RNA metabolism in mycobacteria: The role of RNase E

Ph.D. Thesis

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I. Introduction

I.1. Pathophysiology

Tuberculosis (TB) has troubled humankind throughout history. It has been a leading cause of death throughout the world, and still is in low- and middle-income countries. TB is a classic example of airborne infection. Bacilli that reach the respiratory bronchioles and alveoli are generally ingested by alveolar macrophages and removed from the alveoli. Macrophages, even in individuals with no prior immunologic experience with mycobacterial infection, possess some bacterial-killing or growth-inhibitory capability. Thus, killing and digestion of the organism probably follow in the majority of exposures to *Mycobacterium tuberculosis*. However, when many droplet nuclei containing tubercle bacilli reach the alveoli, the number of bacilli ingested by an individual macrophage can overwhelm the microbicidal system of the phagocyte. When the nonspecific defences are insufficient to kill all of the organisms, the surviving bacilli multiply, causing a localized tuberculous pneumonia. In general, in the presence of an effective, specific cell-mediated immune response, this lesion heals spontaneously, leaving only a calcified focus (Ghon lesion), which may be accompanied by calcification in hilar nodes, the two lesions together forming the Ranke complex.

The principal immune response associated with protection against tuberculosis is cell-mediated immunity involving T lymphocytes and macrophages. Although immunization does not prevent infection with *M. tuberculosis*, the growth of the organism within macrophages is reduced by several orders of magnitude. In dramatic contrast, immunodeficiency, such as that produced by Human Immunodeficiency Virus (HIV) infection or selective depletion of T cells or cytokines, especially interferon-γ (IFN-γ), results in a weakening of defences against *M. tuberculosis*. However, because *M. tuberculosis* is a virulent organism, little if any immunodeficiency is necessary for disease development. Thus, TB, which commonly occurs with a lesser degree of immunodeficiency than other HIV-associated infections, has become a sentinel disease for the presence of HIV infection.

I.2. Epidemiology

TB is a major threat to global health, recently exacerbated by the emergence of highly drug-resistant forms of the disease-causing pathogen and synergy with HIV / AIDS (Acquired Immunodeficiency Syndrome). A recent report on the TB pandemic revealed that in 2005, there were almost 9 million new cases and 1.6 million deaths.

Unfortunately, it has been estimated that one third of the world's population is latently infected with *M. tuberculosis*, providing an enormous reservoir for future disease.

I.3. Tuberculosis control

The Stop TB (S TB) Partnership's goal is eliminating TB as a public health problem by 2050. However, the current tools for diagnosing, treating, and preventing TB are inadequate for this task: latent and active TB are most commonly diagnosed using, respectively, a skin test that dates back to 1891 and sputum-smear microscopy from the same era. Despite its widespread use, the BCG vaccine is largely ineffective, at least in preventing adult pulmonary disease. Conventional short-course therapy has not changed for decades. The most frequently recommended and effective combination is isoniazid, rifampicin, pyrazinamide, and ethambutol for 2 months, followed by isoniazid and rifampicin for 4 months. This regimen is very effective for treatment of patients with TB, including those with HIV infection. Failure to complete therapy is associated with longlasting infectious status, relapse, and drug resistance. Unfortunately, acquired rifamycin resistance has arisen in HIV-infected individuals and in those with widespread disease. A growing problem that has had an impact on the success of treatment programs is the emergence of strains of multidrug-resistant TB (MDR-TB), which is defined as resistance to isoniazid and rifampicin, to the drugs used as first-line treatment. Around 400,000 cases of multidrug- resistant *M. tuberculosis* infection occur per year.

Furthermore, essentially untreatable outbreaks of extensively drug-resistant TB (XDR-TB) have begun to appear. XDR-TB is defined as MDR-TB plus resistance to a quinolone and one of the second-line anti-TB injectable drugs (Amikacin, Kanamycin, and Capreomycin).

New antituberculotic drugs are needed to improve the treatment of patients with multidrug-resistant TB, and might enable the duration of treatment to be shortened.

