Genetics and Growth Regulation in *Salmonella enterica*

JESSICA M. BERGMAN
Abstract

Most free-living bacteria will encounter different environments and it is therefore critical to be able to rapidly adjust to new growth conditions in order to be competitively successful. Responding to changes requires efficient gene regulation in terms of transcription, RNA stability, translation and post-translational modifications.

Studies of an extremely slow-growing mutant of Salmonella enterica, with a Glu125Arg mutant version of EF-Tu, revealed it to be trapped in a stringent response. The perceived starvation was demonstrated to be the result of increased mRNA cleavage of aminoacyl-tRNA synthetase genes leading to lower prolyl-tRNA levels. The mutant EF-Tu caused an uncoupling of transcription and translation, leading to increased turnover of mRNA, which trapped the mutant in a futile stringent response.

To examine the essentiality of RNase E, we selected and mapped three classes of extragenic suppressors of a ts RNase E phenotype. The ts RNase E mutants were defective in the degradation of mRNA and in the processing of tRNA and rRNA. Only the degradation of mRNA was suppressed by the compensatory mutations. We therefore suggest that degradation of at least a subset of cellular mRNAs is an essential function of RNase E.

Bioinformatically, we discovered that the mRNA of tufB, one of the two genes encoding EF-Tu, could form a stable structure masking the ribosomal binding site. This, together with previous studies that suggested that the level of EF-Tu protein could affect the expression of tufB, led us to propose three models for how this could occur. The stability of the tufB RNA structure could be affected by the elongation rate of tufB-translating ribosomes, possibly influenced by the presence of rare codons early in the tufB mRNA.

Using proteomic and genetic assays we concluded that two previously isolated RNAP mutants, each with a growth advantage when present as subpopulations on aging wild-type colonies, were dependent on the utilization of acetate for this phenotype. Increased growth of a subpopulation of wild-type cells on a colony unable to re-assimilate acetate demonstrated that in aging colonies, acetate is available in levels sufficient to sustain the growth of at least a small subpopulation of bacteria.

Keywords: tufA, tufB, EF-Tu, ppGpp, Stringent response, RNase E, RNA turnover, Post-transcriptional regulation, rpoB, rpoS, Growth in stationary phase

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Omnia mirari etiam tritissima.

Find wonder in all things, even the most commonplace.

– Carl Linnaeus
Front cover illustration by Oskar Bergman.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

aa-tRNA  Aminoacyl-tRNA
CoA     Coenzyme A
DNA     Deoxyribonucleic acid
_E. coli_ Escherichia coli
EF      Elongation factor
f-Met   N-Formylmethionine
GASP    Growth advantage in stationary phase
GDP     Guanosine di-phosphate
GTP     Guanosine tri-phosphate
IF      Initiation factor
mRNA    Messenger RNA
nt      Nucleotide
PCR     Polymerase chain reaction
PolyP   Inorganic polyphosphate
ppGpp   Guanosine-3’,5’-bis(diphosphate)
qRT-PCR Quantitative real time PCR
RBS     Ribosomal binding site
RF      Release factor
RNA     Ribonucleic acid
RNase   Ribonuclease
rRNA    Ribosomal RNA
_S. enterica_ Salmonella enterica serovar Typhimurium
tRNA    Transfer RNA
TA      Toxin-antitoxin
TCA     Tricarboxylic acid cycle
tmRNA   Transfer-messenger RNA
ts      Temperature sensitive
UTR     Untranslated region
Introduction

Bacteria can grow almost everywhere. They can be found in fresh and salt water, in soil and sediments all over the world including Antarctica, and in and on host organisms, such as humans and animals (Torsvik et al, 1996; Whitman et al, 1998; Yergeau et al, 2007). When bacteria grow they may frequently encounter new environmental conditions. Some changes occur abruptly, like being excreted from a host into a nutrient-poor and much colder environment. Other changes may be less dramatic, like the relatively small temperature changes encountered in a patient with fever, or the decrease in concentration of a favored nutrient in a complex rich medium in the laboratory. In the face of all of these changes a bacterial cell needs to adapt its physiology rapidly in order to grow as fast and efficiently as possible relative to other competing cells. Since proteins are the main “machines” and structural elements of the cell, the over-all goal of a physiological growth adaptation is to change the amounts and types of proteins being produced (reviewed in Neidhardt, 1999). Protein synthesis depends on many steps, and each of these steps can be targeted to influence the final outcome in terms of the rate or focus of the protein synthesis machinery. Thus, the decision to transcribe a section of DNA into RNA, the stability of the transcribed messenger RNA, the efficiency of translation of mRNA into protein, and the rate and nature of different modifications of the final protein products, are all important steps to protein function that can be modified in the process of growth regulation in response to changing environments. In my PhD studies I have been focusing on aspects of these steps in wild-type and various mutants of Salmonella enterica serovar Typhimurium (hereafter S. enterica). In doing so, I have aimed to gain a deeper understanding of the relationship between gene expression and bacterial growth regulation.

