EF-Tu and RNase E

Essential and Functionally Connected Proteins

DISA L. HAMMARLÖF
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Abstract

The rate and accuracy of protein production is the main determinant of bacterial growth. Elongation Factor Tu (EF-Tu) provides the ribosome with aminoacylated tRNAs, and is central for its activity. In Salmonella enterica serovar Typhimurium, EF-Tu is encoded by the genes tufA and tufB. A bacterial cell depending on tufA499-encoded EF-Tu mutant Glu125Arg grows extremely slowly. We found evidence that this is caused by excessive degradation of mRNA, which is suggested to be the result of transcription-translation decoupling because the leading ribosome is ‘starved’ for amino acids and stalls on the nascent mRNA, which is thus exposed to Riboendonuclease RNase E. The slow-growth phenotype can be reversed by mutations in RNase E that reduce the activity of this enzyme.

We found that the EF-Tu mutant has increased levels of ppGpp during exponential growth in rich medium. ppGpp is usually produced during starvation, and we propose that Salmonella, depending on mutant EF-Tu, incorrectly senses the resulting situation with ribosomes ‘starving’ for amino acids as a real starvation condition. Thus, RelA produces ppGpp which redirects gene expression from synthesis of ribosomes and favours synthesis of building blocks such as amino acids. When ppGpp levels are reduced, either by over-expression of SpoT or by inactivation of relA, growth of the mutant is improved. We suggest this is because the cell stays in a fast-growth mode.

RNase E mutants with a conditionally lethal temperature-sensitive (ts) phenotype were used to address the long-debated question of the essential role of RNase E. Suppressor mutations of the ts phenotype were selected and identified, both in RNase E as well as in extragenic loci. The internal mutations restore the wild-type RNase E function to various degrees, but no single defect was identified that alone could account for the ts phenotype. In contrast, identifying three different classes of extragenic suppressors lead us to suggest that the essential role of RNase E is to degrade mRNA. One possibility to explain the importance of this function is that in the absence of mRNA degradation by RNase E, the ribosomes become trapped on defective mRNAs, with detrimental consequences for continued cell growth.

Keywords: bacterial growth, translation, EF-Tu, RNase E, mRNA, RNA degradation

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Till min familj
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†These authors contributed equally.

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<tr>
<td>aa-tRNA</td>
<td>aminoacyl-tRNA</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EF</td>
<td>Elongation Factor</td>
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<tr>
<td>f-Met</td>
<td>N-Formylmethionine</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>IF</td>
<td>Initiation Factor</td>
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<td>mRNA</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>ppGpp</td>
<td>Guanosine tetraphosphate</td>
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<td>RF</td>
<td>Release Factor</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNase E</td>
<td>Riboendonuclease E</td>
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<tr>
<td>r-protein</td>
<td>ribosomal protein</td>
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<td>RRF</td>
<td>Ribosomal Release Factor</td>
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<tr>
<td>r-RNA</td>
<td>ribosomal RNA</td>
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<tr>
<td><em>S. typhimurium</em></td>
<td><em>Salmonella enterica</em> serovar Typhimurium</td>
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<tr>
<td>TIR</td>
<td>Translation Initiation Region</td>
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<tr>
<td>tmRNA</td>
<td>translation-messenger RNA</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
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<tr>
<td>ts</td>
<td>temperature-sensitive</td>
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<td>UTR</td>
<td>Untranslated region</td>
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Introduction

Bacterial growth

The ability of a bacterium to adjust to different growth conditions is fundamental for its survival. It is commonly known in the field of microbiology that there is a simple correlation between the rate at which bacteria grow and the energy supplied (Monod, 1965). Energy is mainly consumed in the production of proteins that assist in all tasks of the cell, i.e. to replicate DNA, transcribe RNA, produce protein, and to act as building blocks in the growing cell (Schaechter et al., 1958). The macromolecular machines responsible for protein production, i.e. translation, in the cell are known as ribosomes. Upon a nutritional shift in the bacterium’s environment, the number of ribosomes is adjusted to supply the cell with the desired amount of protein.

In a nutritionally rich medium the cell uses most of its energy to build ribosomes, and thus transcription and translation are focused on genes for ribosomal RNA (rRNA), and ribosomal protein (r-protein), and the translation factors. To meet the demand for ribosomes at fast growth rates, 7 \textit{rrn} operons in the genome of gamma-proteobacteria, e.g. \textit{Salmonella}, encode the same three rRNAs: 23S, 16S and 5S [reviewed in (Nomura & Morgan, 1977)]. Each of the operons also encodes at least one transfer RNA (tRNA), which brings amino acids to the ribosome in the form of a ternary complex together with the Elongation Factor Tu (EF-Tu) and GTP. In a rapidly growing cell, more than 50\% of the transcripts being made originate from the \textit{rrn} operons (Wagner, 2000), and the net result is that under such conditions rRNA and tRNA make up 95\% of total RNA in the bacterial cell. During optimal growth conditions there are tens of thousands of actively translating ribosomes in each cell.

If the supply of nutrition decreases, the expression pattern of the bacteria is changed in a way that the new requirements for continued growth are met. Usually this means that the transcription is focused on biosynthetical genes, to ensure the supply of nucleotides and amino acids (Bremer & Dennis, 1996).

The aim of this thesis is to further dissect the biological role of EF-Tu, a major determinant for bacterial growth rate, and its functional connections to other proteins, under different growth conditions. One of the functional partners identified, Riboendonuclease E (RNase E) has also turned out to be very
central in this thesis. As one of the most important enzymes in the turn-over of RNA, RNase E contributes to post-transcriptional gene regulation that allows the cell to rapidly adapt to changes in its environment. Our work has been aided by the use of the well-characterized model organism *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S. typhimurium*), which easily can be genetically manipulated.

Translation in bacteria

The ribosome

The ribosome is composed (volume-wise) of two thirds rRNA and one third r-protein, and its enzymatic activity lies within the RNA moiety. This special feature was identified when it was shown that the peptidyl transferase centre, responsible for forming the peptide bonds between the amino acids during polypeptide synthesis, is entirely composed of 23S rRNA (Nissen et al, 2000).

The ribosome consists of two subunits. In bacteria these are called 30S, which is small and flexible, and 50S, which is the larger and more rigid one. Together they make up the functional 70S ribosome. The system with two units probably rose from the need for adequate flexibility in the structure, as progression of tRNA and mRNA through the translation machinery involves major restructuring of the interactions in the ribosome (Maguire & Zimmermann, 2001). The 30S subunit consists of the 16S rRNA, which base-pairs with the Shine-Dalgarno sequence in the Translation Initiation Region (TIR) of mRNA during the initiation phase of protein synthesis, together with 21 r-proteins. The 50S subunit contains the 23S rRNA, which forms the peptidyl bonds during polypeptide synthesis, the small 5S rRNA, and 33 r-proteins. The ribosome core, in which tRNAs bind, is almost entirely composed of rRNA (Yusupov et al, 2001).

As the enzymatic activities of the ribosome lie within the RNA moieties, the main function of the 54 r-proteins is apparently to organize and hold the rRNAs in place. It has been shown in atomic resolution structures of 30S and 50S subunits, that most r-proteins interact with multiple RNA elements (Ban et al, 2000; Schluenzen et al, 2000; Wimberly et al, 2000). It is important to note that although the r-proteins are not directly responsible for the enzymatic activities of the ribosome, the ribosome cannot be put together, or function, without its protein partners. Furthermore, there are exceptions to the exclusively structural role of r-proteins, one being the r-protein S12, which is involved in mRNA decoding (Moore & Steitz, 2002). The primary architectural role of the r-proteins suggests that they have been added later during evolution to this macromolecular machine, streamlining the process of protein synthesis.
The translation cycle

The ribosome uses mRNA as blueprint and aminoacyl-tRNAs (aa-tRNAs) as intermediaries in the translation reaction. Amino acids are attached to tRNAs by their cognate aa-tRNA synthetases (An & Friesen, 1980). For efficient and correct translation, the ribosome is supported by protein factors specific for the initiation (IF-1-3), elongation (EF-Tu, EF-G) and release (RF1-3) steps of the translation cycle.