I.4. The importance of RNA metabolism

M. tuberculosis can survive and even replicate in naive macrophages and thus must assimilate carbon and produce energy in the phagosome. *M. tuberculosis* is among the most successful microorganisms that adapt to long-term residence in macrophage phagosomes. Adaptive intracellular gene expression involves genes that are associated with virulence, the general stress response, changes in cell wall structure and alternative-carbon-source utilization pathways and their regulation. The turnover of mRNA plays an important role in the regulation of gene expression.

In *E. coli*, a key enzyme of RNA decay is RNase E. It is an essential enzyme, responsible for the initial rate-limiting cleavage in the decay of many mRNAs, as well as playing an important role in 5S and 16S rRNA processing.

In our laboratory in accordance with other researchers, we believe that RNase E the main enzyme of RNA metabolism is a potential target of drug aiming.

I.5. RNase E and the degradosome it organises

RNase E was originally discovered as an RNA processing enzyme. Later it was found that its gene, *rne*, and *ams* (altered messenger stability) are identical, suggesting a key role for RNase E in bacterial RNA processing and decay. Subsequent studies revealed that RNase E controls the stability of many, if not the most, mRNAs in *E. coli* as well as being involved in the processing of tRNA precursors and degradation of small regulatory RNAs. Moreover, RNase E has the ability to affect protein degradation through its role in maturation of the 3' end of tmRNA (SsrA).

Our current knowledge about this enzyme mainly originates from studies of *E. coli* RNase E, which is a site-specific ribonuclease that preferentially cleaves in A/U-rich single-stranded regions of structured RNAs.

E. coli RNase E is indispensable for cell viability. Structural data obtained for an N-terminal polypeptide representing the evolutionarily conserved catalytic domain of this protein have revealed the presence of discrete folds with putative functions in RNA recognition and cleavage. Moreover, these data and others indicate that the catalytic domain exists as a homotetramer, which has two non-equivalent subunit interfaces organizing the active site of the enzyme. In contrast with the N-terminal catalytic domain, the C-terminal part of *E. coli* RNase E shows very little or no similarity to the equivalent regions of other RNase E homologues. This part of the *E. coli* enzyme contains an extra RNA-binding domain(s) and multiple sites for interactions with the 3' to 5' phosphorolytic exonuclease PNPase (polynucleotide phosphorylase), the RNA helicase RhlB and the glycolytic enzyme enolase to form the *E. coli* multienzyme complex referred to as 'degradosome'. Interestingly the RNA degradosome proteins comprise parts of the bacterial cytoskeleton.

E. coli RNase G is encoded by the *rng* gene and was initially termed CafA owing to its effects on the formation of cytoplasmic axial filaments observed upon RNase G overproduction *in vivo*. Further studies have revealed that this protein has an endoribonucleolytic activity and is involved in maturation of 16S rRNA *in vivo*. Although RNase G functionally overlaps with RNase E and shares 35% identity with and 50%

similarity to the N-terminal catalytic part of RNase E, this endoribonuclease cannot fully compensate RNase E deficiency in *rne*^{ts} mutant strains.

Owing to their overlapping functions and apparently common origin, RNase E- and RNase G-like proteins are believed to belong to the same family of RNase E/G endoribonucleases that are predicted to exist in many bacteria, including pathogenic species. Although their biochemical properties and biological functions have not yet been investigated in detail in human pathogens, such studies can offer important insights concerning the role of these endoribonucleases during infection and disease development. Recent results demonstrated the importance of RNase E in bacterial pathogenicity. In yersinia, the RNase E activity impacts the Type Three Secretion System and plays a general role in infectivity. Magnesium homeostasis is very important for the intracellular survival of salmonella. The Mg^{2+} riboswitch targets the mgtA transcript for the degradation by RNase E when cells are grown in high Mg^{2+} environments.

Given the central role of RNase E in RNA processing and decay in *E. coli*, it is conceivable that RNase E-dependent regulation of RNA stability in mycobacteria might also represent a very important mechanism adjusting the cellular metabolism to environmental changes. These considerations prompted us to purify and characterize the RNase E/G homologue (RNase E/G) from the intracellular pathogen *M. tuberculosis*.