Bacterial growth

Growth of a bacterial population after its introduction to a new environment goes through several phases. Depending on genotype, cell density, the availability of nutrients and oxygen and the temperature, the length of any particular phase may vary. A typical growth curve is outlined in Figure 1 (Finkel, 2006). The first phase is the lag phase, where the bacterial cells initially adapt to the new environment (Rolfe et al, 2012). This phase is followed by
the *exponential growth phase*, in which each cell divides at a constant and maximum rate supported by the particular medium (Neidhardt, 1999). When nutrients become limiting the population goes into the *stationary phase*. This transition from exponential growth to stationary phase is an organized event that will be discussed in more detail below. The stationary phase is followed by a *death phase* where the viable population of cells is decreased. Many species subsequently continue into a so-called *long-term stationary phase*, where the total number of viable cells can stay relatively constant for a long time but where there may be constant turnover of cells (Finkel, 2006; Navarro Llorens et al, 2010). The long-term stationary phase lasts either until the population dies out or until the conditions change for the better and the surviving cells can start re-adapting and enter into the exponential growth phase again.

![Figure 1. Typical growth curve of an *S. enterica* population with the different phases indicated.](image)

**Transcription in bacteria**

The process of copying the genetic information encoded in DNA to RNA is called transcription. This is carried out by the RNA polymerase (RNAP), whose catalytic core consists of two $\alpha$ subunits, one $\beta$ subunit, one $\beta'$ subunit and one $\omega$ subunit. To start transcription, the $(\alpha_2\beta\beta'\omega)$ core enzyme needs to interact with a $\sigma$ subunit, which recognizes the promoter of a gene (Figure 2). This holoenzyme bound to the promoter is called the open complex. During transcription, the $\beta\beta'$ heterodimer forms the active site where DNA is decoded and RNA is polymerized, illustrated in Figure 3 (Darst et
Transcription can be regulated in different ways, among which differences in the affinity of RNA polymerase for different promoter sequences, and the relative concentrations of different sigma factors, play important parts.

**Figure 2.** The RNA polymerase holoenzyme ($\alpha_2\beta\beta'$ωσ) bound to the -35 and -10 elements of a DNA promoter.

**Figure 3.** The RNA polymerase core enzyme ($\alpha_2\beta\beta'$ω) transcribes DNA into RNA. The double-stranded DNA is melted into a transcription bubble around the RNAP.

**Transcription in stationary phase and the role of RpoS**

When nutrients become limiting or when the cell density gets too high, bacterial cells enter into the stationary phase of the growth cycle. This involves several alterations at the various levels of growth control, resulting in changes in metabolism, cell-to-cell communication, growth rate and cell shape. Many of these changes are governed by the transcriptional sigma factor RpoS (also known as $\sigma^5$ or $\sigma^{38}$) (Hengge-Aronis, 2002; Navarro Llorens et al, 2010). *S. enterica* has seven different sigma factors with different affinities for different promoters. Thereby the relative concentrations of the different sigma factors in the cell, which varies with growth conditions, will be
one factor directing the RNA polymerase to particular sets of genes (Ishihama, 2000). At the onset of stationary phase, the cellular concentration of RpoS is increased three- to fourfold because of an increased rpoS transcription, an increased stability and translation of the rpoS mRNA, and a reduced rate of proteolysis of RpoS protein (Ishihama, 2000; Hengge-Aronis, 2002).

With more RpoS in the cell, the sigma factor competition for interaction with RNA polymerase shifts from being dominated by the housekeeping σ70 towards increased interactions with RpoS. This leads to a transcriptional shift that changes the physiology of the cell from a state of growing as fast as possible on unlimited nutrients, to a state of slower growth, with less translation and metabolism, increased protein turnover and quorum sensing, and a more rigid cell envelope. These changes help protect the cells from external stresses and allow them to use amino acids from degraded proteins as building blocks for new proteins (Navarro Llorens et al, 2010).