Translation is initiated when 16S rRNA (as part of the 30S subunit), aided by ribosomal protein S1, binds the TIR or Shine-Dalgarno sequence, sometimes preceded by a 5’-Untranslated region (5’-UTR), of an mRNA. Because transcription and translation are coupled processes in bacteria, translation can be initiated before the nascent mRNA is completed. Formylmethionine (fMet-tRNA$_{\text{Met}}$), encoded by the start codon, is brought into the peptidyl site (P-site) of the 30S subunit with the aid of IF-1, which recognizes the anticodon, and IF-2, which recognizes the formyl group of fMet-tRNA$_{\text{Met}}$. IF-3 is already bound to 30S and prevents association with 50S until the mRNA and tRNA$_{\text{Met}}$ are bound. The full 70S complex with 50S is only formed after correct anticodon-initiator codon pairing has been completed.

After the 70S subunit is formed, another complementary aa-tRNA can enter the acceptor site (A-site), in the form of a ternary complex with EF-Tu (aa-tRNA•EF-Tu•GTP). Hydrogen bonds and EF-Tu hold the tRNA in place between the 30S and 50S subunits. In this position, the anticodon of the tRNA is in contact with the mRNA codon, but the amino acid is prevented from being in contact with the peptidyl transferase centre in the 23S rRNA. The codon-anticodon match selects the correct aa-tRNA, but the binding energy of this interaction is not strong enough to assure that correct tRNA is selected. In subsequent proofreading steps on the ribosome, near-cognate and non-cognate aa-tRNAs are likely to be rejected. The GTPase activity of EF-Tu is activated if the correct tRNA is positioned in the A-site. When that happens, GTP on EF-Tu is hydrolysed and EF-Tu•GDP is released from the ribosome. The aa-tRNA then moves fully into the ribosomal P-site. The formation of the peptide bond between the carboxyl group on the formylmethionine or amino acid in the P-site, and the amino group on the incoming amino acid in the A-site, is subsequently catalyzed by 23S rRNA. The tRNA carrying the nascent peptide in the A-site is moved to the P-site by the transllocase Elongation Factor G (EF-G). The now naked tRNA moves to the exit site (E-site), which allows a third aa-tRNA to enter the ribosome. How the tRNA in the E-site finally leaves the ribosome is not completely known.

Translation proceeds along the mRNA in the following way, with the ribosome moving one codon at a time in a 5’ to 3’ direction, until any one of the three termination codons is encountered and positioned in the A-site. The ribosome stalls on this codon, which is also recognized by protein release...
factors (RFs). They assist in the termination of translation by freeing the polypeptide chain from the ribosome. RF-1 interacts with termination codons UAG and UAA while RF-2 interacts with codons UAA and UGA. RF-3 releases RF-1 or RF-2 from the ribosome. Other factors, e.g. Ribosomal Release Factor (RRF), EF-G and IF-3, act to break up the translation complex at the end of a protein synthesis cycle. The 30S and 50S subunits dissociate so that the ribosome can be recycled and participate in a new round of protein synthesis. In bacteria the stop codon is usually not at the very end of the mRNA, which typically contains a 3'-UTR. After synthesis of a protein, the formyl group on the first methionine residue of the polypeptide is removed by peptide deformylase, and usually also the methionine by methionine aminopeptidase.

Interestingly, EF-Tu, EF-G and the RFs are similar in structure, which allows them to bind to overlapping sites on the ribosome, while performing very different tasks. This is a case of so-called molecular mimicry (Nyborg et al, 1996).

Ribosomes account for as much as 40% of the cell’s energy consumption (Nierhaus, 1991) which is not surprising considering that at least one ATP and two GTP are required for the translation of a single codon in the mRNA. The ribosomes incorporate amino acids in the growing polypeptide chain at the impressive rate of 17-20 amino acids per second (Young & Bremer, 1976). It is crucial that the production of protein is efficient and tightly controlled, so that energy is not wasted on making unnecessary protein products.

Elongation Factor Tu

Elongation Factor Tu (EF-Tu) is the protein responsible for saturating the ribosome with aa-tRNA during translation. EF-Tu is a single polypeptide 393 amino acids long, and the protein is divided into three globular domains: one α/β domain (residues 1 to 200), containing the GDP/GTP binding site, and two β-domains (Kjeldgaard & Nyborg, 1992). All domains are involved in aa-tRNA binding (see Figure 1A).

The most abundant protein in the cell

EF-Tu is encoded by two separated gene copies, tufA and tufB, in Salmonella as well as other gram-negative bacteria, and the protein products are identical (Hughes, 1986; Jaskunas et al, 1975; McClelland et al, 2001). tufA is the last gene in the str operon, which also encodes the genes for ribosomal proteins S12 (rpsL), S7 (rpsG) and EF-G (fusA). tufB is in turn part of an operon with the genes encoding four different tRNAs (thrU, tyrU, glyT and thrT) (An & Friesen, 1980; Hudson et al, 1981; van Delft et al, 1987). The fact
that two gene copies are needed to supply the cell with EF-Tu clearly shows the importance of this translation factor. EF-Tu is one of the most abundant proteins in the cytoplasm, and in a rapidly growing *Salmonella* it accounts for about 9% of total soluble protein (Tubulekas & Hughes, 1993b). When the amount of active EF-Tu in the cell is reduced, so is the bacterial growth rate (Hughes, 1990; Tubulekas & Hughes, 1993b). One active copy of the *tuf* genes results in approximately two thirds the amount of EF-Tu found in the wild-type cell (Hughes, 1990; Tubulekas & Hughes, 1993b). This is enough to support growth; however the growth rate is decreased by approximately 30% (Hughes, 1990).

Being such an abundant protein, it is not surprising that EF-Tu has been suggested to have roles also outside of the translation machinery. A completely different function ascribed to EF-Tu is as part of the cytoskeleton of the cell. It has been shown that EF-Tu forms filaments that stretch throughout the cell (Beck, 1979; Beck et al, 1978; Mayer, 2006). Recently it was demonstrated that EF-Tu forms a helical pattern in *Bacillus subtilis*, and also co-localizes with MreB, an actin-like element of the bacterial cytoskeleton (Defeu Soufo et al, 2010).

**EF-Tu during translation**

EF-Tu significantly increases the rate of aa-tRNA binding to the ribosome and also dramatically reduces the likelihood of reading mistakes. EF-Tu•GTP has a high affinity for charged aa-tRNA, and these three partners form the ternary complex (EF-Tu•GTP•aa-tRNA) which in turn has a high affinity for the ribosome. The degree to which the ribosomes are saturated with ternary complex is a major determinant of the speed and accuracy of translation, and thus of the bacterial growth rate (Tubulekas & Hughes, 1993a).

The ternary complex, like all translational G proteins, binds to the 50S subunit (Moazed et al, 1988). When the aa-tRNA is positioned in the A-site of the ribosome, with the anticodon of the aa-tRNA interacting with the mRNA codon, GTP is rapidly hydrolyzed to GDP (Bilgin et al, 1992). Binding of tRNA is followed by a series of conformational changes in 30S, tRNA and EF-Tu. The catalytic His\(^{84}\) of EF-Tu is positioned into the GTPase active site and GTP is hydrolysed to GDP (Schmeing et al, 2009; Voorhees et al, 2010). EF-Tu•GDP has a conformation different from EF-Tu•GTP, with low affinity for the ribosome and, as a consequence, the complex is released (Berchtold et al, 1993). Elongation Factor Ts (EF-Ts) aids in recycling of EF-Tu as it catalyzes the exchange of GDP to GTP on EF-Tu, increasing the rate of this event by several orders of magnitude. After recycling EF-Tu can again bind aa-tRNA to form the ternary complex (Kaziro, 1978) (see Figure 2).
Figure 1. The structure of EF-Tu in its GTP conformation. (A) Binding partners of EF-Tu are indicated: GDPNP, (a GTP analogue, blue), tRNA (purple), and kirromycin (yellow). The tufA499-encoded substitution Gln125Arg is indicated in magenta. (B) The tufA499-encoded substitution Gln125Arg (magenta) and the amino acids substituted in the intragenic suppressors of the slow-growth phenotype associated with tufA499 (yellow). *Illustration made in Molscript (L. Liljas 2011)*

Figure 2. The reaction cycle of EF-Tu. *Left hand circuit:* EF-Tu feeds the elongating 70S ribosome with aa-tRNA in the form of ternary complex. When the tRNA anticodon pairs with the mRNA codon, conformational changes lead to activation of the EF-Tu GTPase (indicated as a '*' in the figure). GTP is hydrolysed to GDP and EF-Tu•GDP dissociates from the ribosome. *Right hand circuit:* EF-Ts recycles EF-Tu by exchanging GDP for GTP. The recharged EF-Tu•GTP has high affinity for aa-tRNA. The resulting ternary complex can again supply the ribosome with its aa building block.
Kirromycin-resistant mutants of EF-Tu

Much can be learned by studying spontaneous mutations giving rise to kirromycin resistance, as this antibiotic specifically targets EF-Tu. Kirromycin binds EF-Tu (see Figure 1A) and blocks recycling of this factor by preventing the EF-Tu•GDP complex from leaving the ribosome thus inhibiting translation (Wolf et al, 1974). Kirromycin resistance is almost always achieved by mutations in either of the *tuf* genes. The phenotype is recessive, i.e. it is only detectable when only one active gene copy is present or both the gene copies are mutated.