The difference between pathogenic and saprophytic mycobacteria on the level of RNA metabolism is yet not known.

II. Aims

To learn more about RNase E proteins that function in different mycobacterial species, we cloned, overexpressed and purified putative mycobacterial RNase E/G homologues from *M. bovis* BCG, from *M. tuberculosis* and *M. smegmatis*.

I. The present study was designed to identify proteins associated with RNase E from:

   a. *M. tuberculosis*

   b. *M. smegmatis*

II. Biochemical characterisation of RNase E/G from *M. tuberculosis*.

   a. To study the 5'-end-dependence of this endoribonuclease

   b. To examine the quaternary structure of the mycobacterial RNase E

   c. To study if it can cleave a putative 5S rRNA precursor *in vitro*

   d. To compare its substrate specificity to the *E. coli* counterpart

III. Materials and methods

III.1. Bacterial strains and growth conditions

E. coli DH5α, *E. coli* HB101, *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus (DE3)-RIL strains were routinely grown in LB (Luria–Bertani) medium supplemented with the appropriate antibiotic(s). *M. bovis* BCG was grown in Middlebrook 7H9 broth or 7H11 agar (Difco). *M. smegmatis MC2* 155 was grown either in Middlebrook or in LB medium.

III.2. Cloning of mycobacterial RNase E, RNase E/G and Srne

The DNA fragment containing the corresponding gene was amplified by PCR, using conditions recommended by the manufacturer. The amplified DNA was inserted into p6HisF-11d(*icl*). The resulting plasmid was used to overexpress RNase E in *E. coli*. p6HisF11d(*icl*) is a pET-11d (Novagen) based plasmid carrying the 6His and FLAG tags in the N-terminal of the cloned insert.

III.3. Expression of the mycobacterial proteins (RNase E, RNase E/G and Srne) in *E. coli*

For overexpression, *E. coli* HB101(pGP1-4) cells carrying the proper plasmid were grown and treated according to the method of Tabor and Richardson.

III.4. Expression of the mycobacterial proteins (RNase E, RNase E/G) in *M. bovis* BCG or Srne in *M. smegmatis* MC2 155

pET plasmids cannot be used in mycobacteria, for this purpose we created new constructs using pMV262 shuttle plasmid. These plasmids as well as pMV262(*icl*) coding for isocitrate lyase (Icl) were transformed to *M. bovis* BCG, or in case of MSMEG_4626 to *M. smegmatis* by electroporation.

III.5. Purification of RNase E, RNase E/G, Srne and Icl

Bacteria were opened in a MiniBeadbeater-8 cell disrupter (BioSpec Products, Inc, Barlesville, Oklahoma), using glass beads (106 μm, Sigma) and adding a protease inhibitor cocktail (Sigma). Proteins were purified either on an anti-FLAG M2 affinity gel (Sigma), or by using Talon Metal Affinity Resin (BD Biosciences) according to the manufacturer's instructions.

III. 6. Identification of protein bands

The gel slices containing the corresponding polypeptides were cut from the gel and analyzed by mass spectrometry (Biological Research Centre, Szeged). Post source decay analysis was used to confirm the MALDI-TOF results.

III.7. Purification of Rne498

E. coli BL21(DE3) strain was used to overexpress 6His-tagged Rne498, and the overexpressed protein was purified using immobilized metal-affinity chromatography as described previously.

III.8. Purification of *M. tuberculosis* RNase E/G for biochemical characterisation

The pMRG1 construct, verified by sequencing was transformed into the *E. coli* strain BL21-CodonPlus (DE3)-RIL. The cells were disrupted by sonication in a Cell Disruptor W375 (Heat Systems Ultrasonics), and the supernatant was applied to a HisTrap HP (Amersham Biosciences) column to purify 6His-tagged RNase E/G according to the manufacturer's instructions.

