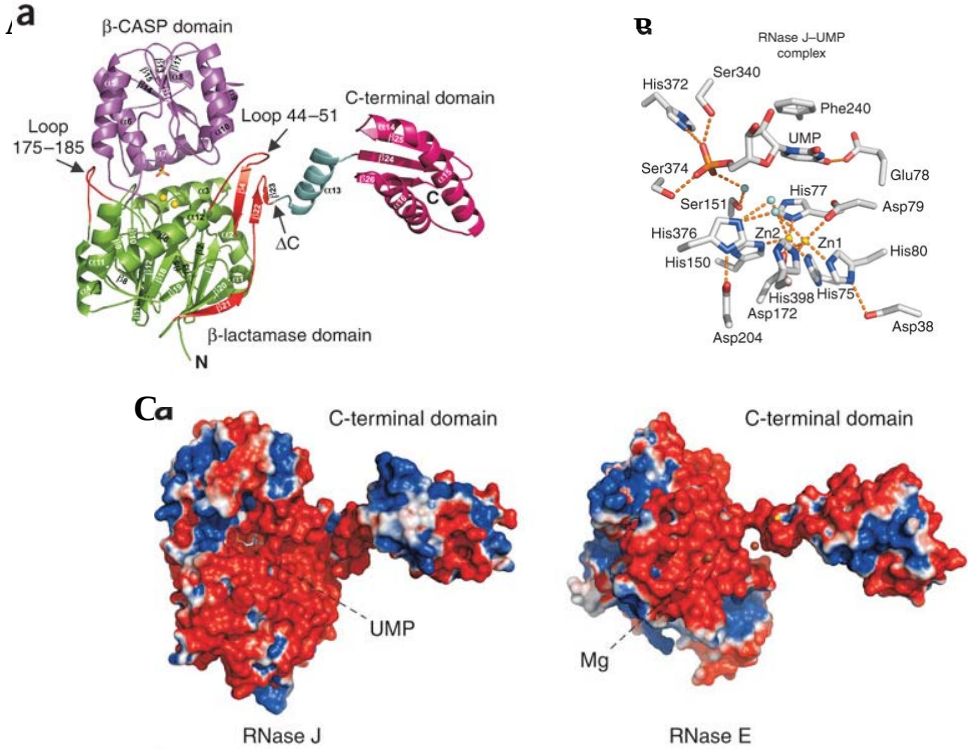


Fig. 6 ***T. thermophilus*** **RNase J. A. Structure of the RNase J monomer.** The β-strands and α-helices are labeled, and the two zinc ions in the active site are represented as yellow spheres. The sulfate ion is shown as a stick model.The N- and C-terminal ends of the protein are indicated by the letters N and C, respectively. **B. The catalytic center of RNase J complexed with a UMP residue.** The UMP phosphate moiety is located in the site occupied by a sulfate ion in the free enzyme and is held in place by interacting with the same residues that coordinated the sulfate. **C. Comparison of the electrostatic surfaces of** ***T. thermophilus*** **RNase J and** ***E. coli*** **RNase E.** Both structures are presented with the active site facing upwards. The UMP residue and the Mg^{2+} ion in the catalytic sites of RNases J and E, respectively, are indicated (Li de la Sierra-Gallay et al., 2008).

Mutations in adjacent catalytic site residues (D78K, H79A) result in severe impairment of both the 5′-to-3′ exonuclease and the endonuclease activities (Li de la Sierra-Gallay et al., 2008). This key finding indicates that there is only one catalytic site on the enzyme, and raises the difficult issue of how an enzyme that appears to be a 5′-binding ribonuclease, with its (apparently) single catalytic site adjacent to this 5′ binding site, can access and cleave endonucleolytically at distal internal sites. One could propose that RNase J1 has a second 5′-end binding pocket, located distal to the catalytic site, which can accommodate a triphosphorylated 5′ end. Binding of a 5′ end at the distal site could be followed by RNA looping such that the endonucleolytic cleavage site is positioned in the binding site that is adjacent to the catalytic site. This is similar to a model that has been proposed for RNase E (Callaghan et al., 2005; Jiang and Belasco, 2004).

Alternatively, RNase J1 might switch from endonucleolytic mode to exonucleolytic mode on the same RNA molecule, without release of the substrate. Since the 5′-monophosphate binding pocket is only one nt distance from the catalytic zinc ions (Li de la Sierra-Gallay et al., 2008), the 5′ monophosphate generated by endonuclease cleavage could immediately slip into the binding pocket to become subject to 5′ exonuclease activity.

In a recent work it has been demonstrated that RNases J1 and J2 form a complex both *in vitro* and *in vivo*, suggesting that this is the primary form of these enzymes in wild-type cells(Mathy et al., 2010). Further, it has been shown that complex formation has dramatic consequences for both the 5′-to-3′ exoribonuclease activity and endonucleolytic cleavage site specificity. RNase J2 alone is an extremely weak 5′-to-3′ exoribonuclease and the association of RNases J1 and J2 alters the enzyme's endonucleolytic cleavage site efficiency and preference.

2.1.B RNase Y

RNase Y, encoded by an essential gene *ymdA*, is a novel discovered endoribonuclease responsible for initiating the decay of all SAM-dependent riboswitches in *B. subtilis* with the exception of *cysH* (Shahbabian et al., 2009). Orthologs of RNase Y occur in about 40% of the currently sequenced eubacteria, with similarities ranging from 56 to 99%. RNase Y is absent from the archea and eukaryotic organisms with a single exception, *Drosophila willistoni*. Few bacteria, like the *Frankia* and *Salinispora* species of Actinobacteria and 25–30% of the *Bacillales* (e.g. *Listeria*) and *Clostridia,* containing RNase Y also have either an RNase E/G like enzyme or RNase J (Shahbabian et al., 2009).

RNase Y initiated the SAM riboswitch turnover with endonucleolytic cleavages and the rate of the cleavages is likely dependent on the intracellular SAM concentration determining RNA folding. The cleavage products then become substrates for the 3′–5′ exonucleases PNPase and RNase R (the aptamer domain and probably the 50 nt 5′ terminal leader fragment) and the 5′–3′ exoribonucleolytic activity of RNase J (Shahbabian et al., 2009).

The chemical decay rate of bulk mRNA was slowed more than two-fold in the RNase Y depletion strain compared with the wild-type strain. The effect of RNase Y on global mRNA stability is thus significant and distinctly greater than the 30% increase in mRNA stability observed in a RNase J1/J2 double-mutant strain (Even et al., 2005).

RNase Y appeared to cleave in single-stranded A or AU rich sequences upstream of a secondary structure, a context resembling the cleavage specificities of RNases J1/J2 (Deikus et al., 2008; Even et al., 2005; Yao et al., 2007) and also *E. coli* RNase E (Callaghan et al., 2005). This enzyme appeared to be very sensitive to the RNA conformation downstream of its cleavage site and finally it preferred 5′ monophosphorylated transcripts for an efficient activity (Shahbabian et al., 2009).

RNase Y has the conserved HD domain conserved in the superfamily of metal-dependent phosphohydrolases (Aravind and Koonin, 1998). The presence of an RNA-binding KH domain (Grishin, 2001) together with the HD domain (**Fig. 7**) suggested that the primary function of this protein is in nucleic acid metabolism. As a putative metalloenzyme RNase Y possesses a distinctive combination of metal-chelating residues, essentially histidines and aspartates (Aravind and Koonin, 1998). Mutation of the His368 and Asp369 that constitute the highly conserved HD motif severely impaired the endonucleolytic activity, indicating that the HD domain is important for the RNase Y.

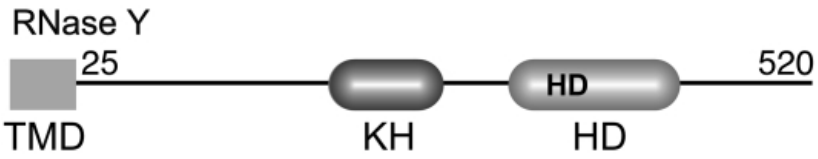


Fig. 7 RNase Y domain structure. The KH and HD domains are indicated together with the HisAsp doublet conserved in HD domain proteins. TMD indicates the N-terminal transmembrane domain (Shahbabian et al., 2009).

The presence of an N-terminal transmembrane domain allow RNase Y (alone or in complex with other proteins) to bind the membrane, in fact Gfp-fusion experiments localized this endoribonuclease to the cell periphery (Hunt et al., 2006). This further extends the analogy to *E. coli* RNase E. In fact, RNase E contains a short domain with the propensity to form an amphipathic α-helix that is necessary and sufficient to bind to membranes (Khemici et al., 2008).

In a recent study, it has been shown that PNPase, RNase J1 and the glycolytic enzymes 6-phosphofructokinase and enolase can interact with the *ymdA* gene product and potentially form a degradosome-like complex *in vivo* (Commichau et al., 2009).

2.1.C Bs-RNase III

The *B. subtilis* homologue of *E. coli* RNase III is Bs-RNase III, which is a 28 kDa protein that is 34% identical to the *E. coli* enzyme and that shows similar cleavage site specificity (Mitra and Bechhofer, 1994). Cleavage occurs at an internal loop sequence in a double-stranded structure that is formed by intramolecular base-pairing. Although the local environment of the site of Bs-RNase III cleavage appears very similar to that of *E. coli* RNase III, there are important differences in their substrate specificity.

The only known endogenous *B. subtilis* RNAs that are substrates for Bs-RNase III are 30S ribosomal precursor RNA (Wang and Bechhofer, 1997) and scRNA (Oguro et al., 1998; Yao et al., 2007).

The fact that the *rncS* gene encoding Bs-RNase III is essential in *B. subtilis* (Herskowitz and Bechhofer, 2000) suggests that one or more critical mRNAs depend on Bs-RNase III for processing to give correct expression, and this may involve endonucleolytic cleavage to control mRNA stability. Rare cells that have a deletion of the *rncS* gene can be recovered, and presumably these have second-site mutations that compensate for the lack of Bs-RNase III.

Very recently, a second RNase III-like protein, called Mini-III, has been described in *B. subtilis*, which is involved in maturation of 23S rRNA (Redko et al., 2008). This enzime is homologous to the catalytic domain of RNase III alone; it lacks the dsRBD characteristic of full-length bacterial RNase III enzymes.

RNase III cleaves rRNA as it is being transcribed and before ribosome assembly is completed while Mini-III is more efficient at cleaving pre-23S rRNA in assembled 50S subunits (Redko et al., 2008). It was shown that a factor stimulating the maturation of the 23S rRNA in assembled 50S subunit by Mini-III was the ribosomal protein L3 bound to the 23S precursor (Redko and Condon, 2009).

2.1.C RNase M5

RNase M5 was identified as responsible of the maturation of the 5S rRNA (Sogin et al., 1977). The ribosomal protein L18 bound to the 5S subunit acts as a cofactor of the maturation reaction (Stahl et al., 1984). RNase M5, coded by the *yabF/rnmV* gene, is highly conserved in low G+C % gram-positive bacteria. This endoribonuclease is not essential for cell viability and when it is absent, the 5S rRNA is not matured. This indicates that no other enzyme can compensate its role of the RNase M5 (Condon et al., 2001).

RNase M5 cleaves the RNA molecules in double stranded regions and no sequence specificity is required to its activity. However, RNase M5 has very few or possibly no mRNA substrates in *B. subtilis* (Condon et al., 2002).

2.1.D. RNase P and RNase Z

RNase P is a widespread essential enzyme (Ellis and Brown, 2009). This endoribonuclease has a heterotetrameric structure composed of two RNA subunits, coded by *rnpB* gene, and two protein subunits, coded by *rnpA* gene (Fang et al., 2001).

RNase P is involved in the maturation of the 5' side of the tRNA precursors. These activity is catalized by the RNA subunit of the enzyme, while the role of the protein subunit is to bind the substrate (Kurz et al., 1998) and to promote the dimerization of the holoenzime (Fang et al., 2001).

It has been thought that the only substrates for RNase P were the tRNA precursors but it was demonstrated that it can cleave also the coenzyme B12 riboswitches (Altman 2005).

The RNase Z, coded by the gene *yqjK/rnz*, is an essential endoribonuclease in *B. subtilis*. It is responsible of the maturation of the tRNAs that lack the 3' end CCA motif. Longer transcripts

(≥ 33 nt) inhibit the RNase Z activity, suggesting that the RNase P has to cleave the tRNA precursor before RNase Z.

2.2 Exoribonucleases

About the 3'-5'exoribonucleolytic activity of RNase J1, we discussed in the section on the endoribonucleases. In this section, we will overview other exoribonucleases present in *B. subtilis*.

2.2.A PNPase

PNPase, encoded by the *pnpA* gene, is the major 3′-to-5′ exonuclease activity involved in *B. subtilis* mRNA turnover. The protein sequence has a high homology (50% identity) to the *E. coli* PNPase (Luttinger et al., 1996), it belongs to the phosphate-dependent exoribonuclease (PDX) family, and close homologues are widely distributed in bacteria (Zuo and Deutscher, 2001). *B. subtilis* PNPase is a large enzyme (molecular weight 77 kDa), and, by analogy to other PNPases, likely functions as a trimer (Symmons et al., 2000). This 3′-5′ exonuclease degrades RNA phosphorolytically, yielding nucleoside diphosphates as products. Under conditions of excess of nucleoside diphosphates, *B. subtilis* PNPase can undergo the reverse reaction and add nucleotides to the 3′ end of an RNA substrate (Mitra et al., 1996).

Despite playing a major role in mRNA decay, a *pnpA* deletion strain of *B. subtilis* is viable. The *pnpA* deletion does result in a number of diverse phenotypes, including cold sensitivity, filamentous growth, tetracycline sensitivity, and competence deficiency (Luttinger et al., 1996; Wang and Bechhofer, 1996).

PNPase binds poorly to the 3′ end of *trp* leader RNA which has a 3′-proximal secondary structure (formed by a transcription terminator sequence) followed by a single-stranded tail of 6 nts (Deikus and Bechhofer, 2007). When the 3′ tail of the substrate is extended to 17 single-

stranded nucleotides, PNPase is able to bind well, although it has difficulty to degrade processively a transcript through secondary structure. This suggested that PNPase does not degrade mRNAs from the native 3′ end, but relies on endonuclease cleavage in the body of the message as an entry site for rapid degradation.

In a recent work it has been shown that in the presence of Mn(2+) and low-level inorganic phosphate P(i), PNPase degrades ssDNA. The limited end-processing of DNA is regulated by ATP and is inactive in the presence of Mg(2+) or high-level P(i). In contrast, the RNase activity of PNPase requires Mg(2+) and P(i), suggesting that PNPase degradation of RNA and ssDNA occur by mutually exclusive mechanisms (Cardenas et al., 2009).

2.2.B. RNase R, RNase PH and YhaM

B. subtilis RNase R is a large (88 kDa) hydrolytic 3′-to-5′ exoribonuclease, a member of the RNR family (Zuo and Deutscher, 2001), whose primary function in *B. subtilis* RNA metabolism is not known. In the absence of PNPase, *B. subtilis* RNase R degrades mRNA that contains secondary structure more efficiently than the other remaining 3′-to-5′ exoribonucleases (Oussenko et al., 2005). In fact, a triple ribonuclease mutant that contains only RNase R (of the four known 3′-to-5′ exoribonucleases) handles mRNA decay better than when RNase R is present with RNase PH and YhaM.

RNase PH is, like PNPase, a phosphorolytic enzyme, and is a member of the PDX family of 3′ exoribonucleases (Zuo and Deutscher, 2001). RNase PH is basically a smaller version (molecular weight of 26.5 kDa) of PNPase, without the large N-terminal domain and KH and S1 domains of PNPase. A medium resolution structure of *B. subtilis* RNase PH has been determined (Harlow et al., 2004), and the functional form appears to be a hexamer. In the absence of the three other known *B. subtilis* 3′-to-5′ exoribonucleases, RNase PH can function

in mRNA decay (Oussenko et al., 2005), but the major role of RNase PH appears to be in tRNA processing (Wen et al., 2005).

YhaM is the fourth known *B. subtilis* 3′-to-5′ exoribonuclease, and it was identified in extracts that were missing PNPase and RNase R (Oussenko et al., 2002). Interestingly, the overall domain structure of YhaM is unique to Gram-positive organisms. While YhaM can participate in mRNA turnover in the absence of other 3′ exonucleases, the true *in vivo* function of YhaM remains unexplored. YhaM has been implicated in DNA replication (Noirot-Gros et al., 2002).

2.2.C NanoRNase

B. subtilis lacks an homolog of the Oligoribonuclease (Orn) of *E. coli*. However, very recently a protein product, YtqI, was identified, demonstrated to be able, *in vitro*, to degrade oligoribonucleotides of 3-mers. Moreover, it has been demonstrated that this protein can complement the *orn* conditional mutant in *E. coli,* when expressed at the same physiological level of the Orn protein. YtqI is not essential in *B. subtilis*, in contrast of the Orn that is essential in *E. coli*. This suggests the presence of other enzymes able to degrade small RNA fragments in *B. subtilis* (Mechold et al., 2007).

2.3 The RNA degradosome in *B. subtilis*

Very recently, a RNA degradosome was identified in *B. subtilis*. This complex is composed of the newly discovered RNase Y, the RNases J1 and J2, the polynucleotide phosphorylase PnpA and the two glycolytic enzymes enolase (Eno), the phosphofructokinase (PfkA) (Commichau et al., 2009) and the DExD box RNA helicase CshA (Lehnik-Habrink et al., 2010). Thus, the principal structure of the *B. subtilis* degradosome is similar to that of other RNA-degrading machines in all domains of life.

In agreement with recently published evidence, RNase Y may perform the initial endonucleolytic attacks on mRNA substrates whereas the exonucleases PnpA and RNase J1/J2 degrade the RNA fragments from the 3′ and 5′ ends respectively (Condon, 2010; Shahbabian et al., 2009; Yao and Bechhofer, 2010).

3. The RNA decay in Mycobacteria

Althought, RNA decay was largely characterized in both gram-negative and gram-positive organisms, little is known about RNA decay in Mycobacteria. Hortologues of endo- and exoribonucleases are present in these organisms in different combination, not found in other species, and this open many questions of how RNA maturation/degradation takes place in Mycobacteria.

Only two RNases have been characterized in *M. tuberculosis*: RNase E and RNase III.

In Mycobateria an RNase E/G homolog is present (Csanadi et al., 2009; Zeller et al., 2007). The only studies on this homolog derive from *in vitro* experiments with the purified proteins of *M. tuberculosis* and *M. smegmatis*. The RNase E/G homolog (MycRne) from the intracellular pathogen *Mycobacterium tuberculosis* is a polypeptide of 953 amino acids. It has been shown that, similar to its *E. coli* homologues RNase E (Callaghan et al., 2005) and RNase G (Briant et al., 2003) polypeptides, purified MycRne can exist in solution in dimeric or tetrameric forms (Csanadi et al., 2009; Zeller et al., 2007).

MycRne has an endoribonucleolytic activity, which is dependent on the 5′-phosphorylation status of its substrates, thereby suggesting that MycRne is a 5′ end dependent endoribonuclease (Zeller et al., 2007). Moreover, this enzyme apparently prefers to cleave sequences enriched mainly in U (but not A) nucleotides.

The catalytic domain of *M. tuberculosis* RNase III has been solved and although globally similar to other RNase III folds, this structure has some features not observed in previously reported models. These include the presence of an additional metal ion near the catalytic site, as well as conserved secondary structural elements that are proposed to have functional roles in the recognition of dsRNAs (Akey and Berger, 2005).

4. Maturation of ribosomal RNA in bacteria

Ribosome are complex ribonucleoprotein (RNP) particles whose primary function is to serve as the site and catalyst for protein biosynthesis. Ribosomes are comprised of two subunits; in prokaryotes, these are 30S and 50S particles, which during translation join together to form the 70S functioning ribosome. The mass of the ribosome is approximately two-thirds RNA and one-third protein. The smaller 30S subunit contains a single 16S rRNA molecule while the 50S subunit contains one molecule each of 23S rRNA and 5S rRNA (Kaczanowska and Ryden-Aulin, 2007).

The rRNA genes in most eubacteria are organized into operons (Brosius et al., 1981; Jarvis et al., 1988; LaFauci et al., 1986). In *E. coli*, seven rRNA operons are present, all with similar structure. The gene for 16S rRNA is near the 5′ end of the operon, followed by the gene for 23S rRNA and the 5S rRNA is nearest the 3′ end. In *E. coli* rRNA operons, a gene for at least one tRNA is located in the spacer region between 16S and 23S rRNA, and depending on the operon, tRNA genes may also be present downstream of 5S rRNA. Sequence analysis revealed that complementary regions flanked both the mature 16S and 23S rRNA sequences, providing the opportunity for extensive base pairing in the RNA transcript (Bram et al., 1980; Young and Steitz, 1978). A very similar gene organization is found in the 10 rRNA operons of *B. subtilis* (Jarvis et al., 1988; LaFauci et al., 1986).

The importance of these secondary structures became apparent from studies of an *E. coli* mutant strain deficient in the endoribonuclease, RNase III. In the absence of RNase III, cells accumulated a 30S rRNA molecule containing all the sequences in the operon (Dunn and Studier, 1973). This molecule can be cleaved by RNase III *in vitro* within the flanking double-stranded regions to generate products containing the sequences of mature 16S and 23S rRNAs as well as additional residues at their 3′ and 5′ ends (Bram et al., 1980; Young and Steitz, 1978). Subsequent work showed that 30S rRNA molecules never exist in wild-type cells; the first RNase III cleavage in the double-stranded region flanking 16S rRNA occurs as soon as this structure can form, and before transcription of the operon is completed (Gegenheimer and Apirion, 1975). Since the RNase III cleavages do not lead to mature 16S or 23S rRNA, these early studies also indicated that other endoribonucleases could separate the 23S and 16S rRNA regions and that additional RNase action could generate partially or fully matured rRNA molecules (King and Schlessinger, 1983). Inasmuch as mature 16S rRNA is made, these data imply that the RNases that normally generate the 5′ and 3′ termini of this molecule can work independently of RNase III action.

Among the most important conclusions to emerge from these early studies is that rRNA maturation events generally occur in the context of an RNP particle, namely the assembling ribosome. In fact, some early studies suggested that protein synthesis by the newly-made ribosome is necessary to complete the final steps of the rRNA maturation process (reviewed in (Stahl et al., 1984)). The close connection between rRNA processing and ribosome assembly was supported by the isolation of numerous assembly mutants in which the rRNAs were not matured completely.

In recent years, our understanding of rRNA maturation has progressed both as a consequence of the discoveries of new Rnases and the generation of mutant strains lacking these enzymes, and from an expansion of information about Rnases initially identified for their roles in other

processes. However, much remains unknown. In the next paragraphs, our current knowledge of the maturation of 16S, 23S, and 5S rRNAs will be described.

4.1 16S rRNA

In *E. coli*, the action of RNase III on rRNA gene transcripts leads to production of a 17S precursor of 16S rRNA (**Fig. 8**). This initial cleavage product contains an additional 115 nucleotides at its 5′ end and 33 nt at the 3′ end that must be removed to generate the mature RNA molecule (Bram et al., 1980; Young and Steitz, 1978).

A major advance in our understanding of 16S rRNA maturation came with the discovery that two endoribonucleases were required to process the 5′ end of 16S rRNA (Li et al., 1999b; Wachi et al., 1999). One was RNase E, already known to be an important participant in both mRNA degradation and 5S rRNA maturation (Apirion and Lassar, 1978). The second enzyme was RNase G (Li et al., 1999b; Wachi et al., 1999). In the absence of either protein, the rate of 5′ processing is slowed dramatically, and when both enzymes are missing, 5′ processing is blocked completely. Detailed examination revealed that in the absence of RNase E, a mature 5′ terminus could be generated by RNase G, although much more slowly. In contrast, in a strain lacking RNase G, the major product that accumulates contains 66 extra 5′ residues (Li et al., 1999b). Over time, a small amount of RNA with a mature 5′ terminus and a product with four or five extra 5′ residues can be generated by the RNase E still present in this strain.

The *in vivo* observations of the roles of Rnases E and G in 5′ maturation were confirmed by *in vitro* analysis (Li et al., 1999b). Ribosome substrates containing rRNA with 115 and/or 66 extra 5′ residues were isolated from *rne rng* or *rng* mutant strains, respectively, and were treated with extracts from wild type, *rng* mutant or RNase G overexpressing strains, or with purified RNase E. These experiments showed that RNase G converted the +66-nt intermediate to the mature 5′ terminus, whereas it acted poorly on the precursor with 115 extra residues.

These studies of 5′ processing also provided some information about 3′ processing of 16S rRNA. In wild type cells, removal of the 33 extra 3′ residues occurs rapidly. This is also true for the *rng* mutant. However, the single or double mutant strains deficient in RNase E process the 3′ terminus more slowly. Thus, while 3′ maturation can proceed in the complete absence of 5′ maturation, it is more efficient when RNase E has already acted at the 5′ terminus. In addition, since no intermediates with less than 33 extra 3′ nucleotides were observed even when 3′ processing was slowed, it is likely that maturation at the 3′ terminus of 16S rRNA occurs by a single endonucleolytic cleavage.

Based on all of these observations, a model can be proposed for 16S rRNA maturation in *E. coli* (**Fig. 8**). RNase III cleavage of the growing rRNA transcript releases the portion containing the 16S rRNA as a 17S precursor with extra residues at each end. This RNA is present in a preribosomal particle, and all subsequent processing events occur in this context. Cleavage by RNase E then shortens the 5′ precursor sequence from 115 to 66 nt. The resulting 16.3S intermediate is also missing the 33-nt 3′ precursor sequence. This has led to the suggestion that the 3′ sequence might be removed first (Wachi et al., 1999). However, since the absence of RNase E slows 3′ processing, it is likely that the preferred route is to first cleave on the 5′ side. In the 17S precursor, 26 of the 33 extra 3′ residues are able to base pair with 5′ precursor-specific nucleotides to form a terminal stem. Once RNase E cleavage occurs, this stem can no longer form, and 3′ maturation could be facilitated. Confirmation of this idea must await identification of the 3′ processing enzyme.