Ribosome stalling

The ribosome stalls on the mRNA quite frequently before it reaches a nonsense codon signalling termination of translation, or it does not encounter a nonsense codon at all before reaching the 3’-end of the mRNA lacking a termination signal. The cell must be able to deal with this kind of situation; otherwise ribosomes will be trapped in non-productive complexes which will lead to growth impairment. In addition, the incomplete polypeptide and possibly defective mRNA need to be degraded. *Trans*-translation is the rescue process developed to handle this detrimental situation [reviewed in (Withey & Friedman, 2002)].

*Trans*-translation

A structural RNA, transfer-messenger RNA (tmRNA), with properties of both tRNA and mRNA, as the name implies, is central for *trans*-translation, a process first described by Keiler and co-workers (Keiler et al, 1996). tmRNA is processed to its mature form by RNase E together with other nucleases (Lin-Chao et al, 1999; Srivastava et al, 1990). It is activated when the ribosome reaches the 3’-end of an mRNA without encountering a stop codon, or when the ribosome stalls on a rare codon. When the ribosome for some reason gets caught on the mRNA (Roche & Sauer, 1999), EF-Tu•GTP•alanine-tmRNA enters the A-site of the ribosome (Barends et al, 2000; Rudinger-Thirion et al, 1999). The positioning of tmRNA in the A-site is aided by the SmpB protein as there is no codon-anticodon interaction during the first step of the *trans*-translation initiation (Karzai et al, 1999; Shimizu & Ueda, 2006). A peptide bond is formed between the polypeptide chain and the alanine linked to tmRNA and the complex is translocated to the P-site. The original mRNA template is released from the ribosome, and the ribosome continues translating the tmRNAs mRNA part until its termination codon is encountered. Then the ribosome is released, and the tagged
polypeptide is degraded by intracellular proteases like ClpX. tmRNA is also involved in the degradation of truncated mRNAs (Yamamoto et al, 2003).

The tmRNA has several protein partners. It interacts with the r-protein S1 (Karzai & Sauer, 2001; Wower et al, 2000), which may be involved in the functional shift between the tRNA and mRNA mode of the tmRNA through its unwinding activity, or in removal of loops in tmRNA that might inhibit the reaction (Bordeau & Felden, 2002; Wower et al, 2000). The tmRNA also interacts with RNase R (Karzai & Sauer, 2001), which is one of the nucleases responsible for degrading defective mRNA released upon the trans-translation event (Ge et al, 2010).

Translational polarity

Transcription and translation are coupled processes in bacteria, i.e. translation of the nascent mRNA starts as soon as the TIR is exposed (and does not wait for completion of the transcript by RNA polymerase). A nonsense mutation would cause premature termination of translation, resulting in dissociation of the ribosome and exposure of *rut*, a recognition motif for the transcription termination factor Rho. Rho binds to the *rut* sequence and interacts with RNA polymerase, causing it to fall off the DNA, i.e. transcription is aborted. In this way, the continued transcription of a defective mRNA is prevented (Bogden et al, 1999; Richardson, 2002).

Stringent response and ppGpp

As previously stated, it is crucial for the bacterial cell to be able to adjust rapidly to new growth conditions. The major coordinator of the rapid and global modification of gene expression required for this ability is guanosine tetraphosphate, ppGpp. About 60 years ago it was shown that when the cell starves for amino acids there is a reduction in the accumulation of rRNA, a phenomenon which was found to be the result of the production of ppGpp (Gallant & Cashel, 1967; Sands & Roberts, 1952). Some bacteria, including *S. typhimurium*, have developed a system with two enzymes regulating ppGpp level in the cell. At the onset of starvation of single amino acids, RelA is activated by uncharged tRNA bound at the A-site of the 50S subunit (Edlin & Broda, 1968; Haseltine & Block, 1973; Neidhardt, 1966; Stent & Brenner, 1961). The other ppGpp-regulating enzyme, SpoT, has both ppGpp synthetase and hydrolase activities. SpoT synthetase is responsible for the basal ppGpp production in the cell during exponential growth and has its maximal activity during multiple amino acid starvation (Lagosky & Chang, 1980). ppGpp can be rapidly degraded by SpoT hydrolysis, an enzyme activity which is down-regulated during multiple amino acid starvation and ener-
During starvation conditions ppGpp redirects RNA polymerase (RNAP) away from promoters of genes encoding stable RNA and ribosomal proteins by binding to the RNAP beta subunit (Reddy et al, 1995). The effect is that fewer ribosomes are produced and that leads to a reduction in the consumption of amino acids. The resulting net amino acid increase allows the actively translating ribosomes to work at a higher speed. At the same time, the genes for amino acid biosynthesis, transport etc. are transcribed to a higher degree due to ppGpp, to make more building blocks for the cell, and to counteract the starvation conditions. Expression of over a thousand genes is affected by ppGpp regulation (Jin et al, 2011; Traxler et al, 2008).

Roles of RNases in the cell

In the bacterial cell there is a wide array of Ribonucleases (RNases), responsible for processing, degradation and quality control of RNA. They have a major role in the post-transcriptional regulation of gene expression and, by their fast action, make sure that the cell can rapidly fine-tune gene expression and adapt to new growth conditions. Individual RNA species differ widely with respect to their stability. It is usually not a particular nucleotide sequence that is recognized by RNases. Instead it is RNA higher-order structure and protection by translating ribosomes that have high impact on the activity of these enzymes. The lack of cleavage specificity probably contributes to the functional overlap of many of these nucleases, which gives rise to redundancy among these enzymes. However, some of them are essential and cannot be replaced, e.g. RNase E. The RNases can be divided into two classes, the endonucleases that cut their RNA substrate internally, and the exonucleases that cut the RNA from one end. Some of the most important members of these classes are presented below.

Some central nucleases in bacteria

One of the most important enzymes in RNA processing and degradation is RNase E (see next chapter). RNase G is a paralogue of RNase E, also depending on an available 5’-end and A/U-rich sequences for binding and degradation of RNA (Jiang et al, 2000). However, RNase G is neither essential (Li et al, 1999; Wachi et al, 1999), nor can it restore growth of a non-viable rne null mutant (Jiang et al, 2000).

RNase III was the first endonuclease identified that specifically cuts double-stranded RNA (Robertson et al, 1968). Roles in both tRNA precursor and rRNA maturation have been attributed to this enzyme (Babitzke et al, 1993). Furthermore, RNase III is involved in the degradation of mRNA.
(Condon & Putzer, 2002) and the decay of sRNA/mRNA complexes (Viegas et al, 2007). Not surprisingly, the RNase III family of enzymes is now thought of as one of the most important factors in controlling RNA stability.

RNase P, which has a universal role in the processing of the 5'-leader sequence of tRNAs, is a ribonucleoprotein with the enzymatic activity associated with the RNA moiety of the complex (Guerrier-Takada et al, 1983).

The endonucleases of the toxin-antitoxin systems, of which RelE is a well-characterized example (Christensen et al, 2001; Gotfredsen & Gerdes, 1998), should also be mentioned in this section. RelE is activated by various stress conditions (Christensen et al, 2001) and specifically cuts mRNA during translation (Hurley et al, 2011; Neubauer et al, 2009; Pedersen et al, 2003).