Growth in stationary phase

Even though there is no net growth in the stationary and extended stationary phases, bacterial populations in these phases are still dynamic. Many cells continue to grow slowly and some subpopulations of cells grow more rapidly, possibly because they are better than other cells at scavenging nutrients from dead cells around them. This phenotype of having a relative growth advantage in stationary phase has been abbreviated GASP and was initially described by Roberto Kolter’s group (Zambrano et al, 1993). It was found that bacteria with a GASP phenotype often had mutations in rpoS, which resulted in RpoS with reduced (but not inactivated) activity. Also mutations in the global transcriptional regulator Lrp, known to affect expression of most of the genes that are upregulated during stationary phase, were found to give a GASP phenotype (Zambrano et al, 1993; Zinser & Kolter, 2004; Finkel, 2006). Based on an increased frequency of rifampicin-resistant mutants appearing in stationary phase bacterial colonies it was suggested that the general mutation rate of the cell increases as a response to the stress of being in stationary phase (Cairns et al, 1988; Bjedov et al, 2003). However, the phenomenon was later explained by natural selection and growth of pre-existing mutant subpopulations within the aging colonies, rather than a general increase in the rate of mutagenesis (Wrande et al, 2008). Thus bacterial populations in stationary and long-term stationary phase are dynamic and include various sub-populations, with some that are growing and others that are dying. This is further examined and discussed in Paper IV.
Post-transcriptional regulation and RNA processing

For a cell to optimize its growth according to the resources available it continuously needs to sense the environment and respond by fine-tuning its gene expression. One level of gene expression control is the post-transcriptional control of RNA stability and modification. The stable species of RNA (tRNAs and rRNAs) need to be processed, and in some cases base-modified, in order to function (Kaczanowska & Rydén-Aulin, 2007; Li & Deutscher, 2002). By controlling this processing, the cell can control the level of functional tRNA and rRNA and thereby the overall rate of protein synthesis. Gene-specific degradation of mRNA, in some cases known to be governed by regulatory small RNAs, adds an extra level of control by determining which gene transcripts are available for translation (reviewed in De Lay et al, 2013; Laalami et al, 2014). It is also necessary for the cell to control the quality of the mRNAs and to degrade faulty RNA species, in order not to waste energy by producing defective proteins, or risk trapping ribosomes on defective mRNAs (Silva et al, 2011).

Ribonucleases

The enzymes responsible for RNA processing are called ribonucleases (RNases). An exoribonuclease cleaves RNA molecules from the end, whereas an endonuclease is able to cleave between two bases in the middle of an RNA molecule. RNases can cleave in the 3'→5' direction or in the 5'→3' direction and can be specific for single stranded or double stranded RNAs (reviewed in Arraiano et al, 2010 and Arraiano et al, 2013).

Ribonuclease E

The endoribonuclease RNase E is important for processing of stable RNAs and for the degradation of mRNAs. Since in most cases RNase E is the first enzyme to cleave an RNA molecule it is central to the processes of post-transcriptional control of gene expression and growth (Jain et al, 2002). The initial cleavage by RNase E has been shown to be the rate-limiting step in degradation of some mRNAs (Luciano et al, 2012). Cleavage by RNase E is greatly enhanced by a 5’ monophosphate group on the 5’ end of the RNA molecule (Mackie, 1998; Celesník & Deana, 2007), although an alternative, direct, entry prior to cleavage also has been suggested and will be discussed below (Bouvier & Carpousis, 2011). It is believed that RNase E preferentially cleaves in A/U-rich stretches of RNA (McDowall et al, 1994).

RNase E has been shown to localize close to the inner membrane in the bacterial cell (Khemic et al, 2008). One study also suggested that a demonstrated interaction between RNase E and the 70S ribosome could be a way to regulate the balance between mRNA degradation and translation initiation.
(Tsai et al, 2012). Both these cases could be examples of intracellular organization to maximize efficiency of the regulation in response to environmental signals.