III.9. In vitro transcription and labelling of 9S RNA

A DNA fragment including the putative *M. bovis* BCG 9S RNA sequence was PCR-amplified from genomic DNA and cloned into the pGEM®-T Easy vector (Promega) at the T cloning site. The resulting plasmid (pMt9S) was used to transcribe 9S RNA with a MEGAscript® T7 kit (Ambion). 9S RNA was 5′-labelled using T4 polynucleotide phosphorylase (Fermentas) and an excess of [γ-^{32}P]ATP (Amersham Biosciences) and subsequently used for cleavage assays.

III.10. Primer extension

Total RNA from *M. bovis* BCG was extracted using the hot phenol method. The 5′-^{32}P-labelled 5S rRNA-specific oligonucleotide 5SPEM (5′-CAGTATCATCGGCGCTGGC-3′) and total RNA were mixed with 5 µl of RT buffer containing 4 units of AMV reverse transcriptase (Promega). The reaction was stopped by adding 10 µl of sequencing dye. The sample was analysed on an 8% sequencing gel along with a concurrently run sequencing ladder.

III.11. Synthetic oligonucleotide substrates

Ribo-oligonucleotides (BR10), (9SA), (OmpC), (A27), (U27), (U27G), (U27A) and (U27C), as well as oligonucleotides (pBR13-Fluor) and (HO-BR13-Fluor) with a fluorescein tag at their 3′ ends, were purchased from VBC Genomics, whereas (U27ab) containing an abasic residue (X) was purchased from Dharmacon.

III.12. RNase E/G cleavage of oligonucleotide substrates and 9S RNA

RNase E/G cleavage reactions were performed as described previously using Rne498 or RNase E/G and 5′-labelled substrates. Ladders (1 nt) were prepared either by partial alkaline hydrolysis, or by partial digestion of each 5′-end-labelled oligonucleotides with S1 nuclease or with RNase T_1.

IV. Results

IV. 1. Expression of RNase E from *M. tuberculosis*

It is a protein consisting of 953 amino acids. Its calculated molecular mass is 103 kDa, but in SDS polyacrylamide gels it migrates as a 150 kDa protein. *M. tuberculosis* H37Rv RNase E displays 100% identity with the full-length *M. bovis* BCG RNase E.

IV. 2. Expression of *M. smegmatis* Srne

Srne is consisting of 1037 amino acids. Its calculated molecular weight is 112.7 kDa; because of the 6His and FLAG tags, our fusion protein is 4 kDa larger. In SDS-polyacrylamide gels it migrates as a 180 kDa protein.

IV. 3. Associated proteins to RNase E from *M. tuberculosis*

RNase E was overexpressed in different bacterial hosts and affinity-purified. Despite the use of protease inhibitors, in addition to the full-length polypeptide, the mycobacterial preparation from *E. coli* also contained its proteolytic forms. DnaK is among the identified copurified proteins. DnaK is involved in many cellular activities, including the heat shock response. Further associated proteins were lipoamide dehydrogenase (NADH), a component of 2-oxodehydrogenase and pyruvate complexes and a mixture of ribosomal proteins L1 (B3984) and S2 (B0169).

In the RNase E preparation isolated from *M. bovis* BCG, GroEL was identified. This is a chaperonin mediating protein folding and assembly. The preparation contained furthermore the products of genes Mb1721 and Mb0825c, respectively. These proteins were found in the database as hypothetical proteins. Mb1721 is equivalent to Rv1695 (100 % identity) from *M. tuberculosis* H37Rv. Gene MT1734 from *M. tuberculosis* CDC1551 codes for the same protein. This protein is an inorganic polyphosphate/ATP-NAD kinase (Ppnk). Mb0825c is equivalent to Rv0802c and MT0822 from *M. tuberculosis* H37Rv and CDC1551, respectively. The protein displays partial similarity with many acetyltransferases and hypothetical proteins. Table I lists the identified proteins and the comparison of the RNase E associated proteins from different bacteria.