PNPase is a degradosomal exonuclease involved in global mRNA decay, and the protein is widely conserved: from bacteria to plants and metazoans (Zuo & Deutscher, 2001). In E. coli, RNase II is the major hydrolytic enzyme that participates in the terminal steps of mRNA degradation (Deutscher & Reuven, 1991) and it degrades RNA in the 3’ to 5’ direction, yielding 5’-nucleoside monophosphates. This activity of the enzyme is blocked by secondary structures (Cannistraro & Kennell, 1999; Spickler & Mackie, 2000).

RNase R, which belongs to the RNase II family, is encoded by the vacB gene (also known as rnr) (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). The fact that this enzyme can degrade also structured RNA makes it unique among exonucleases (Awano et al, 2010; Cheng & Deutscher, 2002; Cheng & Deutscher, 2003). RNase R has an important role in surveillance of the RNA quality of the cell, as it degrades defective tRNAs (Awano et al, 2010; Vincent & Deutscher, 2006) and the rRNAs 16S and 23S (Cheng & Deutscher, 2003). Additionally, RNase R is needed for correct processing of tmRNA (Cairrão et al, 2003).

**Riboendonuclease E**

By the general degradation of mRNA, as well as the processing of many different structural RNAs, Riboendonuclease E (RNase E) is central in both global gene regulation and cellular homeostasis.

RNase E was first discovered as the enzyme responsible for processing 9S into the ribosomal 5S RNA (Ghora & Apirion, 1978; Melefors & von Gabain, 1991), but over the years the enzyme has been shown to take part also in the maturation of tRNAs and tmRNA (Li & Deutscher, 2002; Lin-Chao et al, 1999; Srivastava et al, 1990), and the processing of small RNAs (Davis & Waldor, 2007; Pfeiffer et al, 2009; Prévost et al, 2011). It is defined as the most essential enzyme in the degradation and processing of
mRNA (Mudd et al, 1990; Taraseviciene et al, 1991) and it has also been shown that the enzyme removes poly(A) tails (Walsh et al, 2001).

RNase E is encoded by the gene rne (Chauhan & Apirion, 1991). Two temperature sensitive (ts) mutants of RNase E, rne-1 (Gly66Ser) and rne-3071 (Leu68Phe), have been very useful in characterization of this enzyme (McDowall et al, 1993; Misra & Apirion, 1980).

Organizer and enzyme

The 1061 amino-acid long RNase E can be divided into two roughly equal halves, the N-terminal part which holds the enzymatic activity and the C-terminal protein scaffold. The latter is mainly responsible for the organization of the multiprotein degradosome complex involved in the degradation of mRNA (Callaghan et al, 2004; Kido et al, 1996; McDowall et al, 1993). The other components of the degradosome are PNPase, RNase helicase B (RhlB) and enolase (Miczak et al, 1996). After the initial cut by RNase E, mRNA is polyadenylated and degraded by the exoribonuclease PNPase. The helicase RhlB helps to unwind the RNA (Carpousis et al, 2008) whereas the function of enolase is unknown. The C-terminal part of RNase E is not essential for rRNA processing, but is needed for normal degradation of bulk mRNA (Lopez et al, 1999).

E. coli carrying RNase E truncated for its C-terminal half is viable but growth impaired (Leroy et al, 2002).

While determining the structure of the N-terminal, enzymatic half of RNase E, Callaghan and co-workers took a big step forward in the understanding of the organization and activity of RNase E (Callaghan et al, 2005a). The enzyme is composed of two globular, ‘large’ and ‘small’, domains. The ‘small’ domain, as well as an S1 RNA-binding domain and a Zn-co-coordinating site within the ‘large’ domain, are areas of RNase E involved in organization of the higher order structure of the protein, which forms dimers of dimers. The large domain contains the 5’ sensor responsible for binding the RNA substrate, and apparently RNase H- and DNase I-like domains, in which the latter holds the catalytic site (amino acid residues Asp303 and Asp346, as well as a sequestered Mg$^{2+}$ ion) (Callaghan et al, 2005a) (see Figure 3). RNase E requires Mg$^{2+}$ for its catalytic activity (Callaghan et al, 2005a) and Zn$^{2+}$ for organization of the homo-tetramer (Callaghan et al, 2005b).

From the crystal structure it could be concluded that the 5’ sensor domain and the catalytic site are physically separated, and it was proposed that there must be some reorganization upon binding of the substrate to allow a proper cut (Callaghan et al, 2005a). This hypothesis was confirmed when the RNase E apo-protein was crystallized, and a major conformational change was shown upon catalytic activation (Koslover et al, 2008). The fact that the 5’ sensor and the catalytic site are separated might contribute to the discriminatory ability of RNase E among different substrates. For example, mRNAs
with a 5’-end monophosphate will be cut whereas structural RNAs will not, because structure in the latter might inhibit simultaneous binding at the two RNase E sites (Callaghan et al, 2005a).

Figure 3. The RNase E secondary structure. The N-terminal structural domains and the binding sites of degradosomal proteins RhlB, enolase and PNPase in the C-terminal part of RNase E are shown. The positions of the different mutations we isolated as suppressors of the tufA499-associated growth defect, to the great majority causing amino acid substitutions, are indicated with arrows.

RNase E specificity

Cleavage by RNase E is the first and the rate-limiting step in the degradation of RNA (Jain et al, 2002). The endonuclease cuts single-stranded RNA from the 5’-end. However, the actual site of recognition has not definitively been established (Mackie, 1992; McDowall et al, 1995). It was earlier suggested that RNase E recognizes its target by secondary (hairpin) structure, and that the enzyme then cuts in an A/U-rich sequence (Cormack & Mackie, 1992; Lin-Chao et al, 1994; McDowall et al, 1994). Actually, Callaghan et al later suggested that RNA stem-loop structures both positively and negatively affect the rate of cleavage by influencing downstream sequences: either by masking or revealing RNase E binding and cleavage sites (Callaghan et al, 2005a). Also, the structured region could act as an anchor for the enzyme and make sure that the actual cleavage site is single-stranded, as well as prevent unwanted cleavages elsewhere in the RNA (Mackie & Genereaux, 1993; McDowall et al, 1995). For some substrates, the arginine-rich RNA-binding domain (ARRBD) of RNase E can be required for efficient cleavage (Kaberdin et al, 2000).

A more general characteristic of RNase E specificity is that it depends on an accessible 5’-UTR for the recognition of its substrate (Mackie, 1998; Mackie, 2000). A 5’-end monophosphate increases the rate at which the enzyme cleaves whereas a triphosphate in this position impedes the rate of cleavage (Mackie, 1998). Recently a specific enzyme, RppH, was presented as being responsible for removal of phosphate from the 5’-end of the RNA, and, as such, is also involved in determining the rate at which mRNA is degraded (Celesnik et al, 2007; Deana et al, 2008).
Autoregulation

The essential RNase E always needs to be expressed. However, an excess in activity might lead to detrimental cleavage. Actually RNase E, when present in excess amounts, is itself responsible for the autoregulation. The enzyme binds to the structured and conserved 5'-UTR of the rne mRNA transcript, and thereafter degrades it (Diwa et al, 2000; Jain & Belasco, 1995; Mudd & Higgins, 1993). Inversely, RNase E can boost rne expression when the level of the enzyme is low, as demonstrated by increased β-galactosidase synthesis from an rne–lacZ reporter in the presence of a limited amount of RNase E (Jain et al, 2002). The 5’ phosphate sensor of RNase E is important for autoregulation as mutations in this part of the enzyme result in increased expression of RNase E (Garrey & Mackie, 2011).

Essential function of RNase E

Clearly, the versatile RNase E is involved in most RNA processes in the cell. But is there some function of the enzyme that can be isolated as the essential one? The enzymatic activities of processing of structured RNA (9S to 5S and tRNA) and the degradation of mRNA, as well as autoregulation, can be functionally separated (Perwez et al, 2008). These investigators suggested that initiation of mRNA degradation is not the essential activity of RNase E (Perwez et al, 2008). Rather, they presented data supporting the previous claim that processing of tRNA by RNase E is the activity of the enzyme that is important for cell viability (Li & Deutscher, 2002; Ow & Kushner, 2002). However, others have shown data suggesting that this is not the case (Deana & Belasco, 2004).