As noted earlier, the maturation apparently is endonucleolytic, but neither RNase III, E, nor G appears to be involved (Li et al., 1999b). Likewise, the absence of the known *E. coli* exoribonucleases, alone or in various combinations, does not affect 3′ processing (unpublished observations). Thus, it appears either that the RNase responsible is an as yet undiscovered

enzyme or that some overlapping combination of known Rnases is involved, but they all have not yet been simultaneously removed by mutation.

In a recent work B.W. Davies (Davies et al., 2010) demonstrated that the deletion of the *ybeY* gene in *E. coli* strongly affects the maturation of all three rRNAs causing a particularly strong defect in maturation of the 16S rRNA 5'- and 3'-termini. It has been shown that PNPase and RNase R may act in maturation of the 16S rRNA 3'-terminus and their role may be modulated by YbeY. Moreover, the double mutant Δ*ybeY* Δ*rnr* exhibits additional defects in 16S rRNA metabolism suggesting that the RNase R and YbeY proteins may interact in aspects of rRNA metabolism, such as quality control of rRNA (Davies et al., 2010).

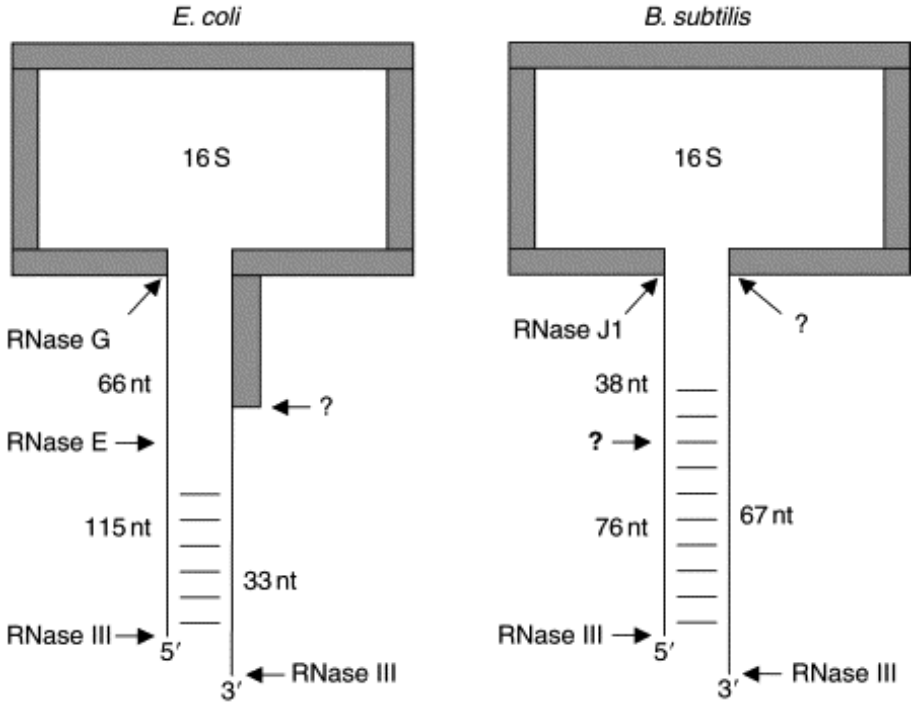


Fig. 8 Diagram of 16S rRNA maturation in *E. coli* and *B. subtilis*. Mature 16S sequences are shown as filled in area, and precursor sequences as a line. Enzymes acting at different positions are named, where known, and denoted with a question mark (?), if unknown. The number of precursor residues present, prior to the action of the RNase immediately above, are indicated.

In another eubacterium, *B. subtilis*, processing of the 16S rRNA proceeds by a different mechanism (**Fig. 8**). *B. subtilis* lacks orthologs of Rnases E and G, indicating that some other

RNase would have to be involved in the 5′ maturation process. The likely candidate is RNase J1 (Britton et al., 2007). Recent work revealed that depletion of RNase J1 also led to the accumulation of 16S rRNA precursors, implicating this enzyme in the processing of the rRNA as well (Britton et al., 2007). The closely-related enzyme, RNase J2, does not appear to be involved.

Primer extension analysis showed that the 5′ ends of the precursors in the RNase J1 mutant corresponded well to both an RNase III cleavage site identified previously at +76 and to a site at +38 (Britton et al., 2007). As in *E. coli*, the accumulated 16S precursors present in the mutant strain were incorporated into 30S and 70S ribosomes, and into small polysomes, suggesting that

they could function *in vivo.* The data also suggested that maturation occurs subsequent to 30S subunit assembly.

In vitro analysis confirmed that purified RNase J1 could act on 70S ribosomes containing the precursor 16S rRNA, establishing that its action is direct (Britton et al., 2007). Most interestingly, subsequent *in vitro* analysis indicated that RNase J1 acted on 16S rRNA precursors as a 5′–3′ exoribonuclease that released 5′ mononucleotides (Mathy et al., 2007). However, this activity was relatively weak and at extended times of incubation actually degraded the 16S rRNA precursor. Moreover, the *in vitro* studies suggest that the mode of action of RNase J1, also being a 5′–3′ exoribonuclease, may be unique among prokaryotic Rnases. What is not yet clear is whether it acts on the +76 as well as the +38 molecule (Li de la Sierra-Gallay et al., 2008).

Although the enzymes required for 3′ maturation of 16S rRNA in *B. subtilis* remain to be elucidated, in the organism *Pseudomonas syringae* (Purusharth et al., 2007) it has been demonstrated that the 3′–5′ exoribonuclease, RNase R, which heretofore had been considered a degradative enzyme (Deutscher, 2006), appears to be responsible for maturation of the 3′ ends of 16S and 5S rRNAs. The *rnr* mutant strain accumulates precursors of 16S and 5S rRNAs at 4 °C,

and these molecules are incorporated into ribosomes. This result is in agreement with the recent work of B.W. Davies (Davies et al., 2010) where he demonstrates that RNase R, in collaboration with the YbeY protein, regulates the 16S rRNA metabolism.

4.2 23S rRNA

In *E. coli*, RNase III-mediated cleavage of the rRNA transcript generates a 23S rRNA precursor containing three or seven extra 5′ residues and seven to nine extra 3′ residues (**Fig. 9**) (King et al., 1986). Earlier studies had suggested that the 5′ residues would be removed endonucleolytically, whereas processing at the 3′ end would be carried out by an exoribonuclease.

The 3′ maturation of 23S rRNA is now known to require the exoribonuclease, RNase T (Li et al., 1999a). In the absence of this enzyme, mutant cells accumulate precursors with extra 3′ residues, and little, if any, mature product is made. Other exoribonucleases may help to shorten the full 3′ extension, but RNase T is required for removal of the last few residues. Nevertheless, even in the absence of RNase T, 23S rRNA products with extra 3′ residues are incorporated into ribosomes, and cell growth is affected only slightly, indicating that final 3′ maturation is not required for functioning ribosomes.

Purified RNase T rapidly and completely converted the 23S precursors in mutant ribosomes to the mature size (Li et al., 1999a). In contrast, naked 23S precursor was converted much more slowly and more product 1 nt shorter than the mature form was generated. These observations demonstrated a direct role for RNase T, and showed that processing is more efficient and more accurate when the rRNA precursor is in the context of the ribosome. The fact that maturation occurs so efficiently with fully assembled ribosomes also suggests that final 3′ maturation is a very late event in ribosome biogenesis. It is also interesting to note that 5′ processing of 23S

rRNA is unaffected by events at the 3′ end. Thus, even though the 3′ and 5′ ends of 23S rRNA are paired, maturation of the 5′ end is an independent process.

It has been recently shown that YbeY is involved in the final step of the 23S rRNA 5' terminus maturation (Davies et al., 2010).

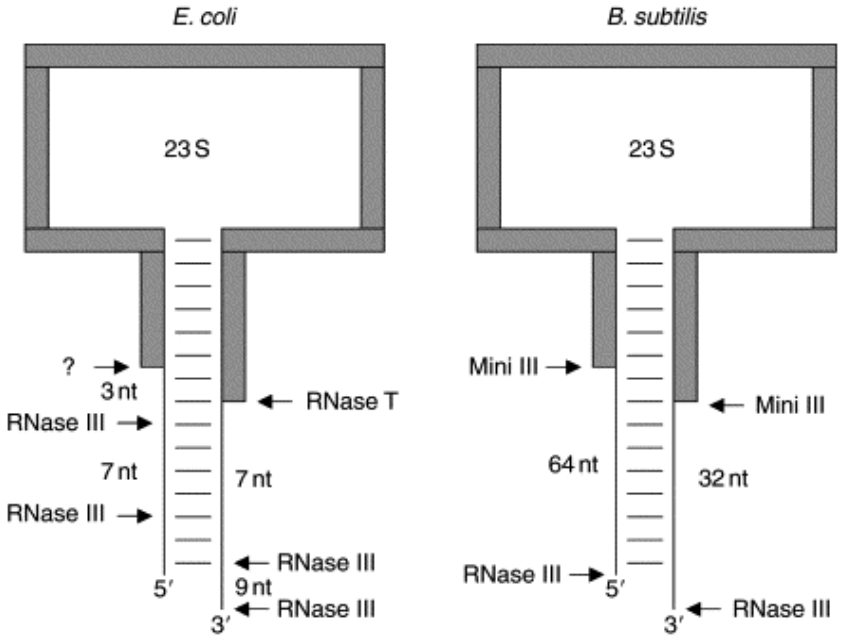


Fig. 9 Diagram of 23S rRNA maturation in *E. coli* and *B. subtilis*. Mature 23S sequences are shown as filled in area, and precursor sequences as a line. Enzymes acting at different positions are named, where known, and denoted with a question mark (?), if unknown. The number of precursor residues present, prior to the action of the RNase immediately above, are indicated.

As with 16S rRNA, final maturation of 23S rRNA in *B. subtilis* differs from that in *E. coli* (**Fig. 9**). A recently discovered member of the RNase III family of enzymes, named Mini-III, was shown to be responsible for maturation of both the 5′ and 3′ termini of *B. subtilis* 23S rRNA (Redko et al., 2008). In the absence of this enzyme, cells accumulate 23S precursors and aberrantly matured forms of 23S rRNA, the latter arising from secondary pathways that apparently utilize exoribonucleases. Although Mini-III can act on naked RNA, its action is much

more rapid on 50S ribosome subunits containing the 23S precursor. Thus, as with most other rRNA maturation enzymes, processing is greatly facilitated by the context of the ribosome. Mini-III is a dimeric enzyme, as is RNase III, and this structure presumably enables it to simultaneously cleave both sides of the double-stranded stalk flanking the mature 23S rRNA in the precursor molecule (Redko et al., 2008).

In some bacterial species, maturation of 23S rRNA proceeds in an unusual manner. Thus, in *Salmonella*, intervening sequences are present which are removed by the action of RNase III (Burgin et al., 1990). Inasmuch as the resulting fragments are not re-ligated, the mature 23S rRNA exists as fragments. In certain α-proteobacteria, an internal transcribed spacer is removed from the 5′ end of 23S rRNA. Cleavages by RNase III and RNase E are involved, but additional unknown Rnases are necessary to generate the mature 5′ terminus (Klein and Evguenieva-Hackenberg, 2002).

4.3 5S rRNA

5S rRNA is located the most 3′ of the three rRNAs in the primary transcript. In *E. coli*, RNase III cleavage releases the 5S rRNA and adjacent sequences as a 9S precursor(Ghora and Apirion, 1978) that is subsequently cleaved by RNase E (**Fig. 10**) (Misra and Apirion, 1979). The resulting product still retains three extra nucleotides at each end that must be removed to generate the mature 5S rRNA. It has been demonstrated that the three residues at the 5′ terminus are probably removed by the action of YbeY (Davies et al., 2010). An RNase E/G type enzyme has also been shown recently to participate in 5S RNA maturation in the hyperthermophilic bacterium, *Aquifex acolicus* (Lombo and Kaberdin, 2008).

E. coli mutant strains lacking RNase T produce no mature 5S rRNA (Li and Deutscher, 1995). In the absence of this enzyme, a processing intermediate containing primarily two extra 3′ residues and a mature 5′ end accumulates, and this molecule is assembled into functioning ribosomes.

Thus, 5′ maturation of 5S rRNA is independent of events at the 3′ terminus, and the presence of extra 3′ residues has little effect on ribosome function. *In vitro*, purified RNase T accurately and efficiently removes the extra 3′ residues from ribosomes derived from RNase T-deficient cells to generate the mature 3′ end, and no further nucleotide removal occurs up to 1 h of incubation. The same experiment carried out with a naked RNA substrate proceeds more slowly, and the final product lacks the normal 3′ terminal residue of mature 5S rRNA (Li and Deutscher, 1995). These data emphasize the importance of the ribosome context for efficient and accurate rRNA maturation. From this discussion, it is obvious that processing of the 3′ termini of 23S and 5S rRNA are strikingly similar. In both cases, only RNase T from among the eight *E. coli* exoribonucleases functions to remove the last 3′ residues to generate the mature 3′ terminus (Li and Deutscher, 1995; Li et al., 1999a). This is understandable because only RNase T is able to efficiently trim residues close to a double-stranded stem (Li et al., 1998).

The similarity in 3′ maturation of 23S and 5S rRNAs extends even further (Li and Deutscher, 1995; Li et al., 1999a). Processing of each molecule appears to be a very late event, such that fully assembled 70S ribosomes, which must be functioning *in vivo* because RNase T-deficient cells grow almost normally (Padmanabha and Deutscher, 1991), are substrates for RNase T *in vitro*. In addition, in each assembled RNA molecule, the extra 3′ residues are accessible to RNase T suggesting that they are not buried within the structure of the ribosome, but are exposed, consistent with the positions of the mature 3′ ends in ribosome crystal structures (Schuwirth et al., 2005). We can conclude that only these residues are exposed in the assembled ribosome or that, once the mature RNA is made, the structure of the ribosome changes to protect against further action by RNase T.

As discussed earlier, 5S rRNA maturation in *B. subtilis* is carried out by a single enzyme, RNase M5, which cleaves in a double-stranded region to generate both the mature 5′ and 3′ termini (**Fig. 10**) (Sogin et al., 1977). The similarity to the recently described RNase Mini-III that acts on 23S

rRNA is evident (Redko et al., 2008), so that in this organism as well, there are striking similarities between 23S and 5S rRNA maturation. Additionally, neither Mini-III nor RNase M5 are essential enzymes, and functioning ribosomes can be made containing precursors to both 23S and 5S rRNA (Condon et al., 2001; Redko et al., 2008), a situation identical to that in *E. coli*.

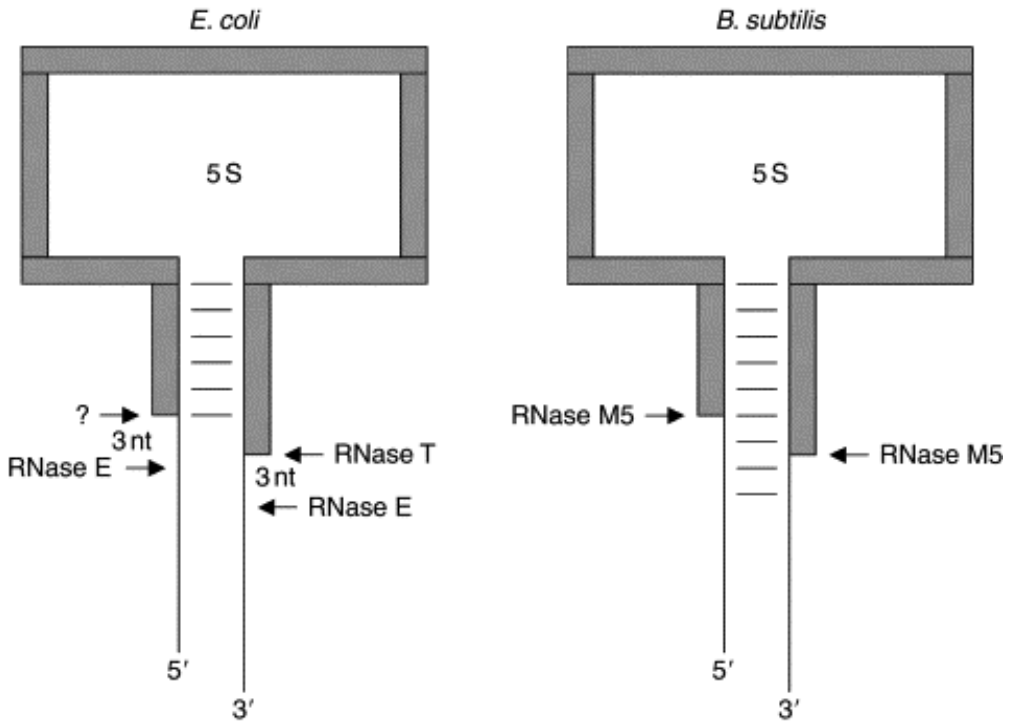


Fig. 10 Diagram of 5S rRNA maturation in *E. coli* and *B. subtilis*. Mature 5S sequences are shown as filled in area, and precursor sequences as a line. Enzymes acting at different positions are named, where known, and denoted with a question mark (?)**,** if unknown. The number of precursor residues present, prior to the action of the RNase immediately above, are indicated.

5. The *furA-katG* region in Mycobacteria

Oxidative stress response appears to be implicated in the intracellular survival of pathogenic mycobacteria and their persistence in the macrophage host. It was demonstrated that the oxidative stress induced mycobacterial KatG catalase-peroxidase that plays a role in the degradation of toxic oxygen compounds (Cole et al., 1998; Pagan-Ramos et al., 1998). Moreover, *M. tuberculosis* KatG plays a role in the innate susceptibility and acquired resistance to the antituberculous drug isoniazid (Pym et al., 2001).

Immediately upstream of the *M. tuberculosis katG* gene an open reading frame encoding for the FurA protein, a transcriptional regulator (Zahrt et al., 2001), is present. The *furA-katG* loci organization appears to be conserved among most of the sequenced mycobacteria (*M. bovis*, *M. leprae, M. smegmatis, M. fortuitum, M. marinum*, etc.) (Cole et al., 1998; Milano et al., 2001; Pagan-Ramos et al., 1998). Moreover, a similar organization is also found in the closely related *Streptomyces coelicolor* (Hahn et al., 2000) and *Streptomyces reticuli* (Ortiz de Orue Lucana and Schrempf, 2000), where a FurS protein regulates expression of a catalase-peroxidase (Ortiz de Orue Lucana and Schrempf, 2000).

Fur-like proteins are transcriptional repressors that exhibit a Fe^{2+}-dependent DNA binding activity and regulate several genes involved in iron metabolism (de Lorenzo et al., 1988; de Lorenzo et al., 1987; Escolar et al., 1999). There is an intimate relationship between iron metabolism and oxidative stress: indeed, the cytotoxic effects of reactive oxygen species are largely mediated by iron. It has been shown that in *Escherichia coli* involved in oxidative stress response, OxyR and SoxRS, activate the expression of Fur, the global repressor of ferric iron uptake (Zheng and Storz, 2000). *E. coli* Δ*fur* mutants are more sensitive to hydrogen peroxide than are Fur-proficient strains (Touati et al., 1995).

Similarly, in *M. tuberculosis* and *M. smegmatis*, the non pathogenic model organism, highly homologous to *M. tuberculosis*, it has been demonstrated that both *furA* and *katG* are induced upon oxidative stress, and that the FurA protein is a transcriptional repressor of the *furA* promoter, located immediately upstream of the *furA* coding sequence (Milano et al., 2001). Moreover, *M. smegmatis furA* knock out mutants exhibited increased levels of both catalase activity and *katG* transcript (unpublished results).

Upstream of the *M. tuberculosis furA* gene, -35 and -10 promoter consensus sequences can be identified that constitute the *furA* promoter. It has been demonstrated that *pfurA* is autoregulated by the FurA protein, which binds to an upstream 30 bp AT-rich DNA region, thus repressing transcription of the downstream operon(Sala et al., 2003). FurA binding is sensitive to peroxide treatment, thus FurA DNA binding is released upon oxidative stress and transcription from *pfurA* activated (Sala et al., 2003).

5.1 Expression of the *furA-katG* region in mycobacteria.

In mycobacteria the *furA* and *katG* genes constitute a single operon (Zahrt et al., 2001) (Master et al., 2001). Two different 5'ends were identified by S1 mapping: the first coincides with the initiation codon of *furA* and is generated by transcription initiation at the *furA* promoter (Pym et al., 2001) (Master et al., 2001) (Sala et al., 2003); the second is 54 bp upstream of *katG* translation start codon.

In *M. smegmatis* specific transcripts of *furA* and *katG* were identified by northern blotting, but a single transcript encompassing both genes was never detected (**Fig. 11**) (Milano et al., 2001). Specific 5' ends were mapped by primer extension (Milano et al., 2001) (Sala et al., 2003): the first was due to transcription initiation from the *furA* promoter; two additional 5' ends were mapped upstream of *katG*, the first one, corresponding to coordinate 443, falls in the final part of the *furA* gene; the second is less intense and corresponds to coordinate 478, located in the *furA-katG* intergenic region (**Fig. 11**).

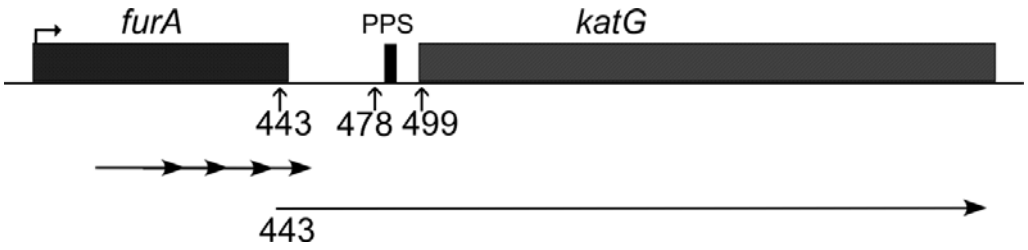
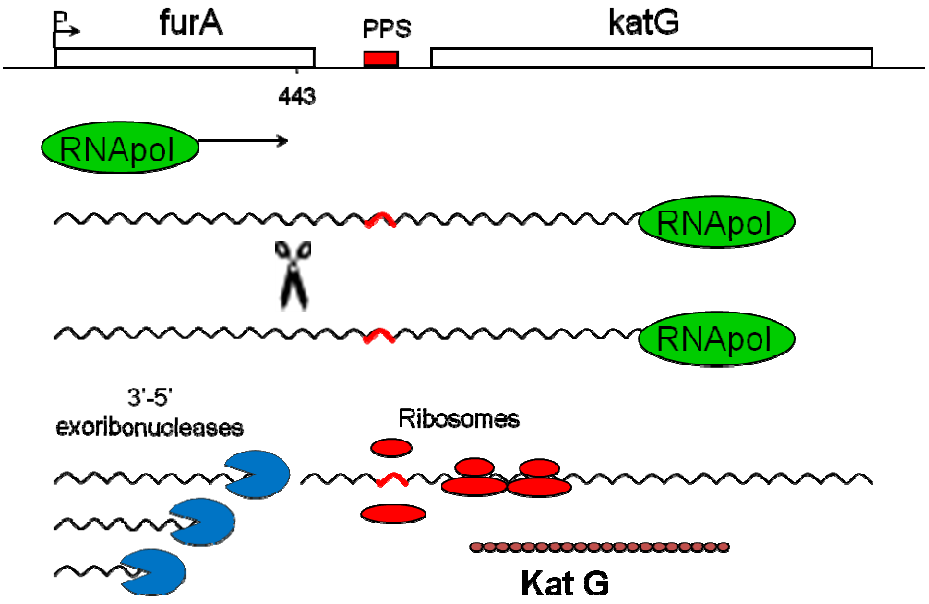


Fig.11 Scheme of the furA-katG operon. Genes are indicated by boxes. The coordinates of the possible 5'ends are indicated at the bottom of the scheme. The mRNAs found by northern blotting are indicated by arrows.

It has been demonstrated by in vitro transcription and by RNA polymerase Chromatin immunoprecipitation that the 5' end at 443 is not produced by transcription initiation, rather by specific processing of longer transcripts, likely starting from the *furA* promoter (Sala et al., 2008). Moreover, it was demonstrated that for processing at 443 to occur, the RNA region must be single-stranded, whereas no specific sequence is required. Further studies showed that a polypurine sequence (which may correspond to the *katG* Shine-Dalgarno sequence), located four bp upstream of the *katG* translation start codon, and the translation of the first *katG* amino acids are important for the downstream transcript stability (Sala et al., 2008). Taken together, all results outline a model for the transcriptional regulation of the *furA-katG* operon: both genes are co-transcribed from the *furA* promoter and the bicistronic transcript is then rapidly processed by an endoribonuclease within a single-stranded region at coordinate 443 to obtain the mature and stable *katG* transcript.

Since the *furA-katG* region in *M. tuberculosis* is highly homologous to the *M. smegmatis* region, we suppose that the same RNA processing is also present in *M. tuberculosis* strain. Thus, identification of the RNAse responsible of this processing may help in unraveling the RNA maturation/decay mechanisms in this important pathogen.



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6. Aim of the project

In this work our intent was to find the endoribonuclease(s) responsible of the maturation of the *katG* 5' end. The studies were focused on the nonpathogenic and fast growing strain *M. smegmatis*, but we are confident that the results may lead to comprehension of the role of the correspondent endoribonucleases present in *M. tuberculosis*.