IV. 4. Srne-associated proteins

Table I lists the identified proteins. Proteolytic fragments of RNase E were found in all bands checked. GroL was the major associated protein. However ribosomal proteins were also present. Among others, a negative regulator of genetic competence and a GTP pyrophosphokinase, which has role in cellular response to starvation,

dimethyladenosine transferase and the universal stress protein were also component of the complex.

No visible differences were seen in the associated proteins when *M. smegmatis* was grown in LB or in Middlebrook media.

E. coli	*M. tuberculosis* RNase E in *M. bovis* BCG	*M. smegmatis*
• Polynucleotide phosphorylase • Enolase • RhlB helicase	• GroEL • Inorganic polyphosphate/ATP-NAD kinase (Mb1721) • N-acetyltransferase (Mb0825c)	• Chaperonin GroL • Ribosomal proteins • Negative regulator of genetic competence • GTP pyrophosphokinase • Proteosome component • EF Tu

Table I.: Comparison of the RNase E associated proteins from different bacteria

IV. 5. Expression and purification of mycobacterial RNase E/G

In *M. tuberculosis* CDC1551, RNase E/G contains 661 amino acids and exhibits 100% identity with the C-terminal part of H37Rv RNase E. This polypeptide with the predicted molecular weight of 67 kDa, has an aberrant mobility (100 kDa) in SDS polyacrylamide gels. Likewise, its overexpression in *M. bovis* BCG and purification results in a preparation that contains a copurified inorganic polyphosphate/ATP-NAD kinase (Ppnk), a truncated form of RNaseE/G and an extra polypeptide, whose identity was not determined.

IV. 6. Expression and purification of mycobacterial RNase E/G for biochemical characterisation

The71 kDa RNase E/G from the *M. tuberculosis* exhibited an aberrant mobility, migrating as a 110 kDa polypeptide. Mass spectrometry analysis did not reveal any contamination of the RNase E/G preparation by *E. coli* RNase E/G polypeptides or other host ribonucleases.

IV. 7. Quaternary structure of *M. tuberculosis* RNase E/G

RNase E was eluted as a complex with a hydrodynamic radius corresponding to a spherical protein with an apparent molecular mass of approx. 325 kDa, suggesting that, it may exist in a tetrameric form.

IV. 8. Cleavage of oligonucleotide substrates

RNase E/G could cut BR10, 9SA and OmpC yielding cleavage patterns that were largely indistinguishable from the analogous patterns generated by its *E. coli* counterpart. A minor difference was only observed in the cleavage pattern of OmpC. Although Rne498 cleaved this oligonucleotide at positions 5 and 6 with equal efficiencies, RNase E/G showed some preference for cleavage at position 5. We also found that, similar to its RNase E/G homologues from *Aquifex aeolicus* and *E. coli*, RNase E/G requires Mg^{2+} ions for its activity.

IV. 9. 5'-end-dependence of *M. tuberculosis* RNase E/G cleavages

RNase E/G cleaves 5'-monophosphorylated substrates faster than the non-phosphorylated ones.

IV. 10. Probing the substrate specificity of *M. tuberculosis* RNase E/G

In order to identify specific sequence determinants of RNase E/G cleavage sites, we used the same oligonucleotide-based approach that was employed previously to characterize its *E. coli* counterpart. By comparing the cleavage patterns of poly(A) and poly(U) oligonucleotides and their mutant variants carrying single-base substitutions of G, C, U or A respectively for adenosine (or uridine), this method allows determination of each nucleotide's effect at multiple positions to the point of cleavage. Unlike *E. coli* RNase E, RNase E/G cleaved A27 inefficiently. In the following assays, we therefore decided to use only U27 (control) and its derivatives, U27A, U27G, U27C and U27ab oligonucleotides, that had a single-base substitution of A, G, C or abasic residue respectively for U at position 14. Similar to cleavage of U27, incubation of U27G with RNase E/G and Rne498 resulted in nearly identical patterns. The major cleavages of U27G at positions U8, U9, U10 and U15 were observed for both enzymes, whereas Rne498 (but not RNase E/G) could additionally cleave this substrate at position U18. In the case of *E. coli* RNase E, it has been reported previously that a G nucleotide, which is 5' and in close vicinity to the scissile bond, is an important determinant of cleavage. Likewise, we found that *M. tuberculosis* RNase E/G cleavage is more efficient if there is a G nucleotide located two nucleotides upstream of the scissile bond. These data are fully consistent with the cleavage patterns of BR10, 9SA and OmpC oligonucleotides, showing that RNase E/G cleavage frequently occurs two nucleotides downstream of a G nucleotide.