RNase E has been shown to form helical structures connected to the cytoskeleton of the cell as well as to the cell membrane (Khemicci et al, 2008; Taghbalout & Rothfield, 2007). Whether this is important for the maintenance of cell structure remains to be tested. If RNase E does contribute to the architecture of the cell, this could potentially provide an alternative explanation for the essentiality of RNase E. However it is possible that this kind of compartmentalization is just a way to organize RNase E and spread it evenly throughout the cell.

Crosstalk between translation and RNA degradation

During cell growth, mRNA is constantly being synthesized and degraded. When the translation machinery is not working properly, RNA degradation is increased to reduce the amount of defective proteins in the cell, mRNAs that are no longer needed are destroyed so that the nucleotides can be recy-
cled, and most importantly, ribosomes are released from aberrant mRNAs so that they instead can be efficiently used on other mRNAs.

The 5′-end of an mRNA has a high impact on its expected half-life. The ribosomal initiation complex has high affinity for an mRNA with a strong Shine-Dalgarno sequence, or with an A/U-rich S1 motif. It has been shown that binding of the ribosome complex protects the mRNA from degradation (Jain & Kleckner, 1993; Komarova et al, 2005). Conversely, if the 5′-UTR is modified in a way that reduces ribosome binding, the stability of the mRNA decreases (Arnold et al, 1998; Komarova et al, 2005; Wagner et al, 1994). The ribosome-stabilizing effect on mRNA has been explained in two ways. One is that increased initiation of translation results in ribosomes being quickly loaded onto the mRNA. The ribosomes then closely follow each other on their translation path across the mRNA and thereby mask potential RNase E cleavage sites (Komarova et al, 2005). Alternatively, the binding of the ribosome complex to the mRNA 5′-UTR, which is the recognition site for RNase E, will out-compete the latter, thereby preventing access of the RNase E mRNA substrate (Arnold et al, 1998; Wagner et al, 1994) (see Figure 4).

Transcription-translation decoupling has been shown to increase the degradation of mRNA. The lacZ mRNA was examined in a system with T7 polymerase, which transcribes eight times faster than the E. coli equivalent. The turn-over rate of the lacZ mRNA was increased in the system. This can be explained as the effect of transcription-translation decoupling, and exposure of RNase E-sites in the naked mRNA (Iost & Dreyfus, 1995). Other studies have also shown that reduced translational activity of an mRNA leads to its destabilization (Deana & Belasco, 2005; Kaberdin & Bläsi, 2006).

Interestingly, ribosomal protein S1, when overexpressed and not necessarily connected to the ribosome, also protects mRNA from degradation by RNases (Delvillani et al, 2011). S1 is known to interact not only with the mRNA and the translation machinery, but also with RNase E and other degradosomal proteins (Draper et al, 1977; Feng et al, 2001), and could therefore contribute to the translation-RNA degradation crosstalk. Also tmRNA is an important mediator in this communication [reviewed in (Withey & Friedman, 2002)].

Model organism S. typhimurium

*Salmonella enterica* (*S. enterica*), a member of the Enterobacteriaceae family, is a flagellated, facultative anaerob, Gram-negative bacterium. Over 2500 serotypes have been characterized, which differ in their H (flagellar), O (LPS), and Vi (capsular) antigens (Murray et al, 2009). *S. enterica* serovar Typhi causes typhoid fever, while serovar Typhimurium is the most common
cause of gastroenteritis, or salmonellosis. The ability to invade intestinal mucosa and produce toxins makes *Salmonella* a successful pathogen in animals. The main source of the infective agent is contaminated foods of animal origin. Millions of people are infected every year, resulting in thousands of deaths (WHO, 2005).

In laboratory experiments, usually attenuated, non-virulent strains are used, e.g. *S. typhimurium* LT2.

**Mutant selections**

The conclusions of this thesis result from a combination of classical and molecular genetics techniques. Mutants can easily be isolated in bacteria such as *S. typhimurium*. They reproduce asexually, are haploid (and one must therefore usually only be concerned about a single copy of a gene), and multiply quickly and in high numbers forming visible colonies on agar overnight. These properties of bacterial growth allow isolation of rare mutants arising once in a billion cells. The prerequisite is the presence of a strong selection phenotype, e.g. antibiotic resistance, or the possibility of screening for auxotrophy or conditional mutants. Most spontaneous mutations are deleterious, but in a selection for a specific phenotype only the mutants with this sought-after phenotype will be able to multiply. The different kinds of mutations that can arise are: silent mutations (nucleotide (nt) sequence changed without changing coding sequence), missense mutations (nt change causes amino acid substitution), nonsense mutations (nt change transforms coding codon to a stop codon), deletion mutations (removal of nts), insertion mutations (addition of nts), and inversion mutations (entire section of DNA is reversed). Suppressor mutations, which can be of any of the types mentioned above, can be intragenic or extragenic, and relieve or suppress the effects of another mutation.

*Salmonella* can easily be genetically manipulated, and analysis of the organism over several decades have made it well characterized, resulting in a very useful model system.

**Generalized transduction**

Genetic exchange occurs naturally through three mechanisms: transformation (Griffith, 1928), conjugation (Lederberg & Tatum, 1946) and transduction (Zinder & Lederberg, 1952). Generalized transduction, an event in the life of bacteriophages, has become immeasurably useful as a genetic tool in microbiology. This transfer of virtually any DNA marker from donor to recipient was first discovered and extensively studied in the bacterium *S. typhimurium* with bacteriophage in the 1950s (Zinder & Lederberg, 1952). When a lytic cycle is initiated upon viral infection, DNA (including host DNA for a small subpopulation of the phages) is packaged into the virus.
particle head. The transducing particles are released upon lysis, and are able to infect a recipient cell. If the bacteriophage contains bacterial DNA, there is the possibility for a homologous recombination event that can substitute the recipient DNA with the donor DNA. A P22 phage HT- mutant has been isolated with increased transduction efficiency (Schmieger & Backhaus, 1973). In the research laboratory this phage is used for transfer of genes/mutations between different strains. Very often a linked marker, e.g. a gene encoding antibiotic resistance, is used to select the desired transductants.
Present investigations

EF-Tu and RNase E are functionally connected

EF-Tu is responsible for feeding the ribosome with aa-tRNAs during translation in the form of ternary complex, and is one of the most abundant proteins in the cell and one of the central enzymes determining the rate at which bacteria can grow. Mutations affecting EF-Tu are likely to have a downstream effect on rate of translation and thus bacterial growth. A particular recessive, kirromycin-resistant mutant of EF-Tu (Gln125Arg) encoded by the \textit{tufA499} allele has been shown to cause a drastic reduction in the affinity of EF-Tu for aa-tRNAs, and thereby starves the ribosome for ternary complex (Abdulkarim et al, 1994; Hammarlöf & Hughes, 2008). \textit{tufA499} is also associated with an extreme growth defect. This phenotype was a good starting-out point for a genetic selection of suppressor mutations, as a means to learn more about structure and function of EF-Tu (Paper I).

Our expectation was that suppression would mainly be caused by second-site mutations within EF-Tu, resulting in a functionally pseudo-wild-type protein. This class of internal suppressors was found (see Figure 1B). Intriguingly, a great majority of the suppressor isolates, with growth rates close to that of the wild-type were found to have mutations in \textit{rne}, the gene encoding RNase E (see Figure 3). We showed that the \textit{tufA499} allele is associated with a decrease in the steady-state level of several mRNAs (including \textit{tufA}), without significantly affecting the average mRNA half-lives, and reduced rate of translation elongation. A reduction in EF-Tu protein, equivalent to the reduced level of \textit{tufA} mRNA, could also be seen. These effects were reversed by the \textit{rne} mutations, which were also associated with defective 9S-to-5S processing, diagnostic for mutants of RNase E.