We identified two ribonucleases in *M. smegmatis*, RNAse J and RNAse E, which are also present in *M. tuberculosis*, and constructed mutants in both genes. Our results indicated that RNAse J is not essential, whereas RNse E is. The latter was found to be responsible of *furA-katG* transcript maturation at 443.

Moreover, we characterized *in vitro* the MSMEG_2685 protein found to be an RNase J hortologous gene and successively we analyzed the role of RNAse J and RNAse E in the 5' end maturation of ribosomal RNAs.

RESULTS

1. Maturation of the *furA-katG* transcript

1.1 *In vitro* processing of the *furA-katG* transcript

Our first approach was to try to reproduce *in vitro* the maturation of the *M. smegmatis katG* transcript. An *in vitro* randomly labeled mRNA was synthesized, as indicated in the Experimental procedures, that covers the terminal part of the *furA* gene from coordinate 360, the intergenic region, the first 101 nucleotides of *katG* gene and an additional stem loop sequence added to stabilize the transcript (FK in **Fig. 13A**). As a control we also synthesized a shorter RNA starting at coordinate 443 (443 in **Fig. 13A**). The latter RNA was used as a marker to identify possible RNA cleavage at this coordinate. The obtained FK mRNA was incubated with a crude extract of *M. smegmatis* and the samples separated by electrophoresis on a 6% denaturing polyacrylamide gel (see Experimental procedures). The results are shown in Fig.11B. It appears that incubation with the extract causes degradation of the FK RNA, but only a faint signal appeared at 443, whereas most signals of lower molecular weights were present, probably deriving from non specific activity of ribonucleases present in the crude extract. Further attempts were made, changing the relative ratio of FK RNA to crude extract and the time of reaction, but specific cleavage at 443 could not be visualized. This indicates that the crude extract contains a high amount of ribonucleases and that the terminal stem-loop sequence is not sufficient to stabilize the transcript. Thus we decided to adopt a more direct approach, focalizing our attention on specific ribonucleases present in mycobacteria.

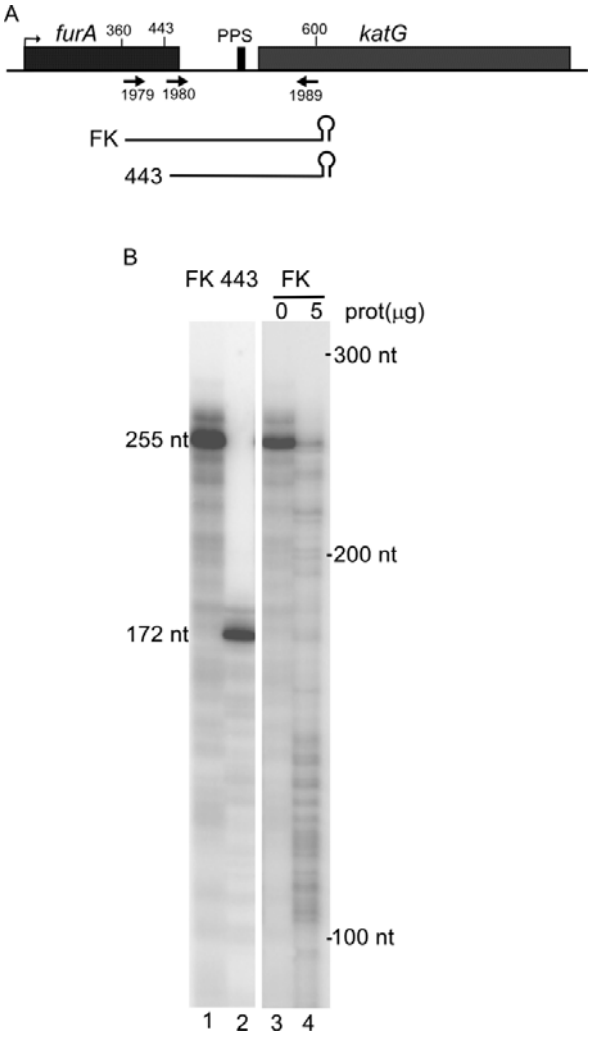


Fig.13 ***In vitro*** **processing assay of the** ***M. smegmatis furA-katG*** **mRNA. A. Schematic representation of the artificial transcripts.** The scheme is not in scale. The template for the FK (255 nucleotides) and the 443 (172 nucleotides) transcripts were obtained by PCR amplification with oligos 1979-1989 or 1980-1989, respectively, on chromosomal DNA. **B. Processing assay with M. smegmatis crude extract.** Random labeled RNA syntheses were performed in the presence γ^{32}P-UTP. Samples of FK transcript (150 pmol) were incubated in the presence (5 μg, lane 4) or absence (lane 3) of crude extract for 30 minutes at 37°C. The samples were separated by gel electrophoresis on a 6% denaturing polyacrylamide gel. Lanes 1-2: FK (255 nucleotides) and 443 (172 nucleotides) control transcripts. RNA molecular weight markers run in the same gel are indicated on the right.

1.2 Construction of the *rne* and *rnj* conditional mutants

1.2.A Identification of mycobacterial endoribonucleases

First, we decided to search on the *M. tuberculosis* and *M. smegmatis* total genomes, the presence of endo- and exoribonucleases, on the basis of previously characterized genes or by homology analysis with the known *E. coli* and *B. subtilis* ribonucleases (**Tab. 1**). In particular, we decided to concentrate our search on endoribonucleases that cleave single stranded RNA regions, since this was a characteristic feature of the endoribonuclease involved in the *furA-katG* RNA processing(Sala et al., 2008). Between all ribonucleases, we choose two as good candidates: RNase E/G and a RNases J1/J2 orthologs. Both ribonucleases process single-stranded regions on a RNA substrate (Ehretsmann et al., 1992; Even et al., 2005; McDowall et al., 1994).

The other endoribonucleases present in both *M. tuberculosis* and *M. smegmatis*, RNase III, RNase P and RNase Z are either double-stranded RNA (dsRNA) endonucleases or more specifically involved in tRNA maturation processes (Blaszczyk et al., 2004; Pellegrini et al., 2003; Schedl et al., 1976).

In a first attempt we decided to concentrate our attention on RNase E and RNase J. It appeared also interesting that both endoribonucleases are present in Mycobacteria. It was previously demonstrated that in *E. coli*, which lacks RNAse J, RNase E is essential (Jain et al., 2002), whereas in *B. subtilis*, which lacks RNase E, RNase J1 is essential (Even et al., 2005). Until now, it was assumed that gram-negative and gram-positive bacteria follow different RNA degradation/maturation pathways. Mycobacteria in which both nucleases coexist may give some new perspective of the RNA processing picture.

	E. coli	*B. subtilis*	*M. smegmatis*	*M. tuberculosis*
Endoribonucleases				
RNase E	+	-	+	+
RNase G	+	-	-	-
RNase III	+	+	+	+
Mini III	-	+	-	-
RNase M5	-	+	-	-
RNase P	+	+	+	+
RNase Z	+	+	+	+
RNase I	+	-	-	-
RNase Y	-	+	-	-
RNase J	-	+	+	+
Exoribonucleases				
RNase J	-	+	+	+
PNPase	+	+	+	+
RNase T	+	-	_	-
RNase R	+	+	-	-
RNase II	+	-	-	-
RNase PH	-	+	+	+
YhaM	-	+	-	-
Oligoribonuclease	+	-	+	+

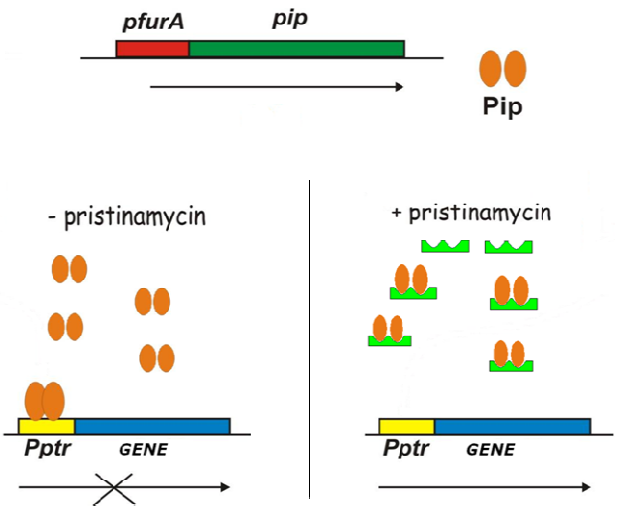
1.2.B Construction of conditional expression mutants in *M. smegmatis* RNAse E and RNAse J orthologous genes

In order to study the possible involvement of the two RNAses in *furA-katG* RNA processing, we decided to construct mutants in the two *M. smegmatis* genes. The genes to be mutated were MSMEG_2685, now renamed *rnj* (see later), whose gene product is homologous to RNase J, and *rne*, encoding RNase E.

Since one or both genes may be essential, we decided to construct conditional expression mutants for either gene. For this purpose we used the inducible system developed by Forti et al. (Forti et al., 2009) based on the *ptr* promoter derived from *Streptomyces pristinaespiralis*, which is repressed by the *Streptomyces coelicolor* Pip protein (Blanc et al., 1995; Folcher et al., 2001; Salah-Bey and Thompson, 1995). The streptogramin pristinamycin I is the inducer of the system (**Fig. 14**) (Barriere and Pribble, 1994). This system was tested in *M. tuberculosis* and in *M. smegmatis* and found to allow a strong induction and to be highly repressed in the absence of the inducer(Forti et al., 2009).

To obtain the conditional mutants, the 5' terminal part of either the *rnj* or *rne* gene was cloned downstream of the *ptr* promoter in plasmid pMYS823 and pMYS820 respectively (see Experimental procedures). The obtained plasmids are unable to replicate in mycobacteria, thus can be maintained only if they integrate into the bacterial chromosome.

M. smegmatis strain mc^2155 was transformed with the plasmids carring the hygromycin cassette and selected for hygromycin resistance. The Hyg^R clones obtained were then tested for the presence of the plasmid by PCR, in order to be sure that the chromosomal copy of the relative gene is under the control of the *ptr* promoter. Clones in which either the *rnj* gene or the *rne* gene were integrated by homologous recombination under the control of the *ptr* promoter were obtained (**Fig. 15**)



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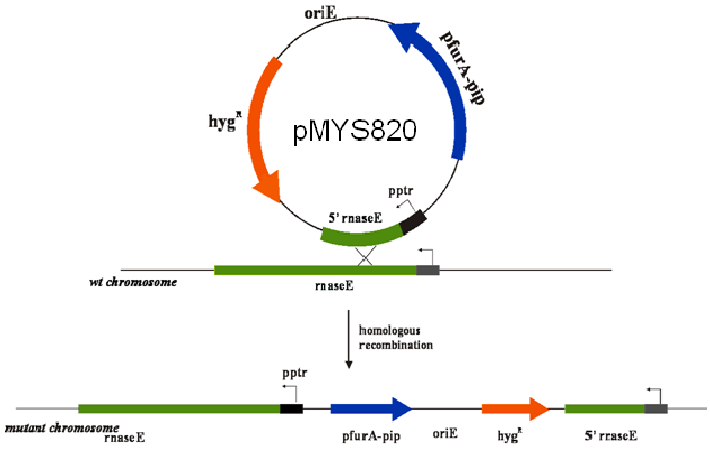

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1.2.C Growth analysis of *M. smegmatis rne* and *rnj* conditional mutant strains

The growth of *rne* and *rnj* mutant strains was analyzed in the presence/absence of inducer: ten-fold serial dilutions of log-phase cultures were plated on solid media. In the presence of the inducer, the mutants grew as the wild-type strain (**Fig. 16A**, I), while in its absence, the *rnj* mutant plated as well as the wild type strain, but the *rne* mutant did not grow (**Fig.16A**, II).

In liquid media we obtained similar results. The *rne* mutant stopped growing about 7 generations after removal of the inducer while the *rnj* conditional mutant grew equally well in the presence or absence of the inducer (**Fig. 16B**). This indicated that in *M. smegmatis* RNase E is essential while RNase J is not.

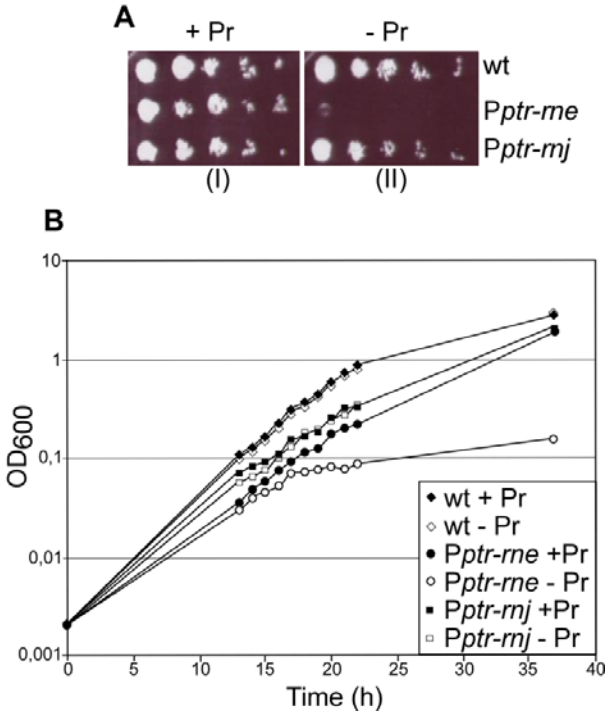


Fig. 16 Growth of conditional expression mutants in *M. smegmatis rnj* and *rne* genes. **A. Growth on solid medium.** Ten fold serial dilutions of log phase cultures of the wild type, *rne* conditional mutant (P*ptr-rne*) and *rnj* conditional mutant (P*ptr-rnj*) strains were replicated on LB-agar in the presence (I) or absence (II) of pristinamycin I (Pr). **B. Growth curves in liquid medium.** Precultures were grown in the presence of pristamycin I to an OD_{600} = 0.5, washed twice and diluted 1:200 in fresh medium, with or without pristinamycin I (Pr); the values reported start from the dilution point (time: 0).

1.2.D Construction of an RNase J null mutant in *M. smegmatis*

In order to definitely confirm that *rnj* is not essential we constructed an RNase J null mutant where the *rnj* gene was partially deleted. The restriction fragment SpeI-KpnI from pMYS824 was utilized to transform *M. smegmatis* and by double crossover, the central region of MSMEG_2685 on the chromosome (903 bp) was substituted by a hygromycin resistance cassette.

In this mutant 418 amino acids out of a total 558 amino acids (positions 140 to 558) were replaced by the hygromycin resistance cassette. The mutant strain was viable, thus confirming that *M. smegmatis rnj* does not encode an essential function in mycobacteria.

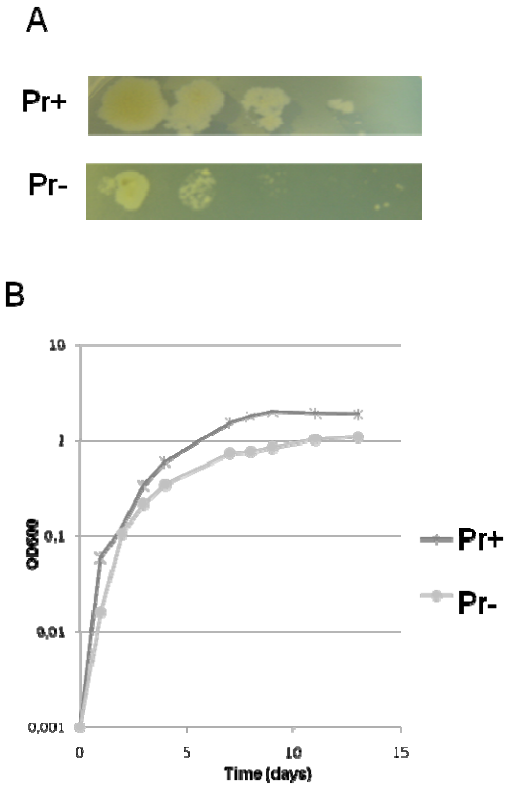
1.2.E Construction of a conditional mutant in *M. tuberculosis rne* gene

In *M. tuberculosis* the *rne* gene has been previously characterized and found to be essential (Sassetti et al., 2003). We decided to construct a conditional mutant, using the same *ptr-pip* inducible system used for the construction of the mutants in *M. smegmatis* (see above). The integration was done by transforming *M. tuberculosis* H37Rv strain with the plasmid pMYT832, and selecting for Hyg^r clones. The transformed colonies were tested by PCR to confirm plasmid integration into the *rne* gene.

The growth of *rne* mutant strain was analyzed in the presence/absence of inducer: ten-fold serial dilutions of log-phase cultures were plated on solid media. In the presence of the inducer, the mutants grew well (Pr +, **Fig. 17A**), while in its absence the *rne* mutant grew poorly (Pr -, **Fig. 17A**).

In liquid media, in absence of the inducer the *M. tuberculosis rne* mutant grew slowly compared to the induced mutant, even if it didn't stop completely to grow (**Fig. 17B**).

The results confirmed that the RNase E function of *M. tuberculosis* is essential.



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1.3 Analysis of the *furA-katG* transcript maturation in the *rne* and *rnj* mutant strains

In order to gain more insights into the respective functions of RNases E and J in the maturation of the *furA-katG* mRNA, we studied the role of these two ribonucleases using the *M. smegmatis* conditional mutant strains.

Primer extension experiments to identify the 5' ends of the transcripts synthesized by *M. smegmatis furA-katG* region were performed using the 244 primer, located 53 bp downstream of the *katG* start codon (**Fig. 18A**; as described in Experimental procedures). As shown previously (Milano et al., 2001), in the wild type strain several signals were present: in particular, signals around coordinate 478 (three bands could be identified), a signal at coordinate 443, and two minor bands at coordinates 328 and 224 (**Fig. 18B**, lanes 1-2). The same signals were also detected in the *ptr/rne* strain in the presence of pristinamycin I (**Fig. 18B**, lanes 3-5). On the contrary, the mutant strain in the absence of pristinamycin I (when RNase E was depleted, although not eliminated) presented only a faint band at coordinate 443; the signal at 478 was intensified, whereas the other signals have the same intensity of the wild type (**Fig. 18B**, lanes 6-9). Moreover, a signal corresponding probably to the 5' end of the *furA* transcript appeared indicating that depletion of RNase E increased the stability of the entire bicistronic transcript. These results suggest that RNAse E is responsible of cleavage at 443.

The apparent intensification of the signal correspondent to the 5' end at the *pfurA* promoter was confirmed by a primer extension experiment using the V26 primer, located 88 bp downstream of the 5' end of *furA* (**Fig. 18A**). The results are shown in Figure 16C. In the wild type strain and in the *ptr-rne* mutant in the presence of pristinamycin I, a faint signal corresponding to the 5' end of *furA* appeared (**Fig. 18C**, lanes 1-2). When RNase E was depleted (**Fig. 18C**, lane 3) a strong signal was evident indicating that the amount of the *furA* transcript is increased.

Finally, the deletion of the RNase J caused only slight effects on the band pattern: a weak increase of the signal at the coordinate 443, the disappearance of the 328 nucleotide long precursor and the appearance of a new 5' end at coordinate 314 (**Fig. 18B**, lanes 10-11).

In conclusion, we demonstrated that RNase E is the endoribonuclease responsible of the *furA-katG* transcript maturation, which cleaves the bicistronic transcript at coordinate 443.

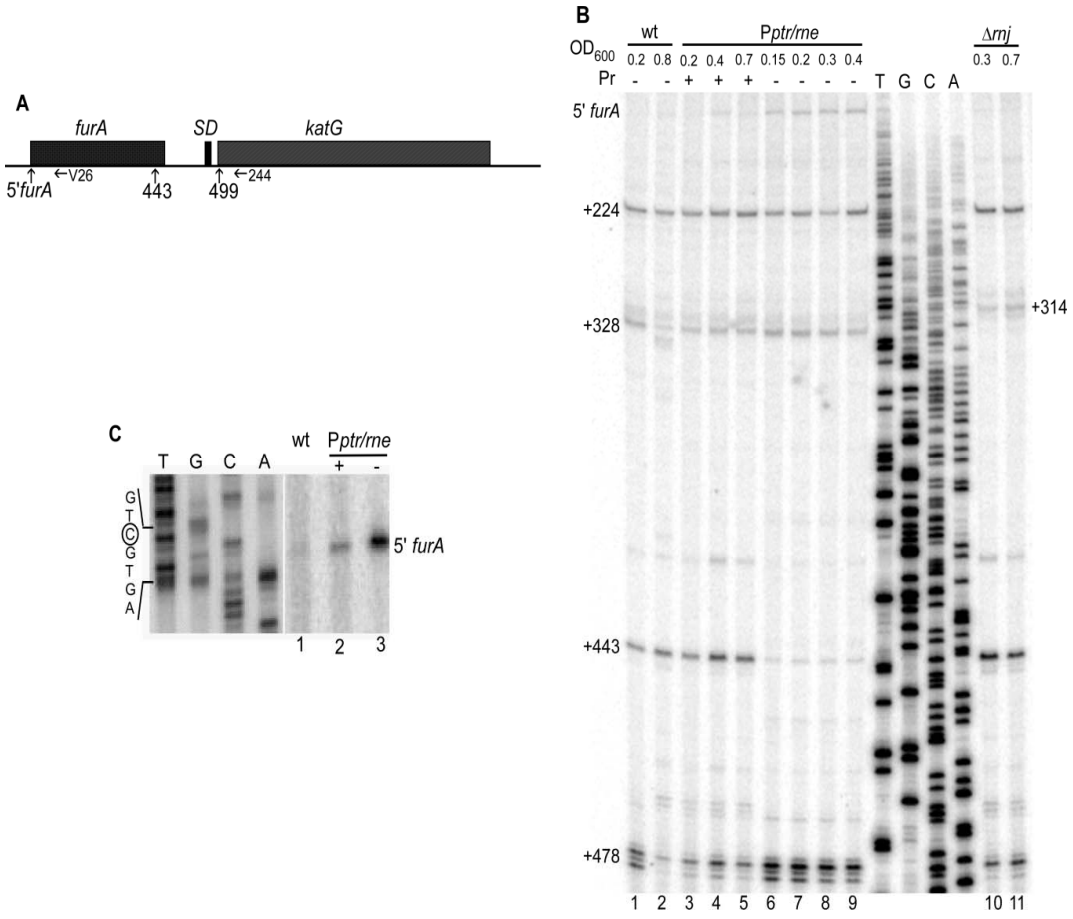


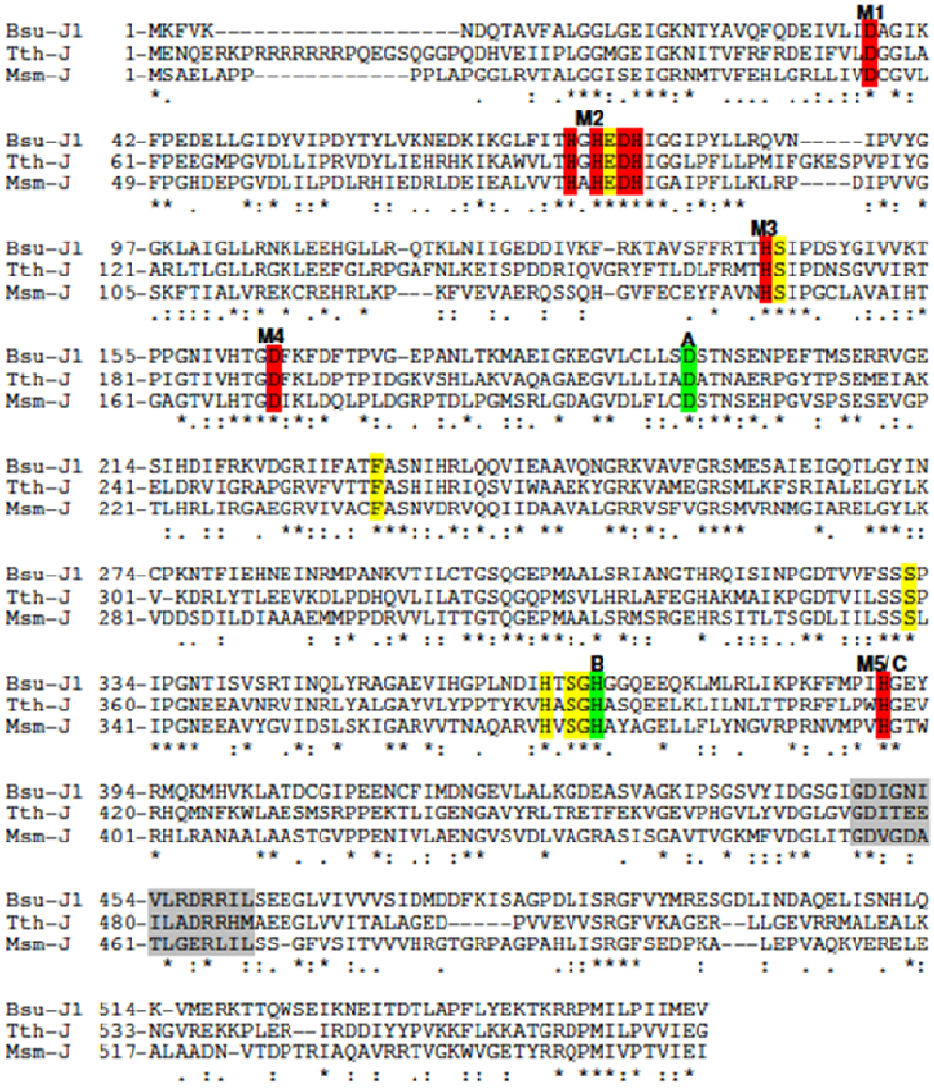
Fig. 18 *In vivo* analysis of *M. smegmatis furA-katG* mRNA processing. The primer extensions were carried out on the wt, RNase E mutant (P*ptr/rne*) in presence (+) or absence (-) of pristinamycin I (Pr) and RNase J (Δ*rnj*) mutant strains. Samples were migrated on a 5% denaturing polyacrylamide gel with sequencing ladders created with the same oligos used for the primer extension. The coordinates are referred to the transcription start point of *furA* (+1). **A. Scheme of *furA-katG* region.** The positions of the oligos are indicate by horizontal arrows. The figure is not in scale. **B. Analysis of *katG* transcript maturation.** The oligo 244 was used for primer extension experiments. For each strain, the analysis was performed at different OD_{600} reported on top of the gel. Numbers at the sides of the gel indicate the position of the signals on the sequence. **C. Identification of the 5' end at P*furA*.** The oligo V26 was used for the primer extension. The sequence around the 5' end of *furA* (marked) is reported at the side of the gel.