In contrast with nearly identical cleavage patterns that were generated by RNase E/G and Rne498 using U27G, the cleavage patterns of U27A, U27C and U27ab showed

relatively low resemblance. Although Rne498 can cut at multiple positions within U27A, RNase E/G cleavage of the same substrate was not observed at many of these locations, especially downstream and upstream of U16. Similarly, the presence of a cytosine at position 14 in U27C strongly inhibits RNase E/G cleavage at nearly all locations, suggesting the overall inhibiting effect of this nucleotide.

Finally, we found that substitution of an abasic residue for the U at position 14 in U27ab decreased the efficiency of RNase E/G cleavage at multiple locations. This finding strongly suggests that the presence of uridines at many locations close to the scissile bond promotes RNase E/G cleavage. On the basis of the above experimental data, we determined and summarized the effects of each nucleotide within RNase E/G cleavage sites Interestingly, according to these data, the C at position +2 in 9SA should confer higher resistance of this oligonucleotide (in comparison with BR10 lacking a cytosine at the equivalent position) to RNase E/G. In contrast, we did not observe any significant differences in the efficiency of cleavage. This may imply that the expected inhibiting effect of the cytosine was efficiently counteracted by other nucleotides, for example by the A and G nucleotides at positions −3 and −2 respectively. In other words, strong enhancing effects of nucleotides at certain positions of different cleavage sites can potentially neutralize or override negative effects of other sequence determinants, resulting in approximately equal efficiency of cleavage. As in the case of *E. coli* RNase E, the efficiency of RNase E/G cleavage of U27 and its mutant variants decreases with the number of the remaining nucleotides that are 5′ to the scissile bond (the 'end-proximity effect'), thereby leading to accumulation of short oligomers that are further resistant to the nucleolytic activity of this enzyme.

IV. 11. 9S RNA processing by *M. tuberculosis* RNase E/G

Although both Rne498 and RNase E/G could cleave *M. tuberculosis* 9S RNA, the cleavages occurred at different locations. Further analysis revealed that RNase E/G cleaved 9S RNA at the position, which nearly coincides with the position of the 5′-end of mature 5S rRNA *in vivo*, which was mapped by primer extension of total RNA.

V.Discussion

It has been more than 30 years since the introduction of a novel compound for the treatment of tuberculosis. New drugs are extremely needed. Agents that reduce the duration and complexity of the current therapy would have a major impact on compliance and overall cure rate. In recent years, our understanding of the tubercle bacillus and its

interaction with the human host has improved dramatically, particularly with the publication in 1998 of the complete genome sequence of *M. tuberculosis* H37Rv. New genetic tools have been developed and we can now ascertain the function of individual genes. Thus, many potential drug targets can be identified.

Mycobacteria are very successful pathogens, which are able to survive under adverse conditions like long non-replicating persistence, intracellular survival. Dynamic regulation of RNA decay and processing should be a very important factor in adapting to environmental changes. Over the past years it became obvious that mRNA degradation is a very important step in regulation of prokaryotic gene expression.

Given the central role of RNase E in RNA processing and decay in *E. coli*, it is conceivable that the RNase E-dependent regulation of RNA stability in mycobacteria might also be a very important mechanism, adjusting the cellular metabolism to environmental changes. Adaptive processes in the RNA metabolism of latent mycobacteria differentially affect the steady-state levels of numerous transcripts, ribosome biosynthesis as well as RNase E/G levels, suggesting an important regulatory role of this enzyme during disease development.