Based on these results we presented a model of the \textit{tufA499} growth defect (see Figure 5):
1. The affinity of EF-Tu$\cdot$GTPs for aa-tRNAs is markedly reduced. This results in ribosomes starving for their ternary complex building blocks.
2. The starved, leading ribosome stalls on mRNA creating a gap between RNA polymerase and itself. Hence, mRNA is more vulnerable to degradation by RNase E.
3. The degradation of mRNAs encoding proteins essential for growth will have a direct negative effect on growth.
An alternative model that was not ruled out for the tufA499 suppression is that the number of ribosomes, as a consequence of defective rRNA processing, is reduced in the rne mutant. As the cell is starving for ternary complex, this would increase the saturation of the remaining active ribosomes. A reduced number of ribosomes would however limit growth, and as the tufA499 rne isolates grow as fast as wild-type this explanation seems less likely.

Why is it that all the suppressors, external to tufA, map in the rne gene? A simple answer could be that the rne gene is very big, more than 3000 nucleotides long. Furthermore, the protein seems to be structurally very flexible. We found mutations causing amino acid substitutions in 50 different positions in the N-terminal part of the enzyme, without any major detrimental effect on bacterial growth rate. Also, the restoration of the tufA499-associated growth defect is, as already mentioned, almost complete in the rne suppressor isolates. Other classes of suppressor mutations might not give such total growth compensation (for example SpoT overexpression or relA inactivation, Paper II), and would thus be disfavoured in our selection procedure. If we were to repeat the selection for suppressor mutations, we could limit the number of cycles for the selection of tufA499 suppressors, as the fast-growing rne suppressors might have been enriched in our study. If instead suppressors with more moderate growth compensation were actively chosen, we might obtain other classes of suppressor mutations. Potential targets for suppression mutations suggested by the model are genes encoding proteins influencing growth, as well as partners of RNase E that are involved in substrate recognition.

From our study we can conclude that RNase E not only cleaves aberrant or ‘old’ mRNA in S. typhimurium. It can also cleave nascent mRNA when exposed. The fact that all of the tufA499 suppressor mutations map in, or in close proximity of, the enzymatic half of the RNase E suggests that it is this activity that is affected by the mutations (rather than physical interactions with the degradosomal proteins through the C-terminal part, for example). However, we do not know whether target recognition, binding, or the actual cleavage efficiency is affected. Structural analysis of different RNase E mutations presented in Paper III sheds some light on this question.

Growth regulation by degradation of tufA mRNA

Among the analysed ribosomal mRNAs, the tufA mRNA seems to be specifically targeted by RNase E in the tufA499 slow-growing strain. When the mutant protein is overexpressed on a plasmid, this markedly increases the fitness of the tufA499 strain. Other mutations (fusA and rpsL) causing slow translation were not compensated by mutations in rne. This rules out a general mechanism of slow translation compensation by rne mutations. Actually, rne also confers higher fitness to a strain with a single (wild-type) tuf
copy, probably by stabilizing the *tuf* mRNA and thus increasing the level of EF-Tu protein, once again stressing the central role of EF-Tu for growth. Altogether, our data suggest a co-operative role for EF-Tu and RNase E in growth regulation by degradation of important mRNAs including the *tuf* mRNA.

![Figure 4.](image)

*Figure 4.* Transcription-translation coupling in the wild-type during normal growth conditions. Ribosomes protect nascent mRNA from excessive degradation by RNase E. Inactive RelA is associated with the ribosome.

**tufA499 increases the ppGpp level**

In Paper II, we extended the analysis of the EF-Tu mutant using a different approach for selection of suppressors of the extreme slow growth. We asked if the mutant could be rescued by overexpression of a wild-type gene other than EF-Tu. In a plasmid chromosome library we found one candidate gene, *spoT*, that restored the growth of *tufA499* when overexpressed, although not to the same extent as the *rne* mutations did.

SpoT is an enzyme with dual activities: it both synthesizes and hydrolyses ppGpp. We showed that the basal level of ppGpp is higher in the *tufA499* strain compared to wild-type during exponential growth, and that upon *spoT* overexpression, the level of ppGpp is reduced. Thus, in this system, the SpoT hydrolase activity is dominant. Alternatively, the overexpressed protein might physically interact with other molecules of SpoT, and thereby inactivate the SpoT synthetase activity. This remains to be tested.
Either way we could conclude that an increased ppGpp level is likely to contribute to the \textit{tufA499}-associated growth defect. To test this hypothesis, we inactivated the \textit{relA} gene, the other ppGpp-producing protein next to SpoT. RelA is known to be activated upon amino acid starvation. Inactivation of \textit{relA} resulted in reduced ppGpp levels, and in agreement with the model it increased the growth rate of the \textit{tufA499} strain. The positive effect on growth by \textit{relA} inactivation was actually more prominent than in cells with overexpressed SpoT. The effect of SpoT overexpression is likely to be complicated due to its opposing activities, which can explain why we do not see more improvement of growth in this strain.

\textbf{Figure 5.} Ribosome starving for ternary complex stalls on mRNA during translation. Defective EF-Tu encoded by the \textit{tufA499} allele is predicted to cause ribosome stalling and transcription-translation decoupling. The growth defect can be reversed, either by mutations in \textit{rne} that reduce the activity of RNase E and thereby reduce degradation of mRNA important for growth, or by SpoT overexpression or \textit{relA} inactivation, which reduce the level of ppGpp.

**EF-Tu mutant causes ‘starvation’ condition**

For survival, the bacterial cell needs to adapt quickly to different growth parameters. During different starvation conditions, one of the global gene expression modulators, ppGpp, ensures that translation is maintained close to the maximum rate by redirecting RNA polymerase from, mainly translation-associated, genes involved in fast growth. Instead transcription of genes in the biosynthesitical pathways is increased, producing amino acids etc. In this
study we show that there is a close connection between the extreme slow growth caused by a mutant EF-Tu and the level of ppGpp in the cell.

The tufA499 allele encodes a mutant EF-Tu that has a weak association with aa-tRNA (Abdulkarim et al, 1994), that reduces the saturation level of the translating ribosomes and thereby the bacterial growth rate (Hammarlöf & Hughes, 2008). We suggest that the reduction in the saturation level of translating ribosomes by ternary complex causes an effect that mimics amino acid starvation on the ribosome. This will activate ribosome-bound RelA to synthesize ppGpp. When the level of ppGpp is reduced, either by overexpressing the SpoT hydrolase activity or by inactivating relA, the cell can stay in the transcriptional ‘fast-growth’ mode. Previously we showed that the detrimental effect of tufA499-encoded EF-Tu is due to ribosome stalling and destabilization of nascent mRNA. From this study we conclude that changes in the transcription pattern by ppGpp also contribute to the extreme slow-growth phenotype of the tufA499-encoded EF-Tu mutant.

RNase E mutants in S. typhimurium

In previous investigations of RNase E and its partners, most research has been done on E. coli. While studying essential proteins and their functions, temperature-sensitive (ts) mutants can be of great use. Usually, by elevating the temperature, to a so-called non-permissive level, the protein is destabilized and therefore dysfunctional. At the permissive temperature the protein is functional and the mutant strain grows as a wild-type. In E. coli, there are two rne ts mutants that are frequently used, encoded by the rne-1 and rne-3071 alleles (McDowall et al, 1993; Misra & Apirion, 1980). From our previous work we knew that mutants of RNase E could readily be obtained by selecting suppressors of the tufA499 growth defect. We screened the collection of rne mutants and encouragingly, two isolates were found to have a cold-sensitive phenotype, whereas one isolate, with the mutation Ile207Ser (rne-6) had acquired a ts phenotype. The cold-sensitive mutants have not been studied further. We enquired if more rne ts mutants could be isolated through the same selection procedure in S. typhimurium. The selection was done at 30°C to allow growth of highly temperature-sensitive strains. Three additional and novel ts mutants of RNase E were identified: Ile207Asn (rne-9), Gly66Cys (rne-10) and Ala327Pro (rne-11) (Paper III). As intragenic ts suppressors previously had been selected for rne-1 in E. coli, we asked if this mutant class also could be selected for the rne ts phenotype in S. typhimurium. We selected suppressors of all four rne ts mutants (Paper III). The possible effects of the complete set of rne mutations on RNase E structure and function were analysed using the available 3-D structure of the N-terminal domain (Callaghan et al, 2005a). For all rne mutations compensating for the tufA499 growth defect, a destabilization effect on RNase E
could be predicted. This affect association of domains or subunits, and in some cases probably negatively influence formation of the RNase E tetramer (Callaghan et al, 2003). None of the mutations directly affected the catalytic site, but some were shown to change amino acid residues that are close to the residues essential for enzymatic activity. In general, the ts suppressor mutations in turn were predicted to stabilize the protein (Paper III).