2. *M. smegmatis* RNase J ortholog characterization

2.1 *M. smegmatis* encodes a protein with high similarity to *B. subtilis* RNases J1/J2

RNases J1/J2 were discovered in *B. subtilis* but orthologous enzymes are present in about half of the sequenced bacterial and archeal genomes (Even et al., 2005). Here, we performed a BLAST search with the *B. subtilis* RNases J1 and J2 sequences against protein sequences encoded by the *M. smegmatis* mc^2155 chromosome. A single gene product (MSMEG_2685) shows a high similarity over its entire length with both RNases J1 and J2 : the 558 amino acids long mycobacterial protein shares 35% identical residues with RNase J1 (555 amino acids, 55% similarity) and 33% identical residues with RNase J2 (555 amino acids, 59% similarity). Analysis of the amino acid sequence revealed the presence of all known motifs important for the ribonuclease activity (Li de la Sierra-Gallay et al., 2008) and clearly categorizes this mycobacterial protein as a member of the β-CASP subfamily of zinc-dependent metallo-β-lactamases (**Fig. 19**) (Callebaut et al., 2002).

Based on the recently resolved 3D structure of the *Thermus thermophilus* RNase J (**Fig. 20A)**, (Li de la Sierra-Gallay et al., 2005) we deduced the 3D structures of *B. subtilis* RNase J1 (**Fig. 20B**) and the putative *M. smegmatis* RNase J (**Fig. 20C**). The three structures are very similar and can practically be superposed. Minor differences observed for the mycobacterial protein with respect to the other two RNases include a somewhat less structured linker (shorter α-helix) and C-terminal domain where only the straight part of the kinked helix is present.


M1
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M3
M4
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B
M5/C

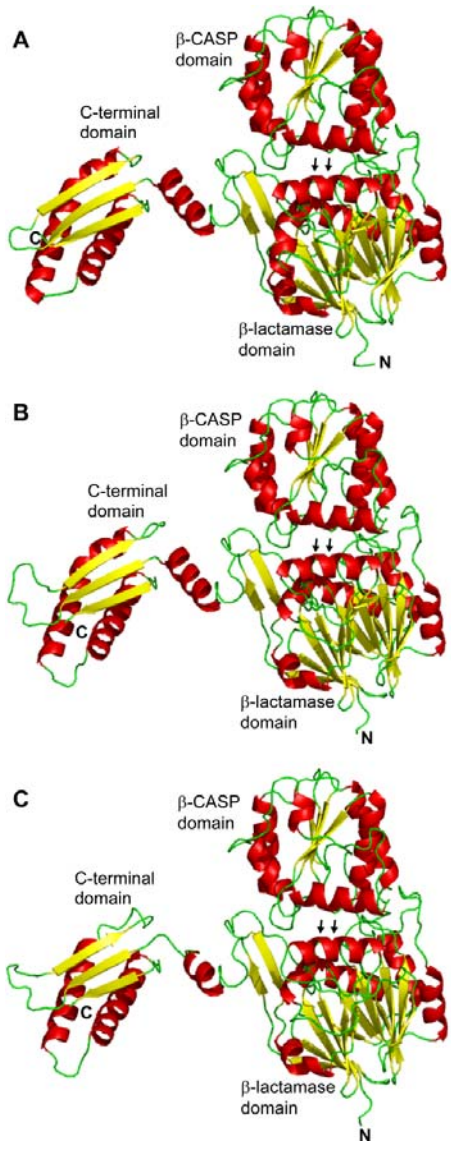


Fig. 20 Three-dimensional structures of the RNase J monomer. The RNase J sequence of (A) *T. thermophilus,* (B) *B. subtilis* and (C) *M. smegmatis* were submitted on the ExPASy proteomics server and the obtained structures were elaborated with the program pyMOL. The N- and C-terminal ends of the proteins are indicated by the letters N and C, respectively. The different domains are indicated. The position relative to the amino acids changed in the mutated protein are indicated by arrows.

2.2 The MSMEG_2685 protein has endoribonucleolytic activity

The *B. subtilis* RNases J1 and J2 were initially identified as 5' end dependent endoribonucleases which can cleave the *thrS* leader mRNA (Even et al., 2005). Since the *B. subtilis thrS* leader is the only well characterized substrate to monitor the endoribonucleolytic activity of RNase J we used it for *in vitro* assays with the *M. smegmatis* enzyme. The *thrS* substrate labeled at its 5' end with γ-^{32}P-GTP was synthesized by *in vitro* transcription of the *thrS* leader region giving a readthrough transcript of 350 bases and a prematurely terminated transcript of 280 bases (Even et al., 2005) as well as some aborted transcripts. It was incubated with the purified MSMEG_2685 protein for up to 20 min (see Experimental procedures). Two major cleavage products, whose signal intensity increased with the time of incubation, were observed (**Fig. 21A**, lanes 2-5). They correspond to a cleavage close to the 5' end of the transcript in an unstructured region, at the approximate positions +3 and +7 (5' GGG/AGAT/AAGAAAGACACACG). These are not the major endonucleolytic cleavage products observed with the *B. subtilis* RNases J1/J2 on a similar substrate (Even et al., 2005; Mathy et al., 2007) but indicate that the MSMEG_2685 protein has endonucleolytic activity.

In order to confirm that the endoribonucleolytic activity was carried by the mycobacterial protein MSMEG_2685, we mutagenized two amino acids, aspartate 85 to lysine and histidine 86 to alanine. These amino acids are conserved in the *B. subtilis* and the *T. thermophilus* RNase Js (**Fig. 19**), and in the 3D structure of the *T. thermophilus* enzyme both are directly implicated in the coordination of the Zn^{+} ion in the catalytic center (arrows, **Fig. 20**). Mutations in either amino acid in *B. subtilis* RNase J1 severely impaired both its endo- and 5'-3' exoribonucleolytic activities(Li de la Sierra-Gallay et al., 2008). The *M. smegmatis* mutated protein was purified and its endonucleolytic activity tested on the *thrS* transcript (**Fig. 21A**, lanes 7-10). No endonucleolytic activity was observed in the presence of the mutated protein, thus confirming that the cleavages of the *thrS* leader were generated by the MSMEG_2685 protein.

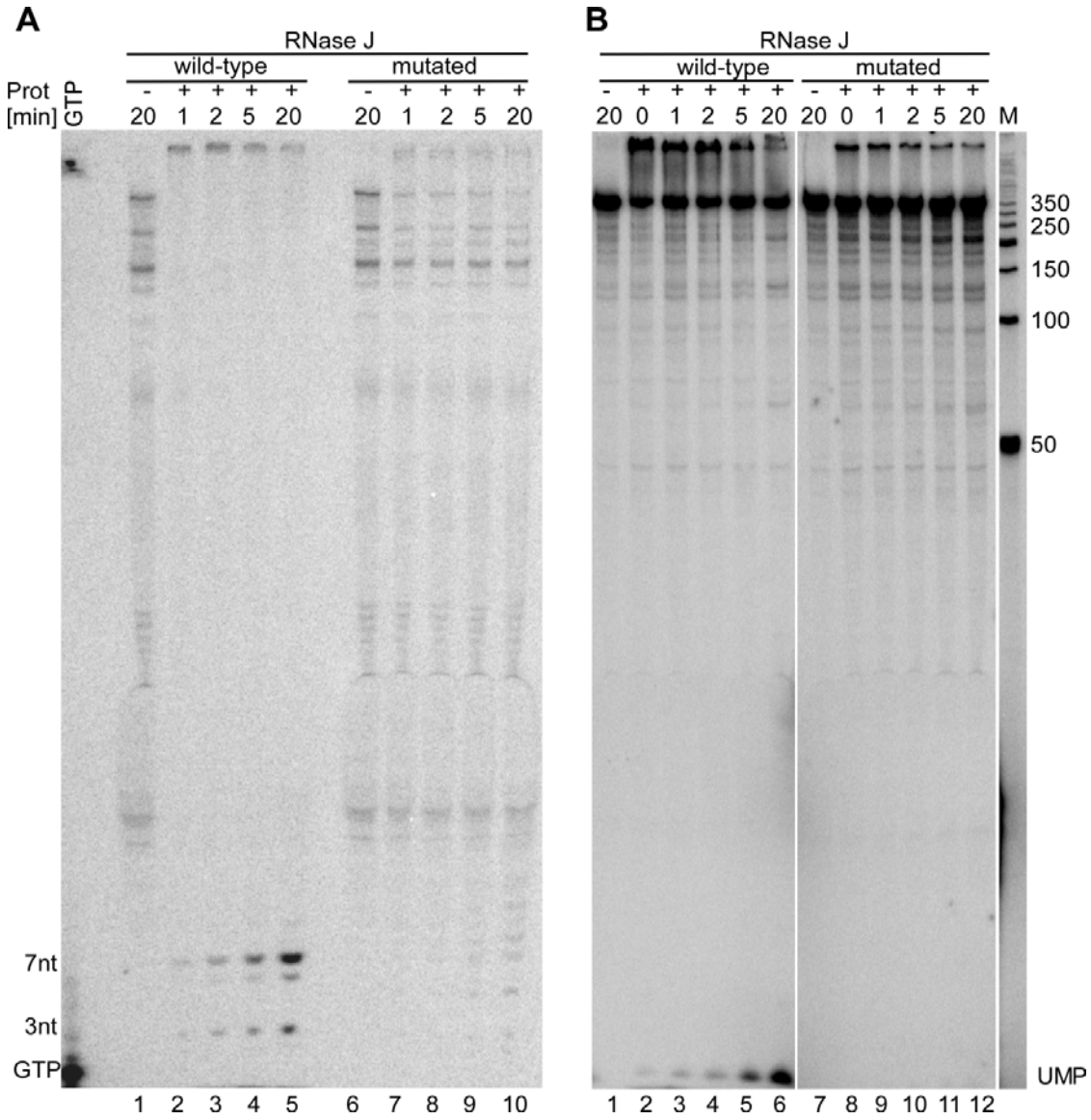


Fig. 21 *M. smegmatis* RNase J endo- and exo-ribonucleolytic activity *in vitro* test. The *B. subtilis thrS* leader transcript obtained by PCR reaction, as described in Experimental procedures, was used as substrate for the reactions. Both the wild type and the mutant RNase J proteins were used. Each reaction was stopped at the time indicated on top of the lanes (in min) and the reaction products were separated on a 20% (w/v) denaturing polyacrylamide gel. **A. Activity on a triphosphate transcript.** The 5'-PPP *thrS* leader transcript labeled at its 5'-end with γ-^{32}P GTP was incubated with the wild-type RNase J (lanes 2-5) and the active site mutated (D85K and H86A) protein (lanes 7-10). As a control the transcript was incubated for 20 min with the reaction buffer (lanes 1 and 6). The positions of the 3 and 7 nucleotide fragments and the GTP position (GTP lane of the gel) are indicated on the left. **B. Activity on a monophosphate transcript.** Internally labeled 5'-monophosphorylated *thr*S leader mRNA (the 350 nt readthrough RNA band was purified) was incubated with wild-type RNase J (lanes 2-6) and the active-site mutant (D85K and H86A) proteins (lanes 8-12). The transcript was incubated for 20 min with the reaction buffer as control (lanes 1 and 7). The numbers at the right of the gel indicate the different size of the ladder and the size of the transcript; UMP indicate the free mononucleotides produced by the exonucleolytic activity of RNase J.

2.3 The mycobacterial RNase J has 5'-3' exoribonucleolytic activity dependent on the 5' phosphorylation state of the substrate.

In contrast to its endonucleolytic activity the exonucleolytic activity of the *B. subtilis* RNase J1 requires a monophosphorylated 5' end substrate (Li de la Sierra-Gallay et al., 2008). We used the same purified 350 bp *thrS* leader read through transcript in its 5'-monophophorylated version and labeled uniformly with α-^{32}P-UMP to detect a potential 5'-3' exoribonucleolytic activity of *M. smegmatis* RNase J. The reaction conditions were identical to those used for the endonucleolytic cleavage assay. The addition of wild-type *M. smegmatis* RNase J to the reaction led to the accumulation of mononucleotides in a time dependent manner (**Fig. 21B**, lanes 2-6). The mutated RNase J had lost all activity (**Fig. 21B**, lanes 8-12).

All data clearly demonstrate that the mycobacterial enzyme MSMEG_2685 has both endo- and exoribonucleolytic activities, similar to the *B. subtilis* RNases J1. We renamed the gene *rnj* and its product RNase J.

3. Ribosomal RNA maturation

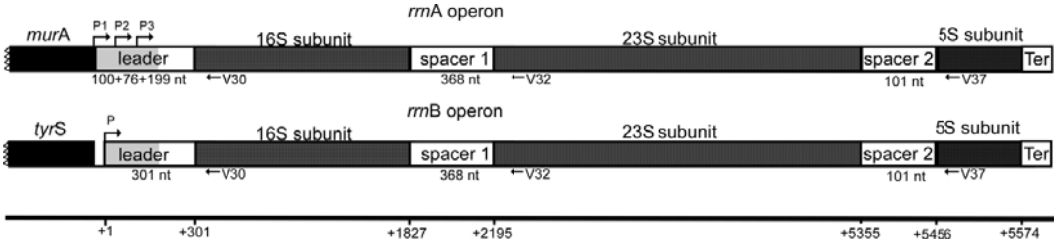
3.1 *M. smegmatis* rRNAs operons

RNase E and RNase J have important roles in ribosomal RNA maturation in *E. coli* and *B. subtilis,* respectively (for a recent review see (Deutscher, 2009)). This again reflects the different set of ribonucleases present in these model organims (see **Table 1**).

In *Mycobacteria*, rRNA processing has not been studied in much detail (Ji et al., 1994a; Ji et al., 1994b; Ji et al., 1994c). In *M. tuberculosis* a single rRNA operon is present (*rrnA*) whereas *M. smegmatis* has two operons (*rrnA* and *rrnB*) (**Fig. 22**). Both operons contain the genes for the 16S, 23S and 5S rRNAs, co-transcribed as a single precursor molecule (Gonzalez-y-Merchand et al., 1996; Ji et al., 1994b). Co- or post-transcriptional folding of the precursor rRNAs is predicted to involve extensive base-pairing between the leader and spacer-1 region (16S), spacer-

1 and spacer-2 (23S), spacer-2 and trailer (5S) as suggested by the secondary structures predicted by the M-fold algorithm (**Fig. 24**) and as found in rRNA operons of other species. The *M. smegmatis rrnA* and *rrnB* operons have identical sequences downstream of position -165 with respect to the 16S rRNA structural gene. Thus the folded precursor structures that are the substrates for a series of maturation reactions to produce the mature rRNAs should be the same for both operons (**Fig. 22**).

Here we studied how the RNases E and J contribute and collaborate in the maturation of the 5' ends of the rRNA precursors in *M. smegmatis.*



3.2 Pre-16S rRNA maturation

Extension of primer V30, located downstream of the 16S 5'-end (**Fig. 22**), showed that in a wild type strain more than 90% of the 16S rRNA exists in its fully matured form (**Fig. 23A,** lane 1), although traces of full-length transcripts originating at the *rrnA* P2 and the *rrnB* promoter could be detected. A significant amount of a precursor 16S rRNA with a 5'-end at position +147 could

also be detected. This processing site lies within a region forming a double stranded stem made up of sequences upstream of the 16S rRNA and sequences of the spacer 1 region (between the 16S and 23S rRNA, **Fig. 22** and **24A**). The presence of a concomitant cleavage observed on the opposite strand within a bulge and shifted by two nucleotides (position +1977, **Fig. 23B**, see below) strongly suggested a maturation by the double-strand specific RNase III (**Fig. 24A**). Indeed, this configuration is reminiscent of RNase III cleavages observed in similar positions in the long processing stalks of 16S rRNA precursors of *E. coli* (Young and Steitz, 1978) and *B. subtilis* (Herskowitz and Bechhofer, 2000; Loughney et al., 1983).

Inactivation of the *rnj* gene had no major effect on 16S maturation (**Fig. 23A**, lane 2) and only caused a weak stabilization of a precursor containing 36 nt additional nucleotides (position +266, see also below).

By contrast, partial depletion of RNase E caused a two-fold increase in full-length precursors and the putative RNase III processing product (position +147) but also a strong accumulation of the +36 nt precursor (position +266, **Fig. 23A**, lane 4). The same precursor was also weakly detected in the *rnj* null mutant (see above **Fig. 23A**, lane 2) suggesting that this transcript is generated through cleavage by a yet unidentified ribonuclease. This accumulation of rRNA precursors in the RNase E depleted strain is consistent with RNase E being able to create the mature 5' end. The putative cleavage site at the beginning of 16S rRNA is composed of a U rich single stranded region (**Fig. 23A**) compatible with the known requirements for RNase E cleavage in *E. coli* (Ehretsmann et al., 1992; McDowall et al., 1994) and *M. tuberculosis*(Zeller et al., 2007).

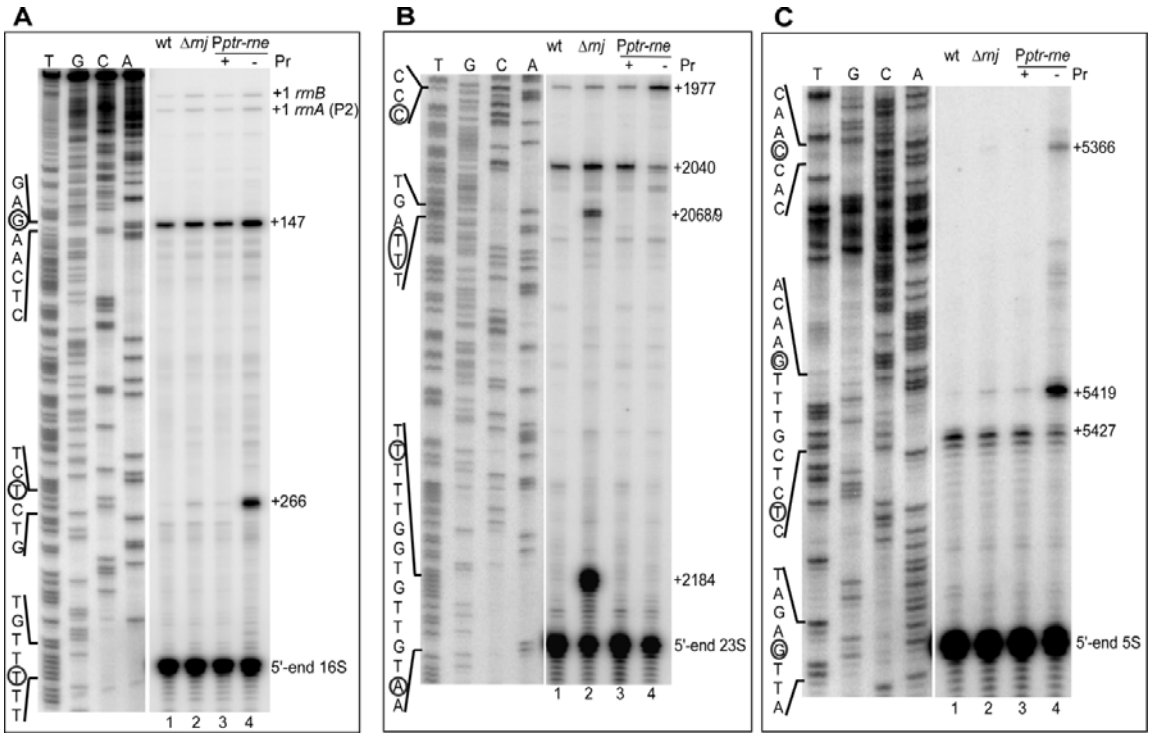


Fig. 23 Analysis of the 5' end maturation of the rRNA precursors. Primer extension experiments were performed as indicated in Experimental procedures on *M. smegmatis* strain mc^{2}155 (wt), the *rnj* null mutant (Δ*rnj*), and the P*ptr-rne* mutant both in the presence and in the absence of the inducer (Pr), as indicated on top of the lanes, using specific primers (V30, V32, and V37, in A, B, and C, respectively). The total RNA of the P*ptr-rne* mutant without pristinamycin I was extracted from cultures after about 7 generations. The extension products were separated on a 5% (w/v) denaturing polyacrylamide gel. The first 4 lanes of each gel report the DNA sequences obtained with the same primers on the *rrnB* operon. On the left, the sequences of the regions surrounding the signals found by primer extension (circled nucleotides), are reported. On the right the dimensions of the signals obtained in the different reaction are indicated (the coordinates are relative to the +1 of the *rrnB* operon). **A. Analysis of the 16S rRNA.** The sequence comprises the first 90 nt of the mature 16S rRNA and all the leader region of the *rrnB* operon. **B. Analysis of the 23S rRNA.** The sequence comprises the last 210 bp of the 3'end of 16S, all the spacer 1 region and the first 71 bp of the 23S rRNA 5'end. **C**. **Analysis of the 5S rRNA.** The sequence comprises the last 103 bp of the 23S rRNA 3'end, all the spacer 2 region and the first 55 bp of the 5S rRNA 5'end.

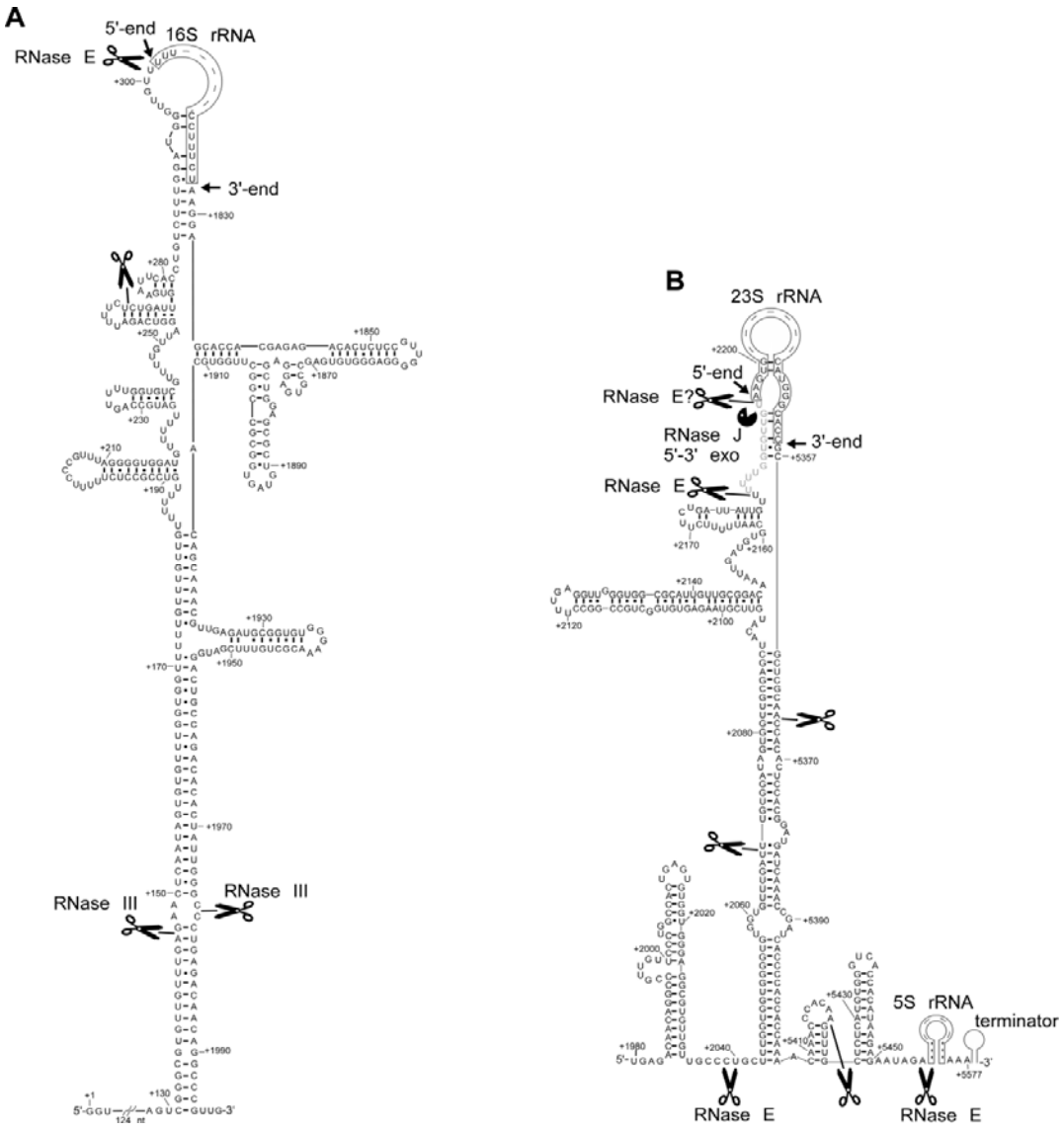


Fig. 24 Secondary structures of the rRNAs precursors. The structures were elaborated from that released by M-fold. All the coordinates are referred to the starting of transcription from the *rrnB* promoter (+1). The scissors indicate the location of the signals found by primer extension. Where known, the name of the RNase responsible of the cleavage is indicated. **A. Pre-16S rRNA structure.** The framed region corresponds to the 16S mature rRNA. The region upstream of the 5'end corresponds to the leader region. The downstream region corresponds to the first half (170 nt from the 3'end of 16S) of the spacer 1 region. **B. Pre-23S-5S rRNA structure.** The circled regions correspond to the 23S and 5S mature rRNAs. The region upstream of the 5'end of 23S corresponds to the second half (215 nucleotides) of the spacer 1 region. The downstream region corresponds to the spacer 2 and the 5S rRNA. In grey is reported the 12 nucleotides region supposed to be degraded by the 5'-3' exonucleolytic activity of the RNase J.