We focus our work on mycobacterial RNase E like proteins; the results are the first steps towards understanding their structure, function and the role of bacterial metabolism.

Although *M. tuberculosis* and related species are considered to be members of Gram positive bacteria, the mechanisms of RNA decay and processing in these organisms are apparently different from the regulatory circuits that control RNA metabolism in Gram positive bacteria with a low GC content such as *Bacillus subtilis*. The latter lacks RNase E and RNase G (CafA) and therefore the mechanisms that regulate RNA stability in *B. subtilis* must be quite different from those taking place in mycobacteria. On the other hand, RNase E can be found in the majority of human pathogens (both Gram positive and Gram negative bacteria), where it seems to be a key enzyme in RNA processing and degradation.

In our work we cloned, expressed and purified the RNase E/G homologue from *M. tuberculosis* (RNase E) and *M. smegmatis* (Srne) and identified associated proteins. RNase E and RNA helicase B play central roles in the cytoskeletal organization of the degradosome in *E. coli*. Polynucleotide phosphorylase and enolase are also important components. In agreement with a previously proposed idea, the associated proteins can be different in other bacteria.

In *M. tuberculosis* RNase E preparation from *E. coli* DnaK was identified. This polypeptide was previously found in the preparations when *E. coli* RNase E was overexpressed upon temperature-induced induction. It was not found in the RNase E complex, when RNase E was isolated without overexpression. In a DnaK(-) background, in an experiment, where the role of molecular chaperones in inclusion body formation was studied, the amount of inclusion body protein was 2.5-fold higher, than in the wild-type strain. In the preparation isolated from *M. bovis* BCG, GroEL, the products of genes Mb1721 (Ppnk) and Mb0825c were identified. Rv1695 (equivalent to Mb1721) has been cloned and expressed in *E. coli*. Ppnk catalyzes the reaction leading to the formation of NADP by the use of ATP or inorganic polyphosphate, playing a crucial role in the regulation of NAD/NADP level and in biosynthetic reactions in the cell. Its association with RNase E may play a regulatory role by helping to adapt to environmental changes. When 6-His-tag labelled proteins from lysates of *M. smegmatis* were purified also GroL was the major associated protein. The copurified proteins from *M. smegmatis* were different from those in *E. coli*, as expected after previous mycobacterial studies, though surprisingly they were not even identical to the associated proteins we found in case of the RNase E/G homologue from *M. tuberculosis*. Interestingly much more proteins were detected in the *M. smegmatis* preparations, than in the ones from *M. tuberculosis*. Although it is not easy to determine whether the associated proteins we report from *M. smegmatis* are specific functional components or occasional contaminants, it is important to note that the overexpression and subsequent purification of isocitrate lyase has yielded nearly pure recombinant protein, indicating that the above described copurification is specific and is not dependent on the presence of the 6HisFLAG tag.

While there is strong homology in the catalytic part of RNase E enzymes among mycobacteria, the C-terminal portions are quite different. In silico, using the CLC Combined Workbench the secondary structure of the RNase E from *M. smegmatis* and *M. tuberculosis* was drawn. Srne is mostly helical protein, containing 30 α-helices and with Pfam analysis 22 potential domains were suggested, with 9 domains more, than what was predicted in the tuberculosis RNase E. The in silico results suggest that there are possible differences among mycobacteria in the structure of the enzyme, which could explain the difference in quality and quantity of the associated proteins.

Despite the use of protease inhibitors in addition to the full-length RNase E its proteolytic fragments were detected in all bands. *E. coli* RNase E is also very sensitive to proteolysis. The presence of PNPase and RhlB helicase could not be detected among the

copurified proteins. The *M. smegmatis* genome contains genes coding for PNPase (MSMEG_2656) and RhlB helicase (MSMEG_1930). The proteins we found as associated proteins take part in protein synthesis, regulation and the stress response.

Our data indicate that, similar to *E. coli*, mycobacterial RNase E/G homologues are able to copurify with other proteins. However, in agreement with the previously proposed idea, the nature of the associated proteins is different when compared to the composition of the *E. coli* RNase E complex. Further studies are needed to address the role of the identified proteins in relation to the specific steps of RNA metabolism in mycobacteria and their regulation by environmental factors.