Although the ts suppressor mutations isolated in this study allows growth of cells harbouring the rne ts alleles at the non-permissive temperature, these suppressed isolates are still stunted in their growth. In line with this observation, we showed that the suppressor mutations only partly restored the activity of RNase E in RNA processing, mRNA degradation, and autoregulation of the rne mRNA. The degree to which the suppressor mutations brought back the different functions of RNase E also varied between the different mutations. Because of these results we could not identify one single defect that could account for the rne ts phenotype.

**tufA499 suppression and rne autoregulation are linked**

Most rne double-mutants could no longer suppress the tufA499 growth defect, whereas, interestingly enough, a few still could. A single enzymatic phenotype of RNase E was associated with the maintained tufA499 suppression ability. These rne ts suppressors did not have significantly improved autoregulation activity of rne mRNA.

We proposed in Paper I that mutations in rne decrease RNase E cleavage of mRNA that is important for growth. Based on the results in the present study, we suggest that this mRNA is likely to have a similar RNase E recognition site as the 5’-UTR of rne (Jain & Belasco, 1995). Neither the 5’-UTR of the rne mRNA, nor this mRNA, will be cut by the mutant RNase E in the tufA499 background. Thus, the rne mRNA will be stabilized, and furthermore the protein product, which allows fast growth, can be made.

In summary, Paper III gave us some more insight into the functional connection between EF-Tu and RNase E. But although such a large set of rne mutations was analysed in terms of their possible effects on RNase E structure and function, in combination with characterization of biochemical properties of the mutant proteins, the essential function of RNase E was still not clear. Thus, we hope that the set of rne mutants presented in this work can be used as a genetic tool in future studies of RNase E in *S. typhimurium*, and will eventually lead to a more complete understanding of RNase E’s functions.
New insights into the essential role of RNase E

Because we had shown that intragenic ts suppressors could be selected in *S. typhimurium*, we raised the question whether also extragenic mutations could suppress the ts phenotype. We isolated 15 independent suppressor isolates for which we could show that none had second-site mutations within *rne* (Paper IV). Therefore, the suppressor mutations must be located in other genes.

Extragenic mutations suppress the *rne* ts phenotype

By genetic mapping and sequencing we showed that all isolates had mutations in one of three different genetic loci: *rpsA*, encoding ribosomal protein S1, *vacB*, encoding RNase R, and within or upstream of the open reading frame of the two genes *STM1551/STM1550*, encoding proteins with homology to the *E. coli* antitoxin RelB and toxin RelE. We could therefore conclude that the *rne* ts phenotype can be suppressed by extragenic mutations in at least three different ways.

We tried to rationalize the mechanisms of suppression in terms of the different functions of RNase E. Mutants of ribosomal protein S1, similar to the one isolated in this study, have been previously studied in *E. coli* (Boni et al, 2000; Schnier et al, 1986; Skorski et al, 2007; Subramanian & Mizushima, 1979). These mutants are associated with a reduced rate of translation initiation and as a consequence the strains harbouring the mutations are found to be growth impaired. The S1 mutant isolated as a suppressor of *rne-6* has a similar growth defect, both in *rne* as well as in the wild-type background. We predict that it is caused by the same negative effect on translation seen in the *E. coli rpsA* mutant.

RNase R is one of the riboexonucleases in the cell responsible for degrading structured RNA. Based on structure-function studies of RNase R (Vincent & Deutscher, 2009), we predicted that the different mutations found in *vacB* are likely to have an effect on either substrate recognition or the catalytic activity of the enzyme, as they all affect amino acids in the nuclease part of the enzyme. The mutations in *vacB* are not associated with any significant growth defect, neither in the *rne* nor in the wild-type background.

The largest, and most intriguing class of ts suppressor mutations, affected the operon encoding *STM1551* and *STM1550*, proteins showing homology to the RelB antitoxin and RelE toxin. Either the mutations caused amino acid substitutions or deletions in the *STM1551* coding region, or major deletions in the 5' upstream region, probably removing the promoter region of the *STM1551/1550* operon. Importantly, none of the mutations in this locus disrupted the coding region of *STM1550*. Thus, we hypothesized that toxin activity of *STM1550* might somehow be involved in the suppression phenotype.
Only on the basis of the location of the suppressor mutations, it was not possible to predict the cause of the suppression phenotype. The amino acid substitutions in STM1551 could increase or decrease binding of the antitoxin to the toxin (Gottfredsen & Gerdes, 1998), or affect autoregulation of the STM1551/1550 transcript (Gottfredsen & Gerdes, 1998), whereas the 5’-UTR promoter deletions could prevent autoregulation by fusing the genes to another promoter leading to increased expression, or completely prevent expression of these two proteins. The fact that one of the mutations, relBEAP6, almost completely removes the STM1551 coding region strongly suggests that the negative regulation of STM1550 is disrupted in this strain. Obviously, we directly had to test the potential toxin activity of STM1550.

RelE is known to specifically cleave translated mRNAs, and it was recently shown that the enzyme cuts from the 5’-end of its substrate, resulting in rapid translation shutdown (Hurley et al, 2011). In the same study it was shown that the RelE toxin cleave some mRNAs, namely lpp, tufA, ompA, ompF, and rpsA, more actively than others (Hurley & Woychik, 2009). By measuring the stability of these mRNAs in the wild-type, rne ts and STM1551-compensated strains, we could show that these mRNAs are stabilized in the rne ts genetic background. The suppressor mutations decrease the half-life of the mRNAs. We interpret this effect as a result of increased expression of the RelE homologue, which cause an increase in degradation of mRNA which is being translated in S. typhimurium.

mRNA - the common denominator

In this study we have shown for the first time that the rne ts phenotype can be suppressed by mutations external of rne. We have isolated such mutations in three different genetic loci, namely rpsA, vacB and STM1551/1550. Obviously, the protein products expressed from these genes must somehow be functionally connected to RNase E. We suggest that the common denominator is mRNA, and more specifically the degradation of aberrant or unnecessary transcripts. If these are not efficiently removed from the cell, the ribosomes might be caught up in unproductive complexes, which is likely to be detrimental for cell growth.

The mRNA-binding protein S1 is strongly associated with the translational machinery, and more specifically the initiation step of translation. As such it could act as a competitor to RNase E for binding to an mRNA substrate. A mutation that reduces the affinity of S1 for mRNA may increase the likelihood of prior binding and cleavage by an RNase E mutant with reduced activity, as is the case with the rne ts mutants.

The genes vacB and STM1550 both encode mRNA-degrading enzymes (RNase R and a RelE homologue). Mutations in these genetic loci can possibly bypass the proposed essential function of RNase E. We suggest that the suppressor mutations in vacB increase the substrate specificity of RNase R
so that it now can degrade aberrant mRNA instead of RNase E. Alternatively, by upregulating the RelE activity, mRNAs can be degraded, also when translation has been initiated. We propose that among many important functions of RNase E, the inability to degrade defective mRNA is the activity that is most central for the rne ts phenotype.

Concluding remarks

As stated in the introduction of this thesis, the capability of a bacterium to adapt to new environments and growth conditions is central for its survival. Several cellular processes and regulatory pathways are needed to achieve this. Continuously changed processing and degradation of RNA are required to assure that an optimal number of ribosomes can translate mRNA into the appropriate protein building blocks that cells require under particular growth conditions. When the growth conditions are changed, i.e. some nutrition is depleted and the cell starves, stress responses are activated. As a result effector molecules, like ppGpp, are produced to rapidly redirect the gene expression of the cell to allow continued growth.

In this thesis we have revealed several connections in this very complex network. We have shown that a severe growth defect resulting from perturbations in the translation machinery, leading to excessive degradation by mRNA, can be overcome by reduced activity in the mRNA degrading machinery. This may be a potential mechanism for growth regulation in the cell. The same translation defect, inadequately sensed as a starvation condition by the cell, leads to activation of the stringent response by ppGpp production. But the following redirection of gene expression, with even less ribosomal building blocks, augments the negative effect on translation and growth. Obviously, also the bacterial cells can make mistakes.