3.3 Pre-23S rRNA maturation

The 23S rRNA structural gene lies downstream of the 16S rRNA gene separated by a 368 nt region (spacer 1, **Fig. 22**) that does not encode any known genes. Extension of primer V32, downstream of the 23S 5'-end, revealed that in the wild type strain about 90% of the 23S rRNA is fully processed (**Fig. 23B**, lane 1). Two minor upstream signals were observed at positions +1977 and +2040. The signal at +1977 (containing 218 nt upstream of the mature 23S 5'-end) is most likely due to RNase III causing a double strand cleavage of the 16S rRNA precursor that separates the 16S and 23S rRNAs on the original transcript (**Fig. 24A**); cleavage of the opposite strand occurs at position +147 as described above.

The 23S precursor RNA with a 5' end at position +2040 was present at significantly lower levels in a strain depleted for RNase E (**Fig. 23B**, lane 4) suggesting that this transcript is generated by an RNase E cleavage. It is located in the single stranded region between two extensive secondary structures of the 23S precursor (**Fig. 24B**). In accordance, the upstream precursor likely generated by RNase III (position +1977) is present at higher levels when RNase E expression is reduced (**Fig. 23B**, lane 4). Furthermore, depletion of RNase E also caused a more than two-fold reduction of the mature 23S rRNA level (**Fig. 23B**, lane 4). This would be in agreement with RNase E being capable of directly generating the 5'-end of mature 23S rRNA.

The absence of RNase J has a major effect on the 23S rRNA maturation. Under these conditions about 50% of the total 23S rRNA accumulates as a +12 nt precursor (position +2184, **Fig. 23B**, lane 2 and **Fig. 24B**). This suggests that about half of the 23S rRNA precursor is cleaved 12 nt upstream the mature 5' end by an endoribonuclease, (probably RNase E), and then degraded to the mature 23S rRNA by the 5'-3' exonucleolytic activity of RNase J.

The *rnj* null mutation also caused a small but significant accumulation of two closely spaced precursors containing an additional 127/128 nucleotides (positions +2068/2069). Together with a signal observed at position +5366 on the opposite strand (see below, **Fig. 23C,** lane 4) we are

probably seeing here the remnants of a precursor generated by RNase III processing within the 23S processing stalk aimed at separating the 23S and 5S rRNAs (**Fig. 24B**).

Depletion of RNase E also causes a 50 % decrease in mature 23S rRNA as well a two-fold reduction of a precursor containing 155 additional nucleotides (position +2040, **Fig. 23B**, lane 4). By contrast, the concentration of the initial precursor following RNase III cleavage is increased under these conditions. This strongly suggests that RNase E generates the +12 nt precursor, a notion corroborated by the sequence and structural context of the cleavage site (single stranded U rich region, **Fig. 24B**). Similarly, the +155 nt precursor (position +2040) is likely also due to RNase E cleavage and then degraded by RNase J, since we observed a two-fold accumulation in the *rnj* mutant (**Fig. 23B**, lane 2).

Half of the 23S rRNA is still found in its mature form in the absence of RNase J suggesting that the mature 5' end can be generated by direct cleavage. Based on our observations the most likely candidate enzyme for this final maturation step, which occurs in a single stranded AU rich environment, is again RNase E.

3.4 Pre-5S subunit maturation

The 5S rRNA structural gene is separated from the upstream 23S rRNA gene by a 101 nt spacer. Primer extensions with the V37 primer in the wild type strain showed that about 95% of the total RNA corresponded to that of the mature 5S rRNA (**Fig. 23C**, lane 1). A precursor rRNA 29 nt longer than the mature 5S rRNA (position +5427) was present in minor quantities. Disruption of the *rnj* gene had no significant impact on the processing of the 5S rRNA (**Fig. 23C**, lane 2). Depleting RNase E also did not substantially reduce the extent of fully processed 5S rRNA (**Fig. 23C**, lane 4). However, under these conditions two new precursor 5S rRNA species appeared. The first (position +5366) likely corresponds to the RNase III generated precursor described above, together with a cleavage on the opposite strand at position +2068/2069 (**Fig. 24B**). The

most prominent precursor detected during RNase E depletion contained 37 additional nucleotides (position +5419). The data are compatible with a direct maturation of the 5S rRNA 5' end by RNase E using either the +37 nt precursor and possibly also longer precursors. Due to the presence of almost wild type levels of mature 5S rRNA under these conditions it is likely that another nuclease besides RNase E is involved in the final processing steps. This unknown nuclease might also account for the +37 nt precursor.

DISCUSSION

4. Discussion

RNases are important enzymes that intervene in the degradation, processing and quality control of all RNA species in the cell. Current knowledge suggests that the RNases E, J and Y are key players in eubacterial RNA metabolism. Individually, orthologues of each of these nucleases are potentially present in about 40 to 50 % of the sequenced eubacterial species (Condon and Putzer, 2002; Even et al., 2005; Shahbabian et al., 2009). In other words, all known eubacteria contain at least one of these three nucleases and several of them have all three (Shahbabian et al., 2009). The presence or absence of these key ribonucleases can provide important insights into the strategies used by different bacterial species to organize their RNA metabolism. For example, the two best studied organisms *E. coli* and *B. subtilis* rely on very different sets of enzymes, namely RNase E in *E. coli* and RNases J1/J2 and Y in *B. subtilis,* which fulfill essential functions in mRNA metabolism and ribosomal RNA maturation in the respective bacteria. Mycobacterial RNA metabolism has not been well studied but these high GC gram positive organisms contain an RNase E/G type enzyme and a putative RNase J ortholog. In addition, a compilation of potential RNase orthologs (**Table 1**) suggests that mycobacteria constitute an interesting class of bacteria that combine key enzymes present in *E. coli* and *B. subtilis*.

Mycobacteria can be divided into two classes : the slow-growers which include pathogens like *M. tuberculosis* and the fast-growers like *M. smegmatis.* It is noteworthy that the putative RNases present in these organisms are the same (**Table 1**). This implies that RNA metabolism is likely to be very similar and studies in *M. smegmatis*, which is nonpathogenic and simpler to handle than *M. tuberculosis*, should be pertinent for both species.

4.1 *In vitro M. smegmatis furA-katG* transcript processing.

Our interest in studying RNA processing moved from identification of the ribonuclease that processes the *furA-katG* transcript in *M. smegmatis* and *M. tuberculosis*. It was demonstrated previously(Sala et al., 2008)that in *M. smegmatis,* cleavage at a specific coordinate 443 required

single stranded RNA and a polypurine sequence downstream. In a first attempt to reproduce RNA cleavage *in vitro* using a crude extract of *M. smegmatis*, we obtained no specific cleavage at the coordinate observed *in vivo*, but degradation of the *furA-katG* transcript to small fragments. Thus we decided to study directly the effects of specific ribonucleases present in *M. smegmatis*. We choose RNAse E and RNase J, both present in *M. smegmatis* and *M. tuberculosis* and involved in single stranded RNA cleavage.

4.2 Mycobacterial mutants in ribonuclease E and J.

We firstly created conditional mutants for *M. smegmatis* RNase E/G type enzyme and the putative RNase J ortholog showing that they are respectively essential and not essential. The result was confirmed by constructing a deletion mutant in *M. smegmatis rnj*, which is fully viable. Furthermore, we constructed also an RNase E mutant in *M. tuberculosis* confirming that it is an essential protein, as previously demonstrated (Sassetti et al., 2003).

4.3 *In vitro M. smegmatis furA-katG* transcript processing.

Maturation of the *M. smegmatis furA-katG* operon in both mutants was analyzed demonstrating that normal processing at 443 occurs in the Δrnj mutant, whereas only a weak signal is present in the *ptr-rne* mutant in the absence of pristinamycin I.

It has to be noted that in our mutant RNase E is not completely absent, since the function of this gene is essential. However, we could demonstrate that depletion of this enzyme causes a severe reduction of the 443 signal and this has an effect on the amount of full length RNA. We suppose that when the RNase E concentration is not sufficient to complete maturation of the *furA-katG* transcript, degradation of the upstream part of the transcript is reduced, likely because of the lack of the free 3' ends to be attacked by the 3'-5' degradative activity of exoribonucleases. Thus, the incomplete maturation at 443 appears to stabilize the full length *furA-katG* transcript. The

apparent intensification of the downstream 478 signal could indicate that in the absence of cleavage at 443, the transcript is processed by a different set of ribonucleases.

Thus we demonstrated that the bicistronic furA-katG operon is processed by RNase E at 443, creating a stable katG transcript and an unstable furA RNA, subject to degradation

A minor role in *furA-katG* RNA maturation can also be assigned to RNase J, in particular this enzyme could be involved in the degradation pathway. In fact, some minor differences in the intermediates were observed in Δrnj compared to the wild type and we demonstrated in this work that beside its endonucleolytic activity, RNase J has a 5'-3' exoribonucleolytic activity that could contribute to the *furA-katG* transcript processing.

4.4 *In vitro* activity of *M. smegmatis* RNAse J

We characterized *in vitro* the MSMEG_2685 protein of *M. smegmatis*. Sequence and 3D structure comparisons of the *M. smegmatis* protein 2685 with known RNases J of *T. thermophilus* and *B. subtilis* (**Fig. 19** and **Fig. 20**) showed good similarities (55% and 59%, respectively with *B. subtilis* RNases J1 and J2), conservation of all important motifs, notably the five classical β-lactamase motifs, the three β-CASP motifs and the amino acids implicated in the coordination of the UMP nucleotide (Li de la Sierra-Gallay et al., 2008). However, this comparison provided no clues as to the major enzymatic activity. It was important to verify that the single putative *M. smegmatis* RNase J ortholog (MSMEG_2685) actually is an RNase and whether it has endo- and/or exoribonucleolytic activities. In fact, in *B. subtilis* both RNase J1 and RNAse J2 show a similar endoribonucleolytic activity (Even et al., 2005), but RNase J2 in contrast to RNase J1 has no significant 5'-3' exoribonucleolytic activity (Mathy et al., 2007). *In vitro* assays with the purified mycobacterial protein showed that it actually has both activities, 5'-3' exo- and endonucleolytic. The exonucleolytic activity is comparable to that of *B. subtilis* J1 when assayed on the same 5' monophosphorylated *B. subtilis thrS* leader substrate (Mathy et al., 2007). This strongly suggests that *M. smegmatis* RNase J, similarly to *B. subtilis* RNAse J1, can

act as a 5'-3' exoribonuclease *in vivo*. Moreover, similarly to RNA J1 of *B. subtilis* (Li de la Sierra-Gallay et al., 2008), also *M. smegmatis* RNase J exoribonucleolytic activity is inhibited by 5'-end triphosphorylated substrate.

Using the *B. subtilis thrS* leader region in its 5'-triphosphorylated form labeled at the 5' end with γ-^{32}P GTP we showed that *M. smegmatis* RNase J also has endonucleolytic activity. The major cleavages were observed at about 3 and 7 nucleotides from the 5' end (**Fig. 21A,** lanes 2-5) in a region supposed to be single stranded(Luo et al., 1998). Cleavage was efficient but occurred at a different site compared to the previously characterized sites for *B. subtilis* RNases J1 and J2 on the same substrate (Even et al., 2005; Mathy et al., 2007). The endonucleolytic activity of RNases J1/J2 is dependent on sequence and structural parameters that are still ill defined but which assure that cleavage *in vivo* apparently only occurs at a rather limited number of sites (Li de la Sierra-Gallay et al., 2008; Shahbabian et al., 2009). Moreover, it is likely that these parameters differ significantly between low GC *B. subtilis* and high GC *M. smegmatis* (43% and 67% GC content, respectively).

We noticed that about half of the exonucleolytic and all of the endonucleolytic substrate was retained in the wells of the 20% polyacrylamide gels used to resolve the exo- and endonucleolytic cleavage products of RNase J activity (**Fig. 21A** and **B**). This probably reflects strong binding of *M. smegmatis* RNase J to its substrate until after cleavage and release of the cleavage products. The sequestration of the substrates was much weaker when the reactions were carried out with a mutant protein where two amino acids (Asp85Lys and His86Ala) in the catalytic center (Li de la Sierra-Gallay et al., 2008) are altered. More importantly, the 5'-3' exo- and endonucleolytic activities of *M. smegmatis* RNase J are almost abolished in this mutant, thus confirming that the ribonucleolytic activities attributed to *M. smegmatis* RNase J are genuine and not due to contamination during protein purification.

RNase E and RNase J1 are essential for viability in *E. coli* and *B. subtilis,* respectively. These ribonucleases are clearly very important for RNA metabolism but the precise reason for their essentiality has not been established, neither for RNase E nor for RNase J1.

We show here that in *M. smegmatis* RNase E is essential while RNase J is not. This also shows that the 5'-3' exonucleolytic activity of RNase J is dispensable, at least in the presence of RNase E. It has been proposed that the major function for the exonucleolytic activity of RNase J in *B. subtilis* is the 5' to 3' degradation of RNA fragments protected by secondary structures (e.g. a transcription terminator) at their 3' ends (Shahbabian et al., 2009). In fact, in the absence of an efficient 3' polyadenylation pathway, as exists in *E. coli* (Hajnsdorf et al., 1995), such RNA fragments can accumulate to very high levels(Shahbabian et al., 2009), especially in an organism like *B. subtilis* where about 3 % of all genes are controlled by modulating premature transcription termination (Merino and Yanofsky, 2005). However, we found no obvious ortholog of the *E. coli* Poly A polymerase in *M. smegmatis* or *M. tuberculosis*. suggesting that mycobacteria have an alternative method of degrading structured RNA fragments.

4.5 Maturation of the 5' end of rRNA in *M. smegmatis*

Finally, we studied how both RNAse E and RNase J share the maturation of the ribosomal RNAs in *M. smegmatis.*

RNase E and RNase J have important roles in ribosomal RNA maturation in *E. coli* and *B. subtilis,* respectively (for a recent review see (Deutscher, 2009)). In Mycobacteria, rRNA processing has not been studied in much detail (Ji et al., 1994b; Ji et al., 1994c).

In this work we demonstrate a pathway for the 5' end maturation of each rRNA subunit, that showed differences from what observed in *B. subtilis* and *E. coli.*

A possible model for rRNA subunits maturation may be proposed:

-16S subunit: i) RNase III cleavage releases the 16S rRNA precursor from the 30S original transcript; ii) an unidentified nuclease cleaves at position +266 leaving 36 nt upstream of the mature 16S rRNA 5' end; iii) this shortened precursor is then processed by RNase E to create the mature 16S rRNA 5' end.

When we compare *M. smegmatis* 16S rRNA maturation to that from *E. coli* and *B. subtilis* (**Fig. 25**) we note that the strategy used resembles more that of *E. coli* than *B. subtilis*. In fact, despite its presence in *M. smegmatis,* RNase J is not involved in any significant way in the intermediate or final steps of 16S rRNA maturation. We can note however one intriguing similarity between the 5' processing pathways of 16S rRNA in these three organisms. In all cases, maturation appears to occur in a two-step mechanism : the initial RNase III precursors are processed by RNase E (*E. coli*, +66 nt) or unknown endoribonucleases (*B. subtilis,* +38 nt and *M. smegmatis*, +36 nt) apparently to create a new 5' end free from the processing stalk and increasing the efficiency of the final maturation steps at both the 5' and 3' extremities. RNase E/G and the 5'-3' exonucleolytic activity of RNase J might be expected to have a similar 5' end dependence and the intermediate processing step would thus facilitate the access of RNase G (*E. coli*) and RNase J1 (*B. subtilis*) to a single stranded monophosphorylated 5'end in order to perform the final maturation step. The fact that RNase J only plays a very minor role compared to RNase E suggests that the new 5' end is not accessible to RNase J or that the requirements for 5' end recognition (e.g. a free 5' monophosphorylated end, not blocked in a secondary structure) are quite different between the two *M. smegmatis* nucleases.

-23S subunit: i) RNase III causes a double strand cleavage of the 16S rRNA precursor that separates the 16S and 23S rRNAs on the original transcript; ii) RNase E is capable of directly generating the 5'end of mature 23S rRNA; iii) in absence of RNase J, 50% of the total 23S rRNA accumulates as a +12 nt precursor, indicating that half of the 23S rRNA precursor is

cleaved by an endoribonuclease, probably RNase E (see below), so that the +12 nt form accumulates because it is no longer trimmed by the 5'-3' exonucleolytic activity of RNase J.

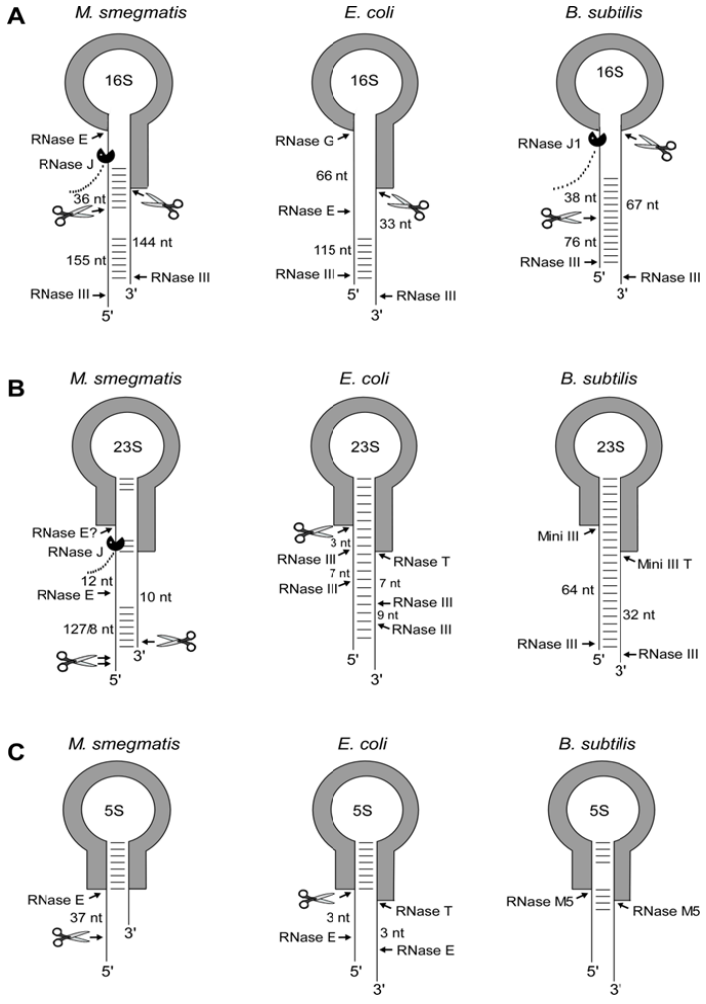
The fact that half of the 23S rRNA is still found in its mature form in the absence of RNase J indicates that the mature 5' end can be generated by direct cleavage. Based on our observations the most likely candidate enzyme for this final maturation step, which occurs in a single stranded AU rich environment, is again RNase E.

Maturation of the 23S rRNA in *M. smegmatis* is thus quite different when compared to both *E. coli* and *B. subtilis* (**Fig. 25**). In fact, neither RNase E nor RNase J, the two nucleases involved in the final steps of 23S rRNA maturation in *M. smegmatis*, participate in the processing of 23S rRNA in *E. coli* or *B. subtilis,* respectively.

-5S subunit: i) RNase J had no significant impact on the processing of the 5S; ii) RNase III probably cleaves and divides the 5S subunit from the 23S subunit; iii) RNase E matures directly the 5' end of the 5S rRNA. However, the presence of almost wild type levels of mature 5S rRNA in RNase E depletion suggests that another nuclease besides RNase E is involved in the final processing steps. This unknown nuclease might also account for the +37 nt precursor.

More data are needed to fully understand rRNA processing in Mycobacteria particularly at the 3' end. Nevertheless, our experiments clearly show that the mechanisms and enzymes involved in the 5' end processing of all three rRNAs are significantly different compared to what we know from the two model organisms *E. coli* and *B. subtilis.* From an evolutionary and mechanistic point of view it will be interesting to better understand how combinations of RNases present in a given organism influence and direct RNA metabolism. Detailed studies have so far been essentially based on *E. coli* and *B. subtilis* and might have exaggerated differences that do exist but which should probably not be viewed as dogmatic. For example, as we have seen in this study the 5'-3' exonucleolytic activity of RNase J is exploited in mycobacteria but, in contrast to

B. subtilis, is not important for cell survival. Studying RNA metabolism in Mycobacteria should thus be a useful approach to better understand how key enzymes like RNase E and the dual activity RNase J are integrated into a global strategy organizing RNA metabolism in a given organism.



MATERIAL AND METHODS

5.1 Bacterial strains and growth conditions

The prototrophic *M. smegmatis* mc^2155 (Snapper et al., 1990) was used for the construction of the mutants. In the strain called rnj101, the *rnj* (MSMEG_2685) gene was put under the control of the *ptr* promoter by Campbell-type integration of plasmid pMYS823. For strain *rnj102*, the *rnj* (MSMEG_2685) gene was partially deleted by double-crossover recombination with plasmid pMYS824. In the derivative strain *rne101*, the *rne* (MSMEG_4626) gene was put under control of the *ptr* promoter by Campbell-type integration of plasmid pMYS820. *M. tuberculosis* strain H37Rv (laboratory stock) was used for mutagenesis experiments.

The following *E. coli* strains were used: DH10B (Grant et al., 1990) as the host for plasmids construction, strain XL1Blue (Stratagene) for protein mutagenesis and strain BL21-CodonPlus (DE3) (Stratagene) carrying pMYS825 and pMYS841, for overexpression of Hys-tagged proteins.

M. smegmatis mc^2155 was grown in LD (Sabbattini et al., 1995) medium containing 0.2% (vol/vol) glycerol and 0.05% (vol/vol) Tween 80 and supplemented when necessary with hygromycin and pristinamycin I (10 μg/ml).

M. tuberculosis was grown in Middlebrook 7H9 broth or 7H10 agar medium supplemented with 0.05% (vol/vol) Tween 80, 0.2% glycerol and 10% ADN (Albumin, Dextrose, NaCl). When necessary hygromycin (50 μg/ml) and pristinamycin I (0,5 μg/ml) was added.

E. coli was grown in LB medium supplemented when necessary with neomycin (25 μg/ml), chloramphenicol (20 μg/ml) and IPTG (20 mM/ml) for the overexpression of the heterologous protein.

5.2 Plasmids

pMYS820 and pMYS823 : the 850 bp fragment of *rne* gene starting at position +2 with respect to the initiation codon was PCR amplified with the oligos V12/V13 and the 846 bp fragment of MSMEG_2685 gene starting at position +4 with respect to the initiation codon was PCR amplified with the oligos V14/V15, respectively, and the fragments were ligated as NcoI-SphI fragments into the respective sites of the integrative plasmid pAZI9479 (Forti et al., 2009) downstream of the *ptr* promoter. Campbell-type integration of pMYS820 or pMYS823 on the *M. smegmatis* chromosome renders *rne* and MSMEG_2685 expression pristinamycin I dependent.

pMYS824: A 1063 bp PCR fragment of the MSMEG_2685 5' region (oligos FG2361/FG2362) beginning at the position -644 with respect to the initiation codon of the gene (with an additional stop codon at the end) was ligated as a SpeI-HindIII fragment into the plasmid pjsc284 (derivative of pYUB854 (Bardarov et al., 2002)), downstream of the hygromycin resistance cassette. On the same plasmid, a 1023 bp PCR fragment of the MSMEG_2685 3' region (oligos FG2363/FG2364) beginning respectively at the +1323 with respect to the initiation codon of MSMEG_2685 was ligated as a XbaI-KpnI fragment upstream of the hygromycin resistance cassette.

pMYS825: the coding sequence of the MSMEG_2685 gene amplified with the oligos FG2360/V7, containing an additional N-terminal his-tag was cloned in plasmid pKYB1 (New England Biolabs) between the sites NdeI and MfeI.

pMYS841 : derivative of pMYS825, but the MSMEG_2685 coding sequence carries mutations that cause two amino acid changes in the active site (D85K & H86A), obtained with the oligos V34-V35.

pMYT832: the 860 bp fragment of *rne* gene starting at position +7 with respect to the initiation codon was PCR amplified with the oligos 2393/2394. The fragment was ligated as NcoI-SphI fragments into the respective sites of the integrative plasmid pAZI9479 (Forti et al., 2009) downstream of the *ptr* promoter. Campbell-type integration of pMYT832 on the *M. tuberculosis* chromosome renders *rne* expression pristinamycin I dependent.