To learn more about the function and biochemical properties of the RNase E/G homologue from *M. tuberculosis* (RNase E/G), we cloned, purified and characterized a polypeptide including the centrally located catalytic domain as well as the flanking C-terminal part of this protein, since the full-length *M. tuberculosis* RNase E is difficult to purify in large amounts because of its tendency to form insoluble aggregates upon overexpression.

RNase E/G homologues from Gram-negative bacteria are known to preferentially cleave 5′-monophosphorylated substrates over non-phosphorylated or 5′-triphosphorylated ones. By employing oligonucleotide substrates, we have also shown that RNase E/G has an endoribonucleolytic activity, which is dependent on the 5′-phosphorylation status of its substrates, thereby suggesting that RNase E/G is a 5′-end-dependent endoribonuclease. Thus our findings suggest that the so-called 5′-end-dependence is apparently a common feature of RNase E/G homologues from both Gram-negative and Gram-positive bacteria. This property of RNase E/G and other RNase E/G homologues implies that these enzymes more efficiently cleave already damaged or partially degraded forms of cellular RNAs (i.e. RNA species that usually carry 5′-monophosphate groups) rather than primary transcripts, which are naturally triphosphorylated.

Given that RNase E and RNase G are involved in maturation of ribosomal and transfer RNAs in *E. coli*, we anticipated that this function of RNase E/G-like proteins might be also conserved in mycobacteria. Indeed, we found that RNase E/G was able to cleave mycobacterial 9S RNA, a putative precursor of its cognate 5S rRNA, and the cleavage occurred in close vicinity to the mature 5′-end of 5S rRNA, thereby suggesting a role for this enzyme in rRNA processing. Although the internucleotide bonds at the 5′-end of 5S rRNA that are generated by RNase E/G *in vitro* and during rRNA processing *in vivo*

were not exactly the same, this minor (one or two nucleotide) difference could be well explained by the action of ancilliary factors or associated ribosomal proteins that can affect RNase E/G cleavage *in vivo* and therefore slightly influence the selection of the scissile bond(s).

Taken together, our findings expand the current knowledge about complexforming and biochemical characteristics of RNase E/G enzymes and provide a basis for further analysis of their putative functions in mycobacteria.

VI. Conclusions

TB is a major threat to global health, recently exacerbated by the emergence of highly drug-resistant forms of the disease-causing pathogen and synergy with HIV / AIDS New antituberculotic drugs are needed to improve the treatment of patients with multidrug-resistant TB, and they might enable the duration of treatment to be shortened.

Mycobacteria are very successful pathogens; they are able to survive under adverse conditions. Dynamic regulation of RNA decay and processing should be a very important factor in adapting to environmental changes. Over the past years it became obvious that mRNA stability is a very important step in regulation of prokaryotic gene expression.

Given the central role of RNase E in RNA processing and decay in *E. coli* and other bacteria, it is conceivable that the RNase E-dependent regulation of RNA stability in mycobacteria might also be a very important mechanism, adjusting the cellular metabolism to environmental changes.

We focus our work on mycobacterial RNase E like proteins. We cloned RNase E from *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis*, expressed and purified the proteins, identified associated proteins and characterised enzyme activities. Our results are the first steps towards the understanding of RNA metabolism in mycobacteria and could make finding new drug targets possible. Further studies are needed to address the specific roles of the associated proteins in relations to the specific steps of RNA metabolism in mycobacteria and their regulation by environmental factors.

VII. New statements of the thesis

- RNase E was cloned for the first time from *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis*.
- We were the first to express and to purify these proteins and also to identify the associated proteins in mycobacteria.

- It was published for the first time, that the RNase E/G homologue from *M. tuberculosis* has enzymatic activity and we characterised the enzyme activities.
- We were the first to show that RNase E/G was able to cleave mycobacterial 9S RNA, a putative precursor of its cognate 5S rRNA.

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