Finally we have presented, to our knowledge, the most extensive mutational analysis of the RNase E. Additionally we have showed for the first time that cells can bypass the essential function of RNase E. We claim that this is to remove excess mRNA from the cell that otherwise would trap ribosomes, resulting in global negative effects on protein synthesis and growth.

Salmonellabakterier kan leva både i mag-tarmkanalen hos värddjur och fritt i naturen, t.ex. i sjöar. För att en sådan cell ska kunna anpassa sig till olika tillväxtförutsättningar krävs en kontinuerlig tillverkning och nedbrytning av DNA, RNA (en ”kopia” av DNA) och proteiner. I denna avhandling har jag fokuserat på cellens proteintillverkning och RNA-nedbrytning. Studier av ett antal mutanta, det vill säga genetiskt förändrade, salmonella-stammar, som har dokumenterade förändringar i sin funktion avseende proteintillverkning och RNA-nedbrytning, har lett till ökad insikt om hur dessa livsnödvändiga processer hänger samman.

Proteintillverkning bestämmer tillväxthastighet

I alla celler, både bakteriers, växter och djurs, är det ribosomer som tillverkar proteiner genom en process som kallas translation. Begreppet translation kommer av att ribosomen ”översätter” budbärar-RNA (en kopia av generna i cellens DNA, som fungerar som en mall i proteintillverkningen), genom att koppla samman aminosyror till ett protein. Aminosyror är proteins minsta beståndsdelar, och proteiner är ansvariga för att utföra cellens olika funktioner.
Ribosomen byggs upp av ett stort antal proteiner och RNA. Ribosomer är även beroende av ett stort antal assisterande proteiner. En av dessa är Elongeringsfaktorn Tu, EF-Tu, som är ansvarig för att förse ribosomen med aminosyror. EF-Tu är det protein som det finns mest av i cellen. Varje EF-Tu-molekyl binder en aminosyra tillsammans med ett så kallat transport-RNA. Varje typ av aminosyra har sitt specifika transport-RNA, vilket i sin tur kan binda till en specifik sekvens i budbärar-RNA. På så sätt känner ribosomen igen de aminosyror som den ska koppla samman till ett protein.

För att celler ska fungera väl och kunna föröka sig snabbt krävs att deras proteintillverkning är effektiv och sker korrekt. Proteintillverkningen måste även vara ekonomisk i meningen att det är viktigt att celler tillverkar just de proteiner de behöver under specifika tillväxtförhållanden. Eftersom EF-Tu är det protein som förser ribosomen med dess byggsstenar är det ett av de proteiner som avgör hur snabbt bakteriecellen kan växa. Genom att studera EF-Tu kan man därför lära sig mer om både translation och bakterietillväxt.

Oväntad koppling mellan proteintillverkning och RNA-nedbrytning

I vårt arbete har vi använt oss av en specifik, muterad variant av EF-Tu som i tidigare studier visat sig vara sämre på att binda till aminosyror med transport-RNA. De salmonellabakterier som har ett sådant muterat EF-Tu växer extremt långsamt med en förökningstid som är fyra gånger längre än normalt. Ibland förändrar mutationer aminosyrasekvensen i ett protein, vilket ger upphov till ett mutant protein, som kan ha förändrad funktion. Vi ställde oss frågan om man kunde hitta andra mutationer som kompenserar för den tillväxtdefekt som orsakats av muterat EF-Tu. Sådana kompenserande mutationer kallas även för suppressormutationer. Vi utgick från att vi skulle hitta mutanta former av EF-Tu med återställd funktion. Istället fick vi ett mycket oväntat resultat.

Huvuddelen av de suppressormutationer vi identifierade förändrade nämligen ett helt annat protein, kallat RNase E. RNase E har bland annat till uppgift att bryta ner budbärar-RNA och vi undersökte om förändringarna i detta protein påverkade denna nedbrytning. Vi kunde visa att så var fallet. Frågan uppkom nu om budbärar-RNA även kunde vara kopplingen mellan RNase E och EF-Tu. Intressant nog fanns det betydligt mindre budbärar-RNA i salmonellabakterier med det muterade EF-Tu.

den kommer att haka upp sig. Samtidigt fortsätter RNA-
tillverkningsmaskineriet att arbeta. Detta kan resultera i att det budbärar-
RNA som skulle översatts till protein istället bryts ner av RNase E. Om cel-
len inte förses med dessa nödvändiga proteinprodukter kan den inte fungera
som vanligt. Vi tror att detta resulterar i EF-Tu-mutantens tillväxtdefekt.

Mutant EF-Tu aktiverar svältsignalen

I vårt fortsatta arbete undersökte vi om defekten orsakad av mutant EF-Tu
kunde motverkas genom att förse bakteriecellen med stora mängder av något
protein. Det visade sig att proteinet SpoT, tillverkat i många kopior, kan
kompensera för det defekta EF-Tu. SpoT bryter ner molekylen ppGpp som
produceras i cellen under svältförhållanden. ppGpp hämmar cellens produkt-
ion av ribosomer vilket hjälper cellen att anpassa sig till svält eftersom pro-
teintillverkningen är mycket kostsamt för cellen. Vi tror att salmonellabakte-
rierna misstolkar effekten av att förses med mutant EF-Tu som svält och
därför börjar producera ppGpp. När vi modifierar bakterierna till att ha
mycket SpoT kommer ppGpp att brytas ner och bakteriecellen förblir istället
i ett snabbväxande tillstånd. Denna hypotes stärktes då vi kunde visa att in-
aktivering av ett protein som tillverkar ppGpp, kallat RelA, också har en
positiv effekt på EF-Tu-mutantens tillväxt.

Ledtrådar om varför RNase E är livsnödvändigt

I de två sista studierna i denna avhandling har vi främst fokuserat på
RNase E, som är ett livsnödvändigt protein, och är centrat för cellens an-
passningsförmåga. Vi använde EF-Tu-mutanten för att hitta ett stort antal
mutanter av RNase E. Några av dessa förändrade proteiner visade sig ha en,
för genetiska analyser, mycket användbar egenskap: vid hög temperatur gör
förändringarna i RNase E att proteinet förlorar den struktur som krävs för att
det ska kunna utföra sin livsnödvändiga funktion, och därför dör cellen.
Denna egenskap kan användas för att välja ytterligare mutanter, antingen av
RNase E eller något annat protein, som tillåter tillväxt vid den högre tempe-
raturen. Om man lyckas identifiera dessa kan man lära sig mer om varför
RNase E är livsnödvändigt för bakterien. Trots att detta protein studerats
under många år är detta faktiskt inte känt.

Vi identifierade ett stort antal suppressormutationer inom RNase E, vilka
alla resulterade i att bakterien kunde växa vid den högre temperaturen. Vi
kunde även visa att dessa suppressormutationer återställer många av
RNase Es funktioner. Men trots att så många olika förändringar av RNase E
analyserades i denna studie kunde vi inte dra någon slutliggig slutsats om
RNase Es livsnödvändiga funktion.
Den avslöjades dock av suppressormutationer som förändrade andra protein än RNase E, nämligen ribosomalt protein S1, som behövs för start av proteintillverkning, RNase R, som precis som RNase E bryter ner RNA, samt två protein som liknar E. coli RelE och RelB, som är ett så kallat ”gift- och motgiftssystem”. Vi tror att budbärar-RNA är nyckeln till varför RNase E är livsnödvändigt för bakteriecellen, och presenterade en modell för detta i det sista manuskriptet i denna avhandling.


Vi tror att nedbrytning av budbärar-RNA är den RNase E-funktion som är livsnödvändig. Tack vare att RNase E bryter ner gammalt eller defekt budbärar-RNA kan ribosomer fokusera på de produkter som cellen verkligen behöver.

I denna avhandling har jag avslöjat flera viktiga kopplingar i det mycket komplexa nätverk av två processer, proteintillverkning och RNA-nedbrytning, som ser till att cellen kan anpassa sig till olika tillväxtförhållanden. Arbetet inleddes med studier av defekt protein-tillverkning och avslutas med en slutsats som återigen visar att uppehållandet och regleringen av dessa processer är av yttersta betydelse för bakteriecellens överlevnad.
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