5.3 Preparation of crude extracts

Crude extracts of *M. smegmatis* were prepared from 100 ml log phase cultures (OD_{600} = 0.8). The cells were pelleted, washed in 20 mM TrisHCl pH 8, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 mM KCl, and resuspended in 500 µl of 20 mM TrisHCl pH 8, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 mM KCl, 1 mM PMSF, 10% glycerol. The cells were lysed by sonication (2 × 20 seconds at 40% amplitude). The cell lysate was spun for 10 min at 15000 × g and the supernatant (about 10 mg/ml) was used as the crude extract.

5.4 Crude extract processing assay

The assay mixture (10 µl) containing 20 mM/ml Tris-HCl (pH: 8.0), 1,5 mM/ml DTT, 1 mM/ml $Mg(Ac)_2$, 20 mM/ml KCl, 10 mM/ml K_2HPO_4, 150 pmol/microl random labeled FK mRNA and 5 microg of crude extract. The reaction was incubated at 37 °C for 30 minutes. Reactions were treated with phenol to eliminate the proteins, precipitated and resuspended in 1X gel loading buffer (87.5% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 5 mM EDTA). The control reaction was performed by incubating the substrate with the reaction buffer in the same conditions. The samples and the two FK and 443 control transcripts were loaded on a 6% denaturing polyacrylamide gel and the reaction products were visualized on a Phosphorimager.

5.5 In vitro transcription

In vitro transcription with T7 RNA polymerase was performed as described by the manufacturer (Promega) using a PCR fragment as template. For *thrS* mRNA synthesis, the PCR template was

prepared using oligonucleotides HP128 and HP27 (Even et al., 2005). It contains the T7 promoter, the entire leader sequence and the first 46 nt of the coding sequence. For FK mRNA synthesis, the PCR template was prepared using oligonucleotides 1979 and 1989. It contains the T7 promoter, the 3' region of *furA* gene (from coordinate 361), the entire intergenic region, the first 82 bp of *katG* and a terminal stem-loop added to stabilize the transcript. For 443 mRNA synthesis, the PCR template was prepared using oligonucleotides 1980 and 1989. It contains the T7 promoter, the 3' region of *furA* gene (from coordinate 443), the entire intergenic region, the first 82 bp of *katG* and a terminal stem-loop added to stabilize the transcript. The effective transcript length is due to the chromosomal region amplified, which comprises 2 initial nucleotides at the 5' end and 13 nucleotides long stem loop sequence at the 3' end.

The transcripts were randomly labeled with α-(^{32}P) UTP or 5'-end labeled with γ-(^{32}P) GTP (Even et al., 2005). The 5'-end monophosphorylated transcripts were obtained by including in the reaction mix, GMP at a concentration 50X higher than the GTP (Li de la Sierra-Gallay et al., 2008).

Transcripts were purified by gel filtration on Sephadex G-25 columns (GE Healthcare). Alternatively, the mRNA substrate was purified from unwanted products by elution from a 5% polyacrylamide gel.

5.6 RNase J overexpression and purification

The *M. smegmatis rnj* open reading frame, N-terminal His6-tagged version, was cloned into the pKYB1 vector (New England Biolabs), between the NdeI-MfeI sites to obtain pMYS825. This plasmid was used to create the plasmid pMYS841 carrying the double mutation D85K and H86A by elongation of the oligos V34-V35. The expression of the RNase J wild-type and mutated proteins, was under the control of the T7 promoter. Overproduction of protein were carried out in *E. coli* strain BL21(DE3) CodonPlus (Stratagene).

The proteins were isolated by affinity chromatography on a Ni-NTA Agarose resin (Qiagen), briefly : cell pellet was resuspended in the lysis buffer (20 mM Hepes pH: 8, 500 mM NaCl, 10% glycerol), sonicated and centrifuged at 4°C 10 min. At the same time, 1 ml of resin was aliquoted into the column and equilibrated with lysis buffer (5 x 1 ml and 1 x 5 ml). After centrifugation, the supernatant was mixed with the resin 1 hour at 4°C. The mix was washed twice with 1 x 30 ml of washing buffer (20 mM Hepes pH: 8, 1 mM NaCl, 10% glycerol, 20 mM Imidazole) and 2 x 30 ml of washing buffer (20 mM Hepes pH: 8, 500 mM NaCl, 10% glycerol, 20 mM Imidazole). Finally, the protein was eluted 10 x 1 ml with elution buffer (20 mM Hepes pH: 8, 500 mM NaCl, 10% glycerol, 500 mM Imidazole). The activity in each tube was checked by Bradford assay. The purity was analyzed on a 10% SDS-acrylamide gel.

5.7 RNase J processing assays

The assay mixture (10 µl) containing 20 mM HEPES-KOH (pH: 8.0), 8 mM/ml $MgCl_2$, 100 mM/ml NaCl, 0.24 U/ml RNasin (Promega), 70 pmol/µl of 5' triphosphorylated, ^{32}P-labeled mRNA substrate or 200 pmol/µl of 5' monophosphorylated random labeled mRNA substrate and 500 µmol/µl of purified RNase J protein. The reaction was incubated at 37 °C. For time course experiments, samples of scaled-up reactions (60 µl) were taken at the indicated times. Reactions were stopped by the addition of 5 µl of 3X gel loading buffer (87.5% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 5 mM EDTA). The control reaction was performed by incubating substrate with the reaction buffer in the same conditions. The samples were directly loaded on a 20% polyacrylamide gel and the reaction products were visualized on a Phosphorimage.

5.8 Site-direct mutagenesis

The mutations D85K and H86A in the RNase J protein of *M. smegmatis* were introduced on the pMYS825 plasmid by the Quikchange strategy (Stratagene) with the oligos V34-V35 and the KOD DNA polymerase (Novagen).

5.9 Total RNA isolation

25 ml of *M. smegmatis* cultures (OD_{600} of 0.7-1) were pelleted, washed with 1 ml Tween 80 0.5%, 1 ml TSE buffer (10 mM Tris HCl pH: 8, 100 mM NaCl, 1mM EDTA) and resuspended in 200 µl of STET buffer (50 mM Tris HCl pH: 8, 8% sucrose, 0.5% Triton X-100, 10 mM EDTA, 7 mg ml^{-1} lysozyme) then incubated at 0 °C for 20 min. The cell suspension was added to a tube containing an equal volume of glass beads (0.2 mm diameter) and 200 µl phenol/H_2O, incubated at 100 °C for 3 min and vortexed 5 min at 4°C. The phases were separated by centrifugation (5 min 13000 rpm at room temperature).

The aqueous phase was re-extracted twice with 200 µl phenol/H_2O pH: 8 and 200 µl phenol/$CHCl_3$. The nucleic acids were precipitated with 0.1 vol LiCl 5 M, 3 vol EtOH 100% and dissolved in RNase-free H_2O.

5.10 Primer extension

The maturation of the rRNA components (16S, 23S and 5S) of *rrn* operons was studied by primer extension on the total RNA from the wild type, RNase J knock-out mutant and RNase E conditional expression mutant strains. Briefly, an annealing mix (10 µl), containing 10 µg of total RNA, 1 U/µl RNasin (Promega), 0.5 pmole radiolabeled oligo and 1X ss-hybridization buffer (300 mM NaCl, 10 mM TrisHCl pH 7.5, 2 mM EDTA), was denatured 4 min at 80 °C and incubated 2 h at 50 °C for the annealing. Then, to the annealing mix were added 40 µl of 1.25 X RT-buffer (1.25 mM of each dNTP, 12.5 mM DTT, 12.5 mM TrisHCl pH 8, 7.5 mM $MgCl_2$), 5

U RNasin (Promega) and 10 U AMV Reverse Transcriptase (Finnzymes) and incubated 30 min at 50°C for the extension. All samples were precipitated with 1/10 NaAC 3M, 2.5 Vol EtOH, dissolved in 12 µl stop mix (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Run on a 5% denaturing polyacrilamide gel. Primer extension protocol was adapted from that described by Sambrook J. (Sambrook et al., 1989)

Oligonucleotides used : V30 (complementary to region +347/+367 internal to 16S), V32 (complementary to region +2246/+2268 internal to 23S), V37 (complementary to region +5491/+5512 internal to 5S). All the coordinates are relative to the transcription start (+1) of the *rrnB* operon.

The oligos were radiolabeled with γ-(^{32}P) ATP using T4 Polynucleotide Kinase (New England Biolabs).

5.11 Bioinformatic tools

The RNases research was performed by homology analysis with the BLAST program, aligning the protein known sequences against chromosomal total length of *M. smegmatis* and *M. tuberculosis*.

Protein alignements were done using ClustalW program.

The structures of the different RNAses were released by the ExPASy web server and elaborated with the program PyMOL.

5.12 Oligos used in this work

V7	TCAATGCAATTGTTAGATCTCTATGACGGTCGGGACGATC
V12	CTTCTACCATGGCCGAAGATGCCCATACCGAAGACC
V13	GTAGCTGCATGCTCAGCTGTCCTCGTCGCCGCTGTCG
V14	CTTCTACCATGGCCAGCGCCGAACTCGCGCCGCCACC
V15	GTAGCTGCATGCTCAGTCGTCGACCTTCAGGTAGCCC
V20	CCGAGCTCGAATTCAGATCTCG
V21	CGCCACCAATCCCCATATGGAC
V22	GAGCGAGACCACAACAAGTCC
V23	GGTGTTCGGCGGGTCGTCAGG
V29	GTAGGGAGTAGGGGATTTGACTC
V30	CGACTTGCATGTGTTAAGCAC
V31	GGAGTCGCTAGTAATCGCAG
V32	CTTATCGCAGCCTCCTACGTCC
V34	TCACCCACGCGCACGAGAAAGCCATCGGCGCGATCCCGTT
V35	AACGGGATCGCGCCGATGGCTTTCTCGTGCGCGTGGGTGA
V37	CTTCCGGGTTCGGGATGGGAC
V38	GACCAGACCTGGAAGCCTAG
HP27	CCTTGACTGCTCCATCAGGAAATG
HP128	AGAATTCTAATACGACTCACTATAGGGAGATTAAGAAAGACAC
1979	CTAATACGACTCACTATAGGGCTACGCCATCGACGAGGCCG
1980	CTAATACGACTCACTATAGGGACTTCCCGATCACACCCGTG
1989	GCCGCGTGTTCGCGGCTTGATGCGGCCGAAACC
2361	GGACTAGTCTACGACACCGCCCACAGCGTGC
2362	ATCACCAAGCTTTCACTCGAAGACGCCGTGTTGGCTGC
2363	GTACTGTCTAGAGGTGACGGTCGGCAAGATGTTCG
2364	GTACTAGGTACCCCGATGATCGACAACGAGTTCACC
2393	CTTCTACCATGGCCGACGGTGCCCCACCTTCAGATCC
2394	GTAGCTGCATGCTCAGCCCTTGATCTCAGTGGAGCCAG

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ANNEX: Part of this work has been submitted on Molecular Microbiology**.**

Mycobacterium smegmatis RNase J is a 5'-3' exo-/endoribonuclease and both RNase J and RNase E are required for ribosomal RNA maturation

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Summary

The presence of very different sets of enzymes, and in particular the presence of RNase E and RNase J, have been used to explain significant differences in RNA metabolism between the two model organisms E. coli and B. subtilis. However, these studies might have somewhat polarized our view of RNA metabolism. Here, we have identified a functional RNase J in M. smegmatis that has both 5'-3' exo- and endonucleolytic activity. This enzyme co-exists together with RNase E in this organism, a configuration that enabled us to study how these two key nucleases collaborate. We show that RNase E and RNase J are cooperatively involved in the 5' end processing of 16S and 23S ribosomal RNA. The combination of the two enzymes actually leads to processing pathways for all three ribosomal RNAs that are quite different from those observed in both E. coli and B. subtilis. In addition, the 5'-3' exonucleolytic activity of RNase J, albeit required in vivo for full 23S rRNA maturation, is not essential in M. smegmatis in contrast to B. subtilis. Studying organisms containing different combinations of key ribonucleases can thus significantly broaden our view of the strategies directing RNA metabolism used by various organisms.

Introduction

RNA processing and degradation is a vital process in all organisms and plays an essential role in the control of gene expression and in the adaptation to environmental changes. There are important differences in the mechanisms underlying mRNA decay between Escherichia coli and Gram positive organisms which is reflected by the fact that they contain quite different sets of RNases (Condon & Putzer, 2002).

In the Gram-negative bacterium E. coli, RNase E is the key enzyme initiating mRNA decay. It generally catalyses a rate limiting endonucleolytic cleavage within an AU rich single stranded region causing the rapid 3'-5' exonucleolytic degradation of the fragment upstream of the cleavage site (Carpousis et al., 2009). The strong preference of RNase E in vitro for 5' monophosphorylated RNA substrates (Mackie, 1998) is thought to stimulate the subsequent cleavage of the 3' cleavage products. However, recent data indicate that 5'-end recognition by RNase E might be less important than initially thought (Garrey et al., 2009, Kime et al.). In addition, the preference of RNase E for a 5' monophosphorylated substrate allows, in some cases, for an alternative decay pathway in which internal cleavage by RNase E is triggered by prior conversion of the 5' terminal triphosphate to a monophosphate by the pyrophophorylase RppH (Deana et al., 2008). RNase E also serves as the scaffold for the degradosome complex and inactivation of RNase E increases global mRNA half-life (Carpousis et al., 2009). In addition, RNase E plays a major role in the maturation of ribosomal RNA (Deutscher, 2009).

RNase G is a shorter paralogue of RNase E and only the !-and "-subdivisions of the Proteobacteria have both enzymes (Condon & Putzer, 2002). RNase G only plays a minor role in mRNA metabolism in E. coli (Arraiano et al., 2010). RNase E orthologs in other species can be classified as RNase E/G enzymes because they usually share significant similarity with the catalytic N-terminal half of E. coli RNase E and RNase G (Condon & Putzer, 2002). Despite its central role in mRNA stability in E. coli, RNase E is absent from many bacterial species including most Firmicutes, such as Bacillus subtilis, and even some proteobacteria (Condon & Putzer, 2002 , Shahbabian et al., 2009). These bacteria instead

contain the endo-/5'-3' exoribonuclease RNase J (Even et al., 2005 , Mathy et al., 2007) and/or the endonuclease RNase Y (Shahbabian et al., 2009). For example, in B. subtilis the essential RNase J1 (rnjA) and its paralog RNase J2 (rnjB) have endonucleolytic cleavage specificity similar to that of RNase E (Even et al., 2005). They also possess a 5'-3' exonucleolytic activity (Mathy et al., 2007) which strongly prefers a substrate with a single phosphate at the 5'-end (Even et al., 2005, Li de la Sierra-Gallay et al., 2008, Mathy et al., 2007). This property fitted well with the observation that, in B. subtilis, obstacles such as a stalled ribosome or secondary structure near the 5'-end can strongly stabilize downstream RNA for several kilobases indicative of a strong 5'-3' directionality for mRNA degradation (Agaisse & Lereclus, 1996, Bechhofer & Dubnau, 1987, Hambraeus et al., 2002). Indeed, RNases J1 and J2, which have overlapping substrate specificities, together affect the expression levels of hundreds of genes (Mäder et al., 2008). The exonucleolytic activity of RNase J1 also participates in 16S rRNA maturation (Mathy et al., 2007). On the other hand, in a rnjA/rnjB double mutant (where RNase J1 was depleted but not eliminated) global mRNA half-life was only slightly increased (Even et al., 2005), an observation that is not indicative of an important function for RNases J1/J2 in initiating global mRNA decay. This role could potentially be ascribed to RNase Y, a recently characterized novel endoribonuclease whose depletion increases bulk mRNA stability in B. subtilis to a similar extent as that of RNase E mutants in E. coli (Shahbabian et al., 2009). In addition, the activity of RNase Y is strongly increased by the presence of a 5'-P group on the RNA substrate, another similarity shared with RNase E (Shahbabian et al., 2009). These observations raised the possibility that, after all, mRNA processing and degradation might be more similar between B. subtilis and E. coli than currently assumed, with an endonucleolytic cleavage being the key step in initiating mRNA decay.

In eubacteria the RNases E/G, J and Y probably represent the key enzymes initiating mRNA decay. In this context, mycobacteria are especially interesting to study because sequence analysis shows that they contain an RNase E/G and an RNase J but no RNase Y. This combination has not been found in any of the organisms where RNA metabolism has been

studied in some detail. This opens new perspectives for studying how these two important ribonucleases have coevolved to create an efficient machinery for RNA maturation/degradation. We used Mycobacterium smegmatis, a fast-growing alternative to the pathogen Mycobacterium tuberculosis, which also has functional orthologs for both RNase E and, as we show, RNase J. The mycobacterial RNase E protein has been characterized previously (Zeller et al., 2007). Here we have studied the RNase J ortholog in vivo and in vitro demonstrating that it has similar characteristics to that of RNase J1 of B. subtilis. To analyse in more detail the relative roles of RNase J and RNase E in vivo in M. smegmatis, we created mutants for both of them and studied their effect on the maturation of the ribosomal RNA. Our results show that rRNA maturation in M. smegmatis is different from that in both E. coli and B. subtilis. In addition, we demonstrate that the 5'-3' exonucleolytic activity of RNase J, albeit used for rRNA processing is not essential for cell survival.

Results

M. smegmatis encodes a protein with high similarity to B. subtilis RNases J1/J2

RNases J1/J2 were discovered in B. subtilis but orthologous enzymes are present in about half of the sequenced bacterial and archeal genomes (Even et al., 2005). Here, we performed a BLAST search with the B. subtilis RNases J1 and J2 sequences against protein sequences encoded by the M. smegmatis mc2155 chromosome. A single gene product (MSMEG_2685) shows a high similarity over its entire length with both RNase J1 and J2 : the 558 amino acids long mycobacterial protein shares 35% identical residues with RNase J1 (555 amino acids, 55% similarity) and 33% identical residues with RNase J2 (555 amino acids, 59% similarity). Analysis of the amino acid sequence revealed the presence of all known motifs important for the ribonuclease activity (Li de la Sierra-Gallay et al., 2008) and clearly categorizes this mycobacterial protein as a member of the !-CASP subfamily of zinc-dependent metallo-!lactamases (Callebaut et al., 2002) (SuppFig. 1). Based on the recently resolved 3D structure of the Thermus thermophilus RNase J (Fig. 1A, (Li de la Sierra-Gallay et al., 2008) we deduced the 3D structures of B. subtilis RNase J1 (Fig. 1B) and the putative M. smegmatis RNase J (Fig. 1C). The three structures are very similar and can practically be superposed. Minor differences observed for the mycobacterial protein with respect to the other two RNases include a somewhat less structured linker (shorter #-helix) and C-terminal domain where only the straight part of the kinked helix is present.

The MSMEG_2685 protein has endoribonucleolytic activity

The B. subtilis RNases J1 and J2 were initially identified as 5'-end dependent endoribonucleases which can cleave the thrS leader mRNA (Even et al., 2005). Since the B. subtilis thrS leader is the only well characterized substrate to monitor the endoribonucleolytic activity of RNase J we used it for in vitro assays with the M. smegmatis enzyme. The thrS

substrate labeled at its 5'-end with "-32P-GTP was synthesised by in vitro transcription of the thrS leader region giving a readthrough transcript of 350 bases and a prematurely terminated transcript of 280 bases (Even et al., 2005) as well as some aborted transcripts). It was incubated with the purified MSMEG_2685 protein for up to 20 min (see Experimental procedures). Two major cleavage products, whose signal intensity increased with the time of incubation, were observed (Fig. 2A, lanes 2-5). They correspond to a cleavage close to the 5'end of the transcript in an unstructured region, at the approximate positions +3 and +7 (5' GGG/AGAT/AAGAAAGACACACG). These are not the major endonucleolytic cleavage products observed with the B. subtilis RNases J1/J2 on a similar substrate (Even et al., 2005, Mathy et al., 2010) but indicate that the MSMEG_2685 protein has endonucleolytic activity. In order to confirm that the endoribonucleolytic activity was carried by the mycobacterial protein MSMEG_2685, we mutagenized two amino acids, aspartate 85 to lysine and histidine 86 to alanine. These amino acids are conserved in the B. subtilis and the T. thermophilus RNase Js, and in the 3D structure of the T. thermophilus enzyme both are directly implicated in the coordination of the Zn ion in the catalytic center (see Fig. 1). Mutations in either amino acid in B. subtilis RNase J1 severely impaired both its endo-and 5'-3' exoribonucleolytic activities (Li de la Sierra-Gallay et al., 2008). The M. smegmatis mutated protein was purified and its endonucleolytic activity tested on the thrS transcript (Fig. 2A, lanes 7-10). No endonucleolytic activity was observed in the presence of the mutated protein, thus confirming that the cleavages of the thrS leader were generated by the MSMEG_2685 protein. We renamed the gene rnj and its product RNase J.

The mycobacterial RNase J has 5'-3' exoribonucleolytic activity dependent on the 5' phosphorylation state of the substrate.

In contrast to its endonucleolytic activity the exonucleolytic activity of Bs RNase J1 requires a monophosphorylated 5'-end substrate (Li de la Sierra-Gallay et al., 2008).

We used the same purified 350 bp thrS leader read through transcript in its 5'monophophorylated

version and labelled uniformly with #-32P-UMP to detect a potential 5'3' exoribonucleolytic activity of M. smegmatis RNase J. The reaction conditions were identical to those used for the endonucleolytic cleavage assay. The addition of wild type M. smegmatis RNase J to the reaction led to the accumulation of mononucleotides in a time dependent manner (Fig. 2B lanes 2-6). The mutated RNase J had lost all activity (Fig. 2B lanes 8-12). This demonstrates that the mycobacterial enzyme is a dual activity RNase, similar to the B. subtilis RNases J1.

M. smegmatis RNase J is not essential

RNase J1 in B. subtilis and RNase E in E. coli are both essential enzymes that significantly affect RNA processing and degradation in their respective organisms. Mycobacteria have both an RNase E/G type enzyme and RNase J raising the intriguing question of how these two key enzymes cooperate within the same organism.

We first analyzed whether either or both enzymes are essential in M. smegmatis by creating two conditional mutants in the rne and rnj genes, respectively. Expression of the genes was put under the control of the inducible ptr promoter (Forti et al., 2009). The ptr promoter can be induced by the addition of pristinamycin I, which removes the Pip repressor from the operator region of ptr, thus inducing transcription of the downstream genes (Forti et al., 2009). The mutants were created by constructing plasmids in which the 5' region of the truncated genes is cloned downstream of the ptr promoter (pMYS820 for rne and pMYS823 for rnj). The plasmids also carry the pip repressor gene and a hygromycin resistance cassette for selection of the recombinant clones. M. smegmatis strain mc2155 was transformed and recombinants in which the plasmid integrated by single crossover were selected on hygromycin. In these strains expression of the wild type rne or rnj alleles, respectively, are under ptr control (see Experimental procedures). The growth of both mutant strains was analyzed in the presence/absence of inducer: ten-fold serial dilutions of log-phase cultures

were plated on solid media. In the presence of the inducer, the mutants grew as the wild-type strain (Fig.3A, I), while in the absence, the rnj mutant plated as well as the wild type strain, but the rne mutant did not grow (Fig.3A, II). In liquid media we obtained similar results. The rne mutant stopped growing about 7 generations after removal of the inducer while the rnj conditional mutant grew equally well in the presence or absence of the inducer (Fig. 3B). This indicated that in M. smegmatis RNase E is essential while RNase J is not.

In order to definitely confirm this conclusion we constructed an RNase J null mutant where the rnj gene was partially deleted following a double cross-over event with plasmid pMYS824 (see Experimental procedures). In this mutant 418 amino acids out of a total 558 amino acids (positions 140 to 558) were replaced by the hygromycin resistance cassette. The rnj deletion strain had the same growth rate as the conditional rnj mutant, which is about 30% slower than a wild type strain (data not shown).

The role of RNases E and J in ribosomal RNA maturation

In E. coli RNase E is involved in rRNA maturation (Li et al., 1999), whereas in B. subtilis RNase J is involved at least in 16S subunit maturation. Nothing is known about ribosomal RNA processing in mycobacteria. In order to gain a first insight into the respective functions of RNAse E and J in this process we studied the role of these two ribonucleases using the mutant strains of M. smegmatis.

M. smegmatis has two ribosomal RNA operons, rrnA and rrnB. The two operons differ only in the first part of the leader region, upstream of a putative Box A antiterminator element (Ji et al., 1994b). The mature rRNAs encoded by the two operons are thus identical (Fig. 4). Transcription of the rrnA operon has been shown to initiate predominately at one of three potential promoters (P2), the rrnB operon is transcribed from a single promoter (Gonzalez-y-Merchand et al., 1996).

In order to study the role of RNase J and RNase E on mycobacterial rRNA maturation we carried out primer extension analysis using oligonucleotides complementary to regions close

to the 5'-end of the structural rRNA genes (V30, V32, and V37 in Fig. 4). The primer extension probes hybridize to both the rrnA and rrnB operon transcripts. The profile of ribosomal RNA precursors was analysed in the wild type strain mc2155, the RNase J null and the RNase E conditional mutant (Fig. 5). The cleavage sites observed by primer extension are summarized on the RNA structures in Fig. 6. The major transcripts detected with the three primers corresponded to the mature 16S, 23S and 5S RNAs but additional longer transcripts corresponding to presumed processing sites and intermediates were detected. the relative abundence of these transcripts changed with the presence or absence of RNase J and RNase E (Fig. 5). The detailed interpretation of these results are discussed below in the Discussion section.

Discussion

RNases are important enzymes that intervene in the degradation, processing and quality control of all RNA species in the cell. Current knowledge suggests that the RNases E, J and Y are key players in eubacterial RNA metabolism. Individually, orthologues of each of these nucleases are potentially present in about 40 to 50 % of the sequenced eubacterial species (Condon & Putzer, 2002 , Even et al., 2005, Shahbabian et al., 2009). In other words, all known eubacteria contain at least one of these three nucleases and several of them have all three (Shahbabian et al., 2009). The presence or absence of these key ribonucleases can provide important insights into the strategies used by different bacterial species to organize their RNA metabolism. For example, the two best studied organisms E. coli and B. subtilis rely on very different sets of enzymes, namely RNase E in E. coli and RNases J1/J2 and Y in

B. subtilis, which fulfil essential functions in mRNA metabolism and ribosomal RNA maturation in the respective bacteria. Mycobacterial RNA metabolism has not been well studied but these high GC Gram positive organisms contain an RNase E/G type enzyme and a putative RNase J ortholog. In addition, a compilation of potential RNase orthologs (Table 1)

suggests that mycobacteria constitute an interesting class of bacteria that combine key enzymes present in E. coli and B. subtilis.

Mycobacteria can be divided into two classes : the slow-growers which include pathogens like Mycobacterium tuberculosis and Mycobacterium leprae and the fast-growers like M. smegmatis. It is noteworthy that the putative RNases present in these organisms are the same (Table 1). This implies that RNA metabolism is likely to be very similar and studies in M. smegmatis, which is non pathogenic and simpler to handle than M. tuberculosis, should be pertinent for both species.

Sequence and 3D structure comparisons of the M. smegmatis protein 2685 with known RNases J of T. thermophilus and B. subtilis (Supp Fig. 1 and Fig. 1) showed good similarities (55% and 59%, respectively with B. subtilis RNases J1 and J2), conservation of all important motifs, notably the five classical !-lactamase motifs, the three !-CASP motifs and the amino acids implicated in the coordination of the UMP nucleotide (Li de la Sierra-Gallay et al., 2008). However, this comparison provided no clues as to the major enzymatic activity. It was important to verify that the single putative M. smegmatis RNase J ortholog (MSMEG_2685) actually is an RNase and whether it has endo-and/or exoribonucleolytic activities. In fact, in B. subtilis both RNase J1 and RNAse J2 show a similar endoribonucleolytic activity (Even et al., 2005), but RNase J2 in contrast to RNase J1 has no significant 5'-3' exoribonucleolytic activity (Mathy et al., 2010). In vitro assays with the purified mycobacterial protein showed that it actually has both activities, 5'-3' exo-and endonucleolytic. The exonucleolytic activity is comparable to that of B. subtilis J1 when assayed on the same 5' monophosphorylated B. subtilis thrS leader substrate (Li de la Sierra-Gallay et al., 2008). No mononucleotides were produced with a 5' triphosphorylated RNA. This strongly suggests that M. smegmatis RNase J, similarly to B. subtilis RNAse J1, can act as a 5'-3' exoribonuclease in vivo.

Using the B. subtilis thrS leader region in its 5'-triphosphorylated form labeled at the 5' end with "-32P GTP we showed that M. smegmatis RNase J also has endonucleolytic activity. The major cleavages were observed at about 3 and 7 nucleotides from the 5' end (Fig. 2A, lanes 25) in a region supposed to be single stranded (Luo et al., 1998). Cleavage was efficient but

occurred at a different site compared to the previously characterized sites for B. subtilis RNases J1 and J2 on the same substrate (Even et al., 2005 , Mathy et al., 2010). The endonucleolytic activity of RNases J1/J2 is dependent on sequence and structural parameters that are still ill defined but which assure that cleavage in vivo apparently only occurs at a rather limited number of sites (Shahbabian et al., 2009 , Li de la Sierra-Gallay et al., 2008). Moreover, it is likely that these parameters differ significantly between low GC B. subtilis and high GC M. smegmatis (43% and 67% GC content, respectively). We noticed that about half of the exonucleolytic and all of the endonucleolytic substrate was retained in the wells of the 20% polyacrylamide gels used to resolve the exo-and endonucleolytic cleavage products of RNase J activity (Fig. 2A and 2B). This probably reflects strong binding of M. smegmatis RNase J to its substrate until it after cleavage and release of the cleavage products. The sequestration of the substrates was much weaker when the reactions were carried out with a mutant protein where two amino acids (Asp85Lys and His86Ala) in the catalytic center (Li de la Sierra-Gallay et al., 2008) are altered. More importantly, the 5'-3' exo-and endonucleolytic activities of M. smegmatis RNase J are almost abolished in this mutant, thus confirming that the ribonucleolytic activities attributed to M. smegmatis RNase J are genuine and not due to contamination during protein purification.

RNase E and RNase J1 are essential for viability in E. coli and B. subtilis, respectively. These ribonucleases are clearly very important for RNA metabolism but the precise reason for their essentiality has not been established, neither for RNase E nor for RNase J1.

We show here that in M. smegmatis RNase E is essential while RNase J is not. This also shows that the 5'-3' exonucleolytic activity of RNase J is dispensable, at least in the presence of RNase E. It has been proposed that the major function for the exonucleolytic activity of RNase J in B. subtilis is the 5' to 3' degradation of RNA fragments protected by secondary structures (e.g. a transcription terminator) at their 3' ends (Shahbabian et al., 2009). In fact, in the absence of an efficient 3' polyadenylation pathway, as exists in E. coli (Hajnsdorf et al., 1995), such RNA fragments can accumulate to very high levels (Shahbabian et al., 2009), expecially in an organism like B. subtilis where about 3 % of all genes are controlled by

modulating premature transcription termination (Merino & Yanofsky, 2005, Lathe et al., 2002) However, we found no obvious ortholog of the E. coli Poly A polymerase in M. smegmatis or M. tuberculosis.suggesting that mycobacteria have an alternative method of degrading structured RNA fragments.

RNase E and RNase J have important roles in ribosomal RNA maturation in E. coli and B. subtilis, respectively (for a recent review see (Deutscher, 2009). This again reflects the different set of ribonucleases present in these model organims (Table 1).

In Mycobacteria, rRNA processing has not been studied in much detail (Ji et al., 1994b, Ji et al., 1994a). In M. tuberculosis a single rRNA operon is present (rrnA) whereas M. smegmatis has two operons (rrnA and rrnB). Both operons contain the genes for the 16S, 23S and 5S rRNA, co-transcribed as a single precursor molecule (Ji et al., 1994c, Gonzalez-y-Merchand et al., 1996). Co-or post-transcriptional folding of the precursor rRNAs is predicted to involve extensive basepairing between the leader and spacer-1 region (16S), spacer-1 and spacer-2 (23S), spacer-2 and trailer (5S) as suggested by the secondary structures predicted by the M-fold algorithm (Fig. 6) and as found in rRNA operons of other species. The M. smegmatis rrnA and rrnB operons have identical sequences downstream of position -165 with respect to the 16S rRNA structural gene. Thus the folded precursor structures that are the substrates for a series of maturation reactions to produce the mature rRNAs should be the same for both operons (Fig. 6).

Here we studied how the RNases E and J contribute and collaborate in the maturation of the 5’ ends of the rRNA precursors in M. smegmatis.

Pre-16S rRNA maturation

Extension of primer V30, located downstream of the 16S 5’-end (Fig. 4), showed that in a wild type strain more than 90% of the 16S rRNA exists in its fully matured form (Fig. 5A, lane 1), although traces of full-length transcripts originating at the rrnA P2 and the rrnB promoter could be detected. A significant amount of a precursor 16S rRNA with a 5’-end at

position +147 could also be detected. This processing site lies within a region forming a double stranded stem made up of sequences upstream of the 16S rRNA and sequences of the spacer 1 region (between the 16S and 23S rRNA, Fig. 4 and 6A). The presence of a concomittant cleavage observed on the opposite strand within a bulge and shifted by two nucleotides (position +1979, Fig. 5B, see below) strongly suggested a maturation by the double-strand specific RNase III (Fig. 6A). Indeed, this configuration is reminiscent of RNase III cleavages observed in similar positions in the long processing stalks of 16S rRNA precursors of E. coli (Young & Steitz, 1978) and B. subtilis (Herskowitz & Bechhofer, 2000 , Loughney et al., 1983).

Inactivation of the rnj gene had no major effect on 16S maturation (Fig. 5A, lane 2) and only caused a weak stabilisation of a precursor containing 36 nt additional nucleotides (position +266, see also below).

By contrast, partial depletion of RNase E caused a two-fold increase in full-length precursors and the putative RNase III processing product (position +147) but also a strong accumulation of the +36 nt precursor (position +266, Fig. 5, lane 4). The +36 nt precursor was also weakly detected in the rnj null mutant (Fig. 5A, lane 2) suggesting that this transcript is generated through cleavage by a yet unidentified endoribonuclease. This accumulation of rRNA precursors in the RNase E depleted strain is consistent with RNase E being able to create the mature 5' end. The putative cleavage site at the beginning of 16S rRNA is composed of a U rich single stranded region (Fig. 6A) compatible with the known requirements for RNase E cleavage in E. coli (Ehretsmann et al., 1992 , McDowall et al., 1994) and M. tuberculosis (Zeller et al., 2007).

We propose the following model for 16S rRNA maturation : i) RNase III cleavage releases the 16S rRNA precursor from the 30S original transcript. ii) an unidentified nuclease cleaves at position +266 leaving 36 nt upstream of the mature 16S rRNA 5' end. iii) This shortened precursor is then processed by RNase E to create the mature 16S rRNA 5' end. When we compare M. smegmatis 16S rRNA maturation to that from E. coli and B. subtilis (Fig. 7) we note that the strategy used ressembles more that of E. coli than B. subtilis. In fact, despite its

presence in M. smegmatis, RNase J is not involved in any significant way in the intermediate or final steps of 16S rRNA maturation. We can note however one intriguing similarity between the 5' processing pathways of 16S rRNA in these three organisms. In all cases, maturation appears to occur in a two-step mechanism : the initial RNase III precursors are processed by RNase E (E. coli, +66 nt) or unknown endoribonucleases (B. subtilis, +38 nt and M. smegmatis, +36 nt) apparently to create a new 5' end free from the processing stalk and increasing the efficiency of the final maturation steps at both the 5' and 3' extremities. RNase E/G and the 5'-3' exonucleolytic activity of RNase J might be expected to have a similar 5' end dependence and the intermediate processing step would thus facilitate the access of RNase G (E. coli) and RNase J1 (B. subtilis) to a single stranded monophosphorylated 5' end in order to perform the final maturation step. The fact that RNase J only plays a very minor role compared to RNase E suggests that the new 5' end is not accessible to RNase J or that the requirements for 5' end recognition (e.g. presence of secondary structure) are quite different between the two M. smegmatis nucleases.

Pre-23S rRNA maturation

The 23S rRNA structural gene lies downstream of the 16S rRNA gene separated by a 368 nt region (spacer 1, Fig. 4) that does not encode any known genes. Extension of primer V32, downstream of the 23S 5'-end, revealed that in the wild type strain about 90% of the 23S rRNA is fully processed (Fig. 5B, lane 1). Two minor upstream signals were observed at positions +1979 and +2040. The signal at +1979 (containing 216 nt upstream of the mature 23S 5' end) is most likely due to RNase III causing a double strand cleavage of the 16S rRNA precursor that separates the 16S and 23S rRNAs on the original transcript (Fig. 6A); cleavage of the opposite strand occurs at position +147 as described above (Fig. 6A).

The 23S precursor RNA with a 5'-end at position +2040 was present at significantly lower levels in a strain depleted for RNase E (Fig. 5B, lane 4) suggesting that this transcript is generated by an RNase E cleavage. It is located in the single stranded region between two

extensive secondary structures of the 23S precursor (Fig. 6B). In accordance, the upstream precursor likely generated by RNase III (position +1979) is present at higher levels when RNase E expression is reduced (Fig. 5B, lane 4). Furthermore, depletion of RNase E also caused a more than two-fold reduction of the mature 23S rRNA level (Fig. 5B, lane 4). This would be in agreement with RNase E being capable of directly generating the 5'-end of mature 23S rRNA.

The absence of RNase J has a major effect on the 23S rRNA maturation. Under these conditions about 50% of the total 23S rRNA accumulates as a +12 nt precursor (position +2184, Fig. 5, lane 2 and Fig. 6B). This indicates that half of the 23S rRNA precursor is cleaved by an endoribonuclease, probably RNase E (see below), so that the +12 nt form accumulates because it is no longer trimmed by the 5'-3' exonucleolytic activity of RNase J. The fact that less than half of the 23S rRNA is present in its mature form does not have a dramatic effect on cell growth since an rnj mutant grows with a doubling time of 3 hours compared to 2 to 2.5 hours for a wild type strain. In addition, there is no indication that the observed reduction in growth rate is actually due to impaired 23S rRNA processing. Depletion of RNase E also causes a 50 % decrease in mature 23S rRNA as well a two-fold reduction of a precursor containing 155 additional nucleotides (position +2040, Fig. 5, lane 4). By contrast, the concentration of the initial precursor following RNase III cleavage is increased under these conditions. This strongly suggests that RNase E generates the +12 nt precursor, a notion corroborated by the sequence and structural context of the cleavage site (single stranded U rich region). Similarly, the +155 nt precursor (position +2040) is likely also due to RNase E cleavage and thus accumulates two-fold in the rnj mutant (Fig. 5B, lane 2). Since half of the 23S rRNA is still found in its mature form in the absence of RNase J indicates that the mature 5' end can be generated by direct cleavage. Based on our observations the most likely candidate enzyme for this final maturation step, which occurs in a single stranded AU rich environnement, is again RNase E.

The rnj null mutation also caused a small but significant accumulation of two closely spaced precursors containing an additional 127/128 nucleotides (positions +2068/2069). Together

with a signal observed at position +5366 on the opposite strand (see below, Fig. 5C, lane 4) we are probably seeing here the remnants of a precursor generated by RNase III processing within the 23S processing stalk aimed at separating the 23S and 5S rRNAs (Fig. 6). Maturation of the 23S rRNA in M. smegmatis is thus quite different when compared to both E. coli and B. subtilis (Fig. 7). In fact, neither RNase E nor RNase J, the two nucleases involved in the final steps of 23S rRNA maturation in M smegmatis, participate in the processing of 23S rRNA in E. coli or B. subtilis, respectively.

Pre-5S subunit maturation

The 5S rRNA structural gene is separated from the upstream 23S rRNA gene by a 101 nt spacer. Primer extensions with the V37 primer in the wild type strain showed that about 95% of the total RNA corresponded to that of the mature 5S rRNA (Fig. 5C, lane 1). A precursor rRNA 29 nt longer than the mature 5S rRNA (position +5427) was present in minor quantities. Disruption of the rnj gene had no significant impact on the processing of the 5S rRNA (Fig. 5C, lane 2). Depleting RNase E also did not substantially reduce the extent of fully processed 5S rRNA (Fig. 5C, lane 4). However, under these conditions two new precursor 5S rRNA species appeared. The first (position +5366) likely corresponds to the RNase III generated precursor described above, together with a cleavage on the opposite strand at position +2068/2069 (Fig. 5B). The most prominent precursor detected during RNase E depletion contained 37 additional nucleotides (position +5419). The data are compatible with a direct maturation of the 5S rRNA 5' end by RNase E using either the +37 nt precursor and possibly also longer precursors. Due to the presence of almost wild type levels of mature 5S rRNA under these conditions it is likely that another nuclease besides RNase E is involved in the final processing steps. This unknown nuclease might also account for the +37 nt precursor.

More data are needed to fully understand rRNA processing in Mycobacteria particularly at the 3' end. Nevertheless, our experiments clearly show that the mechanisms and enzymes involved in the 5' end processing of all three rRNAs are significantly different compared to what we know from the two model organisms E. coli and B. subtilis. From an evolutionary

and mechanistic point of view it will be interesting to better understand how combinations of RNases present in a given organism influence and direct RNA metabolism. Detailed studies have so far been essentially based on E. coli and B. subtilis and might have exaggerated differences that do exist but which should probably not be viewed as dogmatic. For example, as we have seen in this study the 5'-3' exonucleolytic activity of RNase J is exploited in Mycobacteria but, in contrast to B. subtilis, is not important for cell survival. Studying RNA metabolism in Mycobacteria should thus be a useful approach to better understand how key enzymes like RNase E and the dual activity RNase J are integrated into a global strategy organizing RNA metabolism in a given organism.

Experimental procedures

Bacterial strains and growth conditions

The prototrophic Mycobacterium smegmatis mc2155 (Snapper et al., 1990) was used for the construction of the mutants. In the strain rnj101, the rnj (MSMEG_2685) gene was put under the control of the ptr promoter by Campbell-type integration of plasmid pMYS823. For strain rnj102, the rnj (MSMEG_2685) gene was partially deleted by double-crossover recombination with plasmid pMYS824. In the derivative strain rne101, the rne (MSMEG_4626) gene was put under control of the ptr promoter by Campbell-type integration of plasmid pMYS820.

The following Escherichia coli strains were used: DH10B (Grant et al., 1990) as the host for plasmids construction, strain XL1Blue (Stratagene) for protein mutagenesis and strain BL21CodonPlus

(DE3) (Stratagene) carrying pMYS825 and pMYS841, for overexpression of Hystagged proteins.

M. smegmatis mc2155 was grown in LD (Sabbatini et al., 1995) medium containing 0.2% (vol/vol) glycerol and 0.05% (vol/vol) Tween 80 and supplemented when necessary with hygromycin (50 μg/ml) and pristinamycin I (10 μg/ml). E. coli was grown in LB medium supplemented when necessary with neomycin (25 μg/ml), chloramphenicol (20 μg/ml) and IPTG (2 mM) for the overexpression of the heterologous protein.

Plasmids

pMYS825. The coding sequence of the MSMEG_2685 gene was amplified with the oligonucleotides FG2360/V7, and cloned into plasmid pKYB1 (New England Biolabs) between the sites NdeI and MfeI. This plasmid expresses an N-terminal His-tagged protein.

pMYS841. Derivative of pMYS825, but the MSMEG_2685 coding sequence carries two mutations (D85K & H86A) obtained using the oligonucleotides V34-V35.

pMYS820 and pMYS823. A 850 bp fragment of the rne gene starting at position +2 with respect to the initiation codon was PCR amplified with the oligos V12/V13 and the 846 bp fragment of MSMEG_2685 gene starting at position +4 with respect to the initiation codon was PCR amplified with the oligos V14/V15, respectively, and the fragments were ligated as NcoI-SphI fragments into the respective sites of the integrative plasmid pAZI9479 (Forti et al., 2009) downstream of the ptr promoter. Campbell-type integration of pMYS820 or

pMYS823 on the M. smegmatis chromosome renders rne and MSMEG_2685 expression pristinamycin I dependent.

pMYS824. A 1063 bp PCR fragment of the MSMEG_2685 5' region (oligonucleotides FG2361/FG2362) starting at position -644 with respect to the initiation codon of the gene (with an additional stop codon at the end) was ligated as a SpeI-HindIII fragment into the plasmid pJSC284 (derivative of pYUB854 (Bardarov et al., 2002) downstream of the hygromycin resistance cassette. On the same plasmid, a 1023 bp PCR fragment of the MSMEG_2685 3' region (oligos FG2363/FG2364) beginning respectively at the +1323 with respect to the initiation codon of MSMEG_2685 was ligated as a XbaI-KpnI fragment upstream of the hygromycin resistance cassette. The restriction fragment SpeI-KpnI from pMYS824 was utilized to transform M. smegmatis and by double crossover, the central region of MSMEG_2685 on the chromosome (903 bp) was substituted by a hygromycin resistance cassette.

Total RNA isolation

25 ml of M. smegmatis cultures (OD600 of 0.7-1) were pelleted, washed with 1 ml Tween 80 0.5 %, 1 ml TSE buffer (10 mM Tris HCl pH: 8, 100 mM NaCl, 1 mM EDTA) and resuspended in 200 µl of STET buffer (50 mM Tris HCl pH 8, 8 % sucrose, 0.5 % Triton X100, 10 mM EDTA, 7 mg ml-1 lysozyme) then incubated at 0°C for 20 min. The cell

suspension was added to a tube containing an equal volume of glass beads (0.2 mm diameter) and 200 µl phenol/H2O, incubated at 100 °C for 3 min and vortexed 5 min at 4°C. The phases were separated by centrifugation (5 min, 13000 rpm at room temperature).
The aqueous phase was re-extracted twice with 200 µl phenol/H2O pH 8 and 200 µl phenol/CHCl3. The nucleic acids were precipitated with 0.1 vol LiCl 5 M, 3 vol EtOH 100% and dissolved in RNase-free H2O.

Primer extension

The maturation of the different ribosomal RNAs was studied by primer extension on the total RNA from the wild type, RNase J knock-out mutant and RNase E conditional expression mutant strains. Briefly, an annealing mix (10 µl), containing 10 µg of total RNA, 1 U/µl RNasin (Promega), 0.5 pmole radiolabeled primer and 1x ss-hybridization buffer (300 mM NaCl, 10 mM TrisHCl pH 7.5, 2 mM EDTA), was denatured 4 min at 80 °C and incubated 2 h at 50 °C for the annealing. To the annealing mix were added 40 µl of 1.25 x RT-buffer (1.25 mM of each dNTP, 12.5 mM DTT, 12.5 mM TrisHCl pH 8, 7.5 mM MgCl2), 5 U RNasin (Promega) and 10 U AMV Reverse Transcriptase (Finnzymes) and the primer was extended for 30 min at 50°C. All samples were precipitated with 1/10 vol NaAc 3M, 2.5 vol EtOH, dissolved in 12 µl stop mix (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Reaction products were separated on a 5% denaturing polyacrylamide gel.
Oligonucleotides used : V30 (complementary to region +347/+367 internal to 16S), V32 (complementary to region +2246/+2268 internal to 23S), V37 (complementary to region +5491/+5512 internal to 5S). All the coordinates are relative to the transcription start (+1) of the rrnB operon.
The oligos were radiolabeled with gamma-(32P) ATP using T4 Polynucleotide Kinase (New England Biolabs).

Site-direct mutagenesis

The mutations D85K and H86A in the RNase J protein of M. smegmatis were introduced on the pMYS825 plasmid by the Quikchange strategy (Stratagene) with the oligonucleotides V34-V35 and the KOD DNA polymerase (Novagen).

In vitro transcription

n vitro transcription with T7 RNA polymerase was performed as described by the manufacturer (Promega) using a PCR fragment as template. For thrS leader mRNA synthesis, the PCR template was prepared using oligonucleotides HP128 and HP27 (Even et al., 2005). It contains the T7 promoter, the entire leader sequence and the first 46 nt of the coding sequence. The transcripts were randomly labeled with #-(32P) UTP or 5'-end labeled with "(32P) GTP (Even et al., 2005). The 5'-end monophosphorylated transcripts were obtained by including in the reaction mix, GMP at a concentration 50x higher than that of GTP (Li de la Sierra-Gallay et al., 2008).

Transcripts were purified by gel filtration on Sephadex G-25 columns (GE Healthcare). Alternatively, the mRNA substrate was purified from unwanted products by elution from a 5% polyacrylamide gel.

RNase J overexpression and purification

The M. smegmatis rnj open reading frame, N-terminal His6-tagged version, was cloned into the pKYB1 vector (New England Biolabs), between the NdeI-MfeI sites to obtain pMYS825. This plasmid was used to create the plasmid pMYS841 carrying the double mutation D85K and H86A using oligonucleotides V34 and V35. The expression of the RNase J wild-type and mutated proteins, was under the control of the T7 promoter. Overproduction of protein was carried out in E. coli strain BL21(DE3) CodonPlus (Stratagene).

The proteins were isolated by affinity chromatography on a Ni-NTA Agarose resin (Qiagen),

briefly : cell pellet was resuspended in lysis buffer (20 mM Hepes pH 8, 500 mM NaCl, 10% glycerol), sonicated and centrifuged at 4°C 10 min. 1 ml of resin was aliquoted into the column and equilibrated with lysis buffer (5 x 1 ml and 1 x 5 ml). After centrifugation, the supernatant was mixed with the resin for 1 hour at 4°C. The mix was washed twice with 1 x 30 ml of washing buffer (20 mM Hepes pH 8, 1 mM NaCl, 10% glycerol, 20 mM Imidazole) and 2 x 30 ml of washing buffer (20 mM Hepes pH: 8, 500 mM NaCl, 10% glycerol, 20 mM Imidazole). Finally, the protein was eluted with 10 x 1 ml with elution buffer (20 mM Hepes pH 8, 500 mM NaCl, 10% glycerol, 500 mM imidazole). The eluates were analyzed on a 10% SDS-acrylamide gel.

RNase J processing assays

The assay mixture (10 microl) containing 20 mM/ HEPES-KOH (pH: 8.0), 8 mM MgCl2, 100 mM NaCl, 0.24 U/ml RNasin (Promega), 70 pmol/µl of 5’ triphosphorylated, 32P-labeled mRNA substrate or 200 pmol/µl of 5’ monophosphorylated random labeled mRNA substrate and 10 pmol/µl of purified RNase J protein. The reaction was incubated at 37 °C. For time course experiments, samples of scaled-up reactions (60 µl) were taken at the indicated times. Reactions were stopped by the addition of 5 µl of 3X gel loading buffer (87.5% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 5 mM EDTA). The control reaction was performed by incubating substrate with the reaction buffer in the same conditions. The samples were directly loaded on a 20% polyacrylamide gel and the reaction products were visualized on a Phosphorimager.

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Figure legends

Fig. 1. Three-dimensional structures of the RNase J monomer. The RNase J sequence of (A) T. thermophilus, (B) B. subtilis and (C) M. smegmatis were submitted on the ExPASy proteomics server and the obtained structures were elaborated with the program pyMOL. The N-and C-terminal ends of the proteins are indicated by the letters N and C, respectively. The different domains are indicated. The position relative to the amino acids changed in the mutated protein are indicated by arrows.

Fig. 2. M. smegmatis RNase J endo-and exo-ribonucleolytic activity in vitro. The B. subtilis thrS leader transcript was used as substrate for the reactions as described in Experimental Procedures. Both the wild type and the mutant RNase J proteins were used. Each reaction was stopped at the time indicated on top of the lanes (in min) and the reaction products were separated on a 20% (w/v) denaturing polyacrylamide gel. **A. Activity on a 5' triphosphorylated transcript.** The 5'-PPP thrS leader transcript labeled at its 5'-end with !-32P GTP was incubated with the wild-type RNase J (lanes 2-5) and a mutant form carrying the active site mutations D85K and H86A (lanes 7-10). As a control the transcript was incubated for 20 min with the reaction buffer (lanes 1 and 6). The positions of the 3 and 7 nucleotide fragments and the position of GTP are indicated on the left. **B. Activity on a 5' monophosphorylated transcript**. A continously labeled 5'-monophosphorylated purified 350 nt thrS leader mRNA (the 350 nt readthrough RNA band was incubated with wildtype RNase J (lanes 2-6) and the active-site mutant (D85K and H86A) protein (lanes 8-12). The numbers at the right of the gel indicate the size of bands of the 50 bp ladder; UMP indicates the position of free mononucleotides produced by the exonucleolytic activity of RNase J.

Fig. 3. Growth of conditional expression mutants for the M. smegmatis rnj and rne genes. A. Growth on solid medium. Ten-fold serial dilutions of log phase cultures of the wild type, rne conditional mutant (Pptr-rne) and rnj conditional mutant (Pptr strains were replicated on

LB-agar in the presence (I) or absence (II) of pristinamycin I (Pr). **B. Growth curves in liquid medium**. Precultures were grown in the presence of pristamycin I to an OD600 = 0.5, washed twice and diluted 1:200 in fresh medium, with or without Pristinamycin I; point 0 on the time scale corresponds to the dilution of the culture. Pr = Pristinamycin I.

Fig. 4. Schematic view of the two rRNA operons (rrnA and rrnB) in M. smegmatis. The two operons have identical sequences except for the first part of the leader region that is shown in grey. The mature rRNA sequences are shown in dark grey, and the sequences removed by processing as white boxes. P1/2/3 and P indicate the promoters directing expression of the rrnA and rrnB operons, respectively (Gonzalez-y-Merchand et al., 1996). The figure is not drawn to scale. The sizes of the leader and spacer regions are indicated below the schemes. The relative positions of the oligonucleotides V30/V32/V37 used for sequencing and primer extensions are shown by arrows. A scale indicating the coordinates of the mature 5' and 3' ends of each rRNA of the rrnB operon is reported at the bottom.

Fig. 5. Analysis of the 5' end maturation of the rRNA precursors. Primer extension experiments were performed on total RNA from a M. smegmatis wild type strain mc2155, the rnj null mutant ($rnj), and the Pptr-rne mutant both in the presence and in the absence of the inducer (Pr), as indicated on top of the lanes, using specific primers (V30, V32, and V37, in A, B, and C, respectively). The total RNA of the Pptr-rne mutant without pristinamycin I was extracted from cultures that stop growing after about 7 generations. The extension products were separated on a 5% (w/v) denaturing polyacrylamide gel. The first 4 lanes of each gel report the DNA sequences obtained with the same primers on the rrnB operon. Sequences surrounding major signals obtained by primer extension are shown on the left, the exact positions are circled. The numbering indicates the position of a signal with respect to the +1 rrnB transcription start. **A. Analysis of the rrnA/B leader regions**. Primer V30 (Fig. 4) was extended to detect 16S 5' precursors. **B. Analysis of the spacer 1 region (Fig. 4**). Primer V31 was extended to detect 23S intermediate rRNA precursors. **C. Analysis of the spacer 2 region**

(Fig. 4). Primer V32 was extended to detect 5S intermediate rRNA precursors.

Fig. 6. Secondary structure models of the M. smegmatis rRNAs precursors. The structures are based on an M-fold analysis with some manual adjustments. All coordinates refere to the the transcription start from the rrnB promoter (+1). The scissors symbol indicates the location of signals observed by primer extension. Where known, the name of the RNase responsible of the cleavage is indicated. **A. Pre-16S rRNA structure**. The framed region corresponds to the 16S mature rRNA. The region upstream of the mature 5' end corresponds to the leader region. The downstream region (170 nt from the 3' end of the 16S rRNA) corresponds to the 5' half of the spacer 1 region. **B. Pre-23S-5S rRNA secondary structure**. The framed regions correspond to the 23S and 5S mature rRNAs. The region upstream of the 5' end of the 23S rRNA (215 nucleotides) corresponds to the 3' half of the spacer 1 region. The sequences downstream of the mature 23S 3' end represent the spacer 2 and the 5S rRNA. The 12 nucleotides shown in grey are most likely degraded by the 5'-3' exonucleolytic activity of RNase J.

Fig. 7. Models of the 5' end maturation of the 16S, 23S and 5S rRNA in M. smegmatis, E. coli and B. subtilis. Simplified models of the precursors for the three ribosomal subunits are presented. The grey boxes represent the mature rRNAs. The positions of the different cleavages are marked by arrows with the name of the enzyme involved or a scissor if unknown. The 5'-3' exoribonucleolytic activity is shown by a pacman symbol.

Table 1 : Occurrence of ribonucleases

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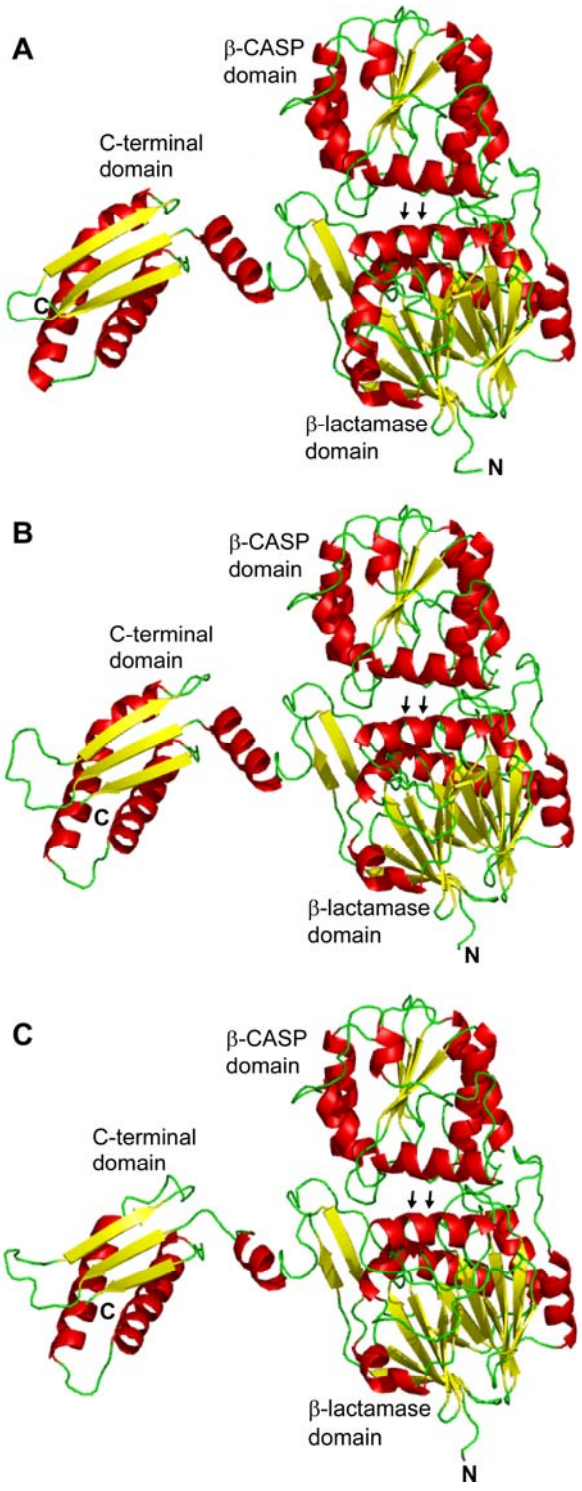
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β-lactamase domain
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C
β-CASP domain
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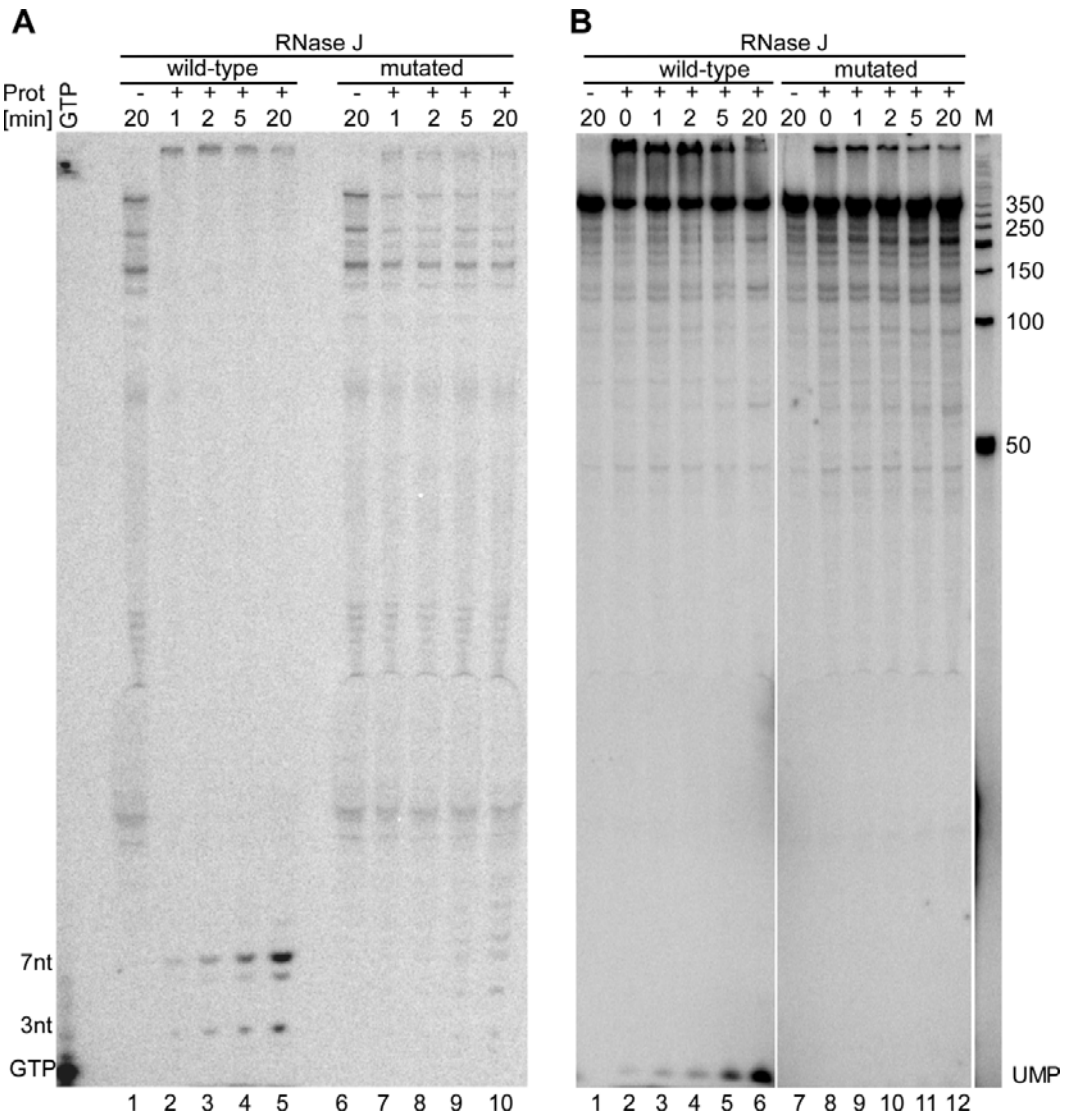
C
β-lactamase domain
N

Fig. 1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mutated
Prot
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3nt
GTP
1 2 3 4 5 6 7 8 9 10
B
RNase J
wild-type
mutated
- + + + + + - + + + + +
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UMP
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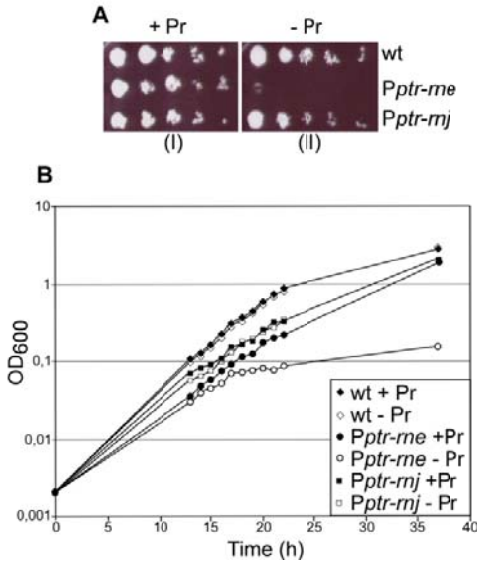
Fig. 2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- Pr
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(II)
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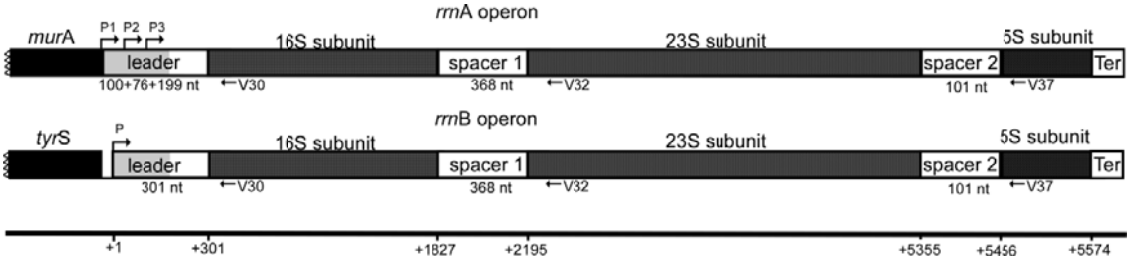
Fig. 3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V32
101 nt
V37
rrnB operon
tyrS
P
leader
16S subunit
spacer 1
23S subunit
spacer 2
5S subunit
Ter
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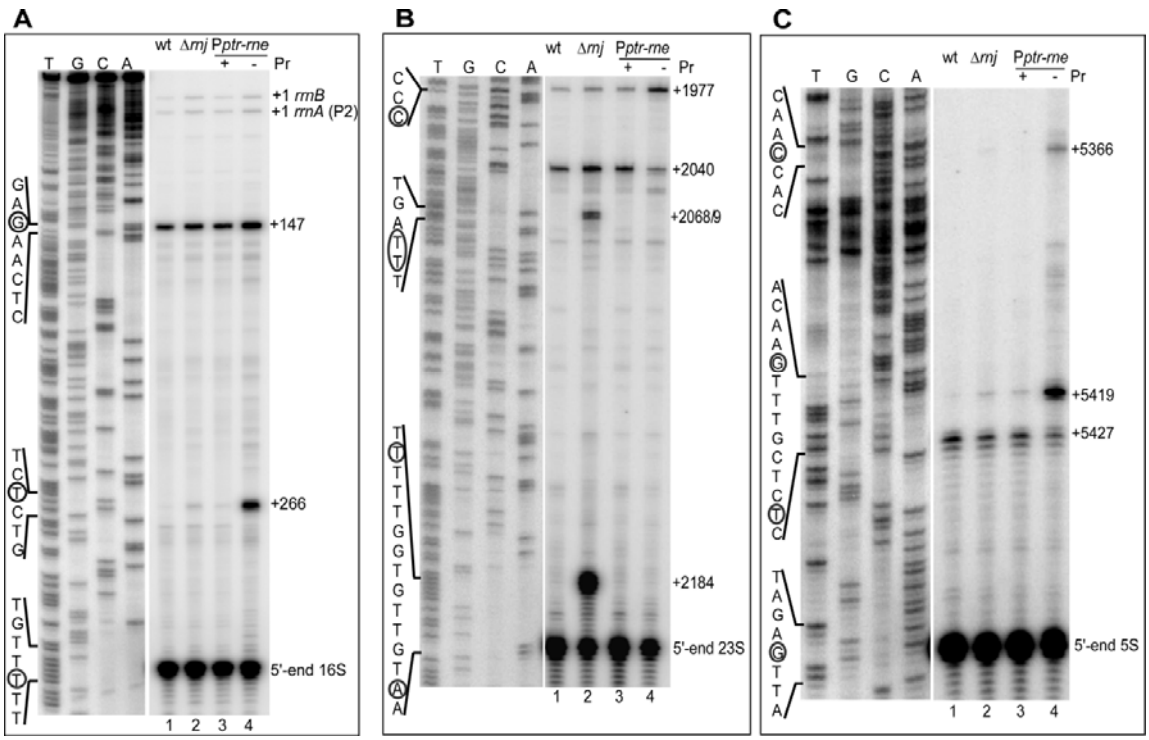
Fig.4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B
wt Δrnj Pptr-rne
+ -
Pr
T G C A
+1977
+2040
+2068/9
+2184
5'-end 23S
1 2 3 4
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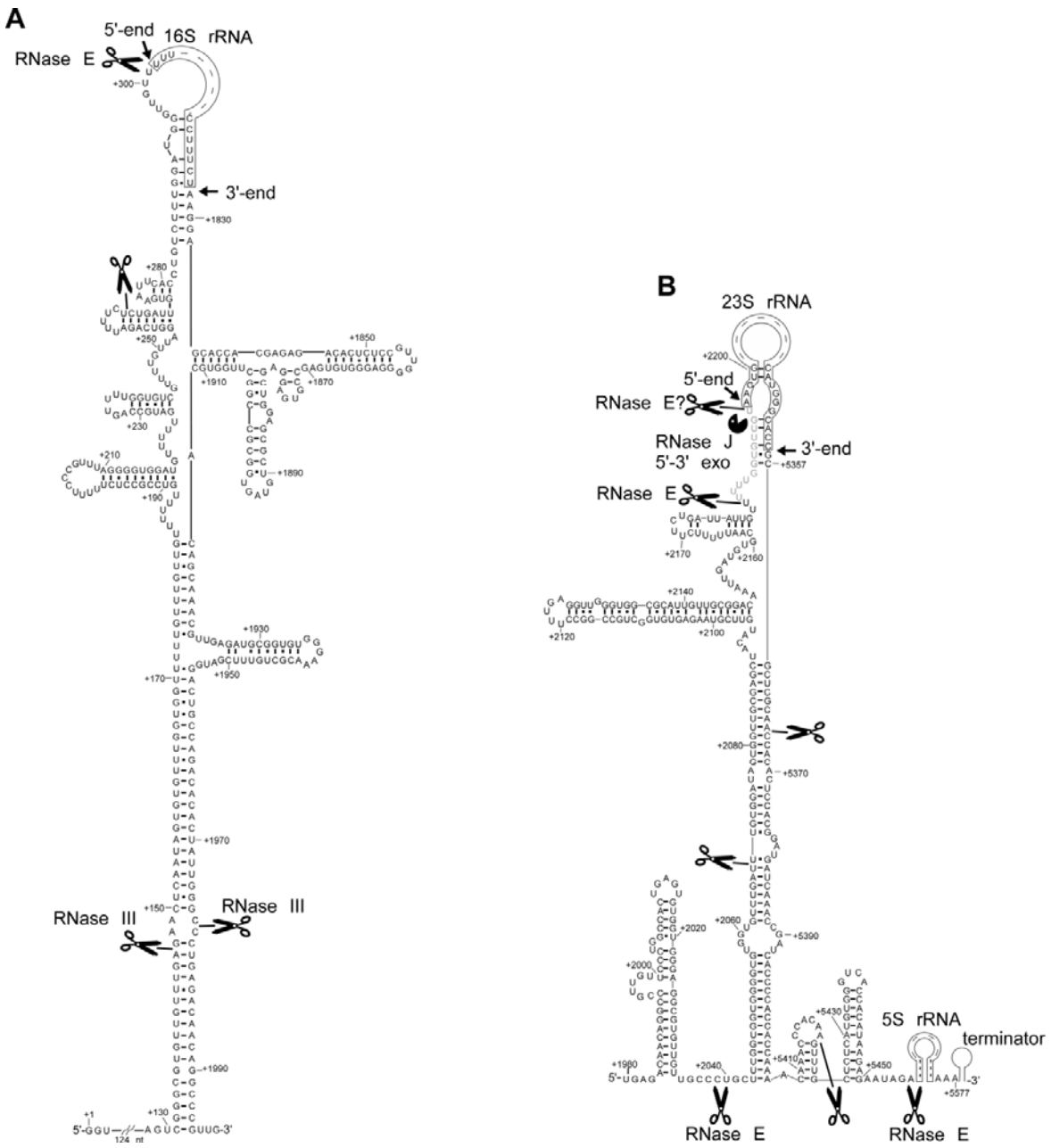
Fig. 5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23S rRNA
5'-end
RNase E?
RNase J
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RNase E
5S rRNA
terminator
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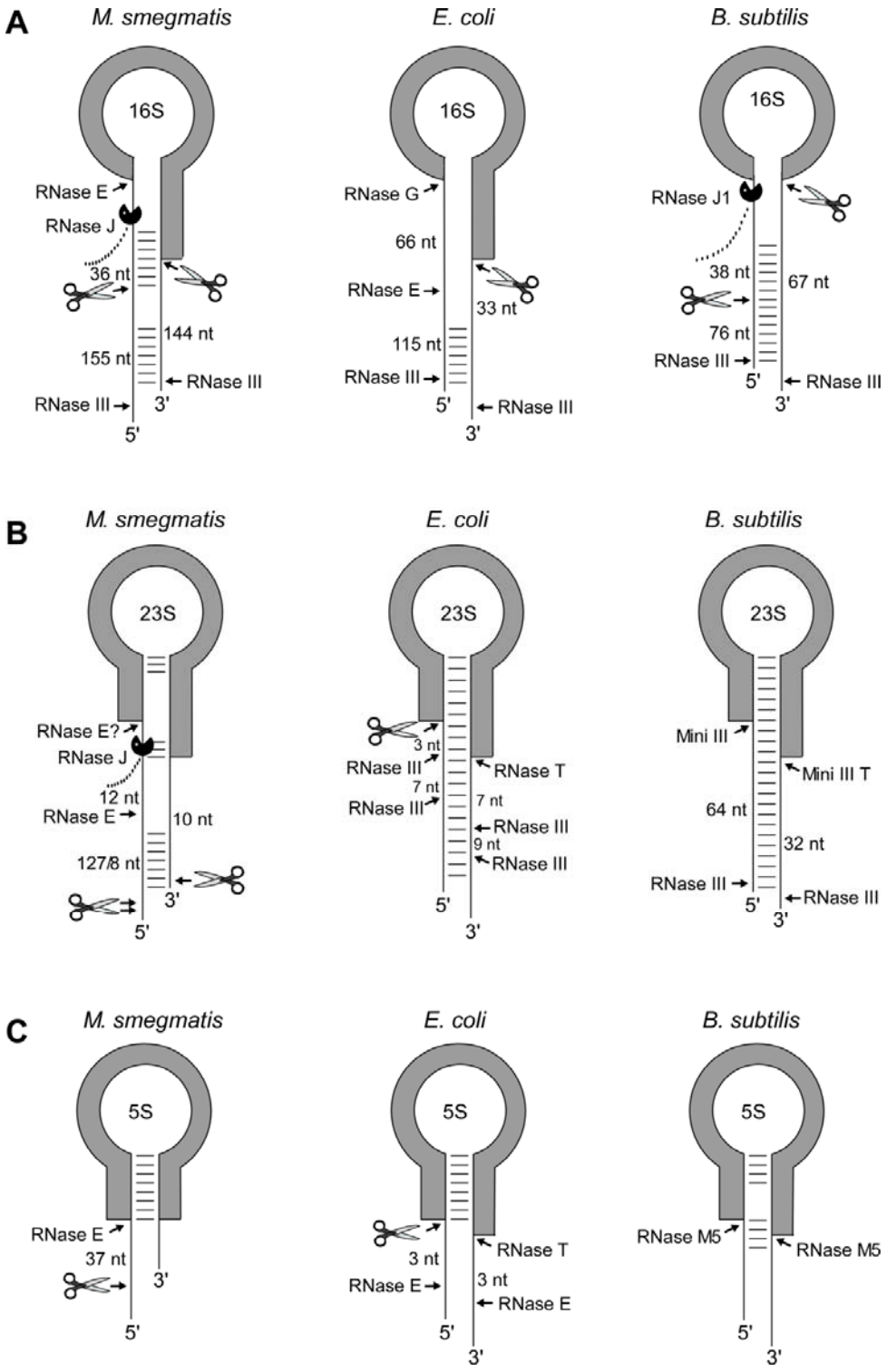
RNase E
RNase E

Fig. 6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RNase III
3'
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E. coli
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RNase G
66 nt
RNase E
33 nt
115 nt
RNase III
5'
RNase III
3'
B. subtilis
16S
RNase J1
38 nt
67 nt
76 nt
RNase III
5'
RNase III
3'
B
M. smegmatis
23S
RNase E?
RNase J
12 nt
RNase E
10 nt
127/8 nt
3'
5'
E. coli
23S
3 nt
RNase III
RNase T
7 nt
RNase III
7 nt
RNase III
9 nt
RNase III
5'
3'
B. subtilis
23S
Mini III
Mini III T
64 nt
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RNase III
5'
RNase III
3'
C
M. smegmatis
5S
RNase E
37 nt
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RNase E
5'
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B. subtilis
5S
RNase M5
RNase M5
5'
3'

Fig. 7