The degradosome and RNase E protein organization

The RNase E polypeptide can be divided into two functionally distinct halves, where the N-terminal part is responsible for the enzymatic capacity and the C-terminal part functions as a scaffold for three other enzymes; PNPase, RNA helicase B and enolase, which together with RNase E form the degradosome. The proteins of the degradosome work together, with RNase E recognizing the 5’ end of a target RNA, undergoing a conformational change and making the initial, endonucleolytic, cut in the RNA. Following this, RNA helicase B unwinds the RNA and PNPase (an exoribonuclease) then degrades the RNA in the 3’ → 5’ direction. The degradosome structure and function is reviewed in (Carpousis, 2007). The function of enolase in the degradosome is not fully understood, but is has been shown to be required for the rapid degradation of the ptsG mRNA in response to cellular accumulation of glucose 6-phosphate, thereby downregulating the expression of the major glucose importer PtsG (Morita et al, 2004). It is not clear what role enolase plays in this degradation, but since enolase carries out the second to last reaction in the glycolysis pathway (and is the last essential enzyme in the pathway) it is tempting to speculate that it could provide a link between glucose availability and RNA turnover.

*E. coli* mutants in which the C-terminal half of RNase E is deleted are viable, although they show a reduced rate of degradation of bulk mRNA and at least one small RNA (Kido et al, 1996; Lopez et al, 1999). However, a cell with a C-terminal deletion in RNase E and a null mutation in *rppH*, the gene encoding the protein responsible for the generation of the 5’ monophosphate, is not viable. Since an *rppH* mutant alone is viable and there is a 5’ binding motif in the N-terminal domain, this suggests two separate pathways for RNase E recognition of RNA targets, with the C-terminal half being important for direct entry (Deana et al, 2008; Anupama et al, 2011). Based on RNA cleavage measurements, direct entry has been suggested to be possible both for mRNA and tRNA targets, in both cases dependent on single-stranded regions of the RNA being processed (Kime et al, 2010; Kime et al, 2014).

Studying RNase E

RNase E function was first studied with the help of a mutant *rne* allele in *E. coli*. The particular mutant RNase E was temperature sensitive, preventing the strain from growing at the non-permissive temperature (42°C), thus defining RNase E as an essential protein for growth (Apirion & Lassar, 1978). It has later been shown that the essential function is associated with the N-terminal, catalytic, half of RNase E (McDowall & Cohen, 1996). Since
RNase E is involved in so many processing and degradation pathways, it has been difficult to establish what precise function (or functions) makes it essential for viability. This issue is still not resolved, but suggestions have been made. Studies on various temperature-sensitive mutants of RNase E have led to the suggestion that the processing of tRNAs is the essential function. This is still not fully understood since overexpression of a mutated version of RNase G can restore viability in a strain lacking RNase E, without restoring the tRNA processing (Li & Deutscher, 2002; Ow & Kushner, 2002; Deana & Belasco, 2004). The essentiality of RNase E is explored in Paper II.

Translation and the bacterial ribosome

Translation of mRNA into proteins takes place on the ribosome. The ribosome can be described as the workbench of the cell, and consists of two subunits, the small (30S) and the large (50S). Both subunits are composed of ribosomal RNA and proteins. The ribosome has been shown to be a ribozyme, i.e. the enzymatic catalysis of polypeptide synthesis is carried out by the rRNA, and the ribosomal proteins are believed to be important for organizing and stabilizing the structures of the rRNA molecules (Cech, 2000). The small subunit in S. enterica is built up of the 16S rRNA and 21 ribosomal proteins, it interacts with the mRNA and is responsible for positioning the codons correctly relative to the active sites in the ribosome. The large subunit (50S) facilitates polypeptide formation and contains the 5S rRNA, 23S rRNA and 33 ribosomal proteins. A detailed structural model has been suggested from the 2.8 Å crystal structure of the Thermus thermophilus 70S ribosome (Selmer et al, 2006). The decoding of the mRNA into proteins, via the tRNA adapter molecules, takes place at the interface between the two subunits, which together form the active, 70S ribosome. At this interface, there are three active sites, the acceptor (A) site where aminoacylated tRNAs enter the ribosome, the peptidyl (P) site where the peptidyl transfer reaction occurs and the exit (E) site where the deacylated tRNA leaves the ribosome (reviewed in Maguire & Zimmermann, 2001 and Kaczanowska & Rydén-Aulin, 2007).

Initiation

Translation is initiated when the ribosomal protein S1 and the 16S rRNA of the small subunit find and interact with the 5’ untranslated region (5’ UTR) of an mRNA. This typically involves a basepairing interaction between the Shine-Dalgarno sequence, situated 4 – 12 nucleotides before the initiation codon at the mRNA, and an anti-Shine-Dalgarno sequence in the 16S rRNA. Since the previous round of translation, the small ribosomal subunit is bound to initiation factor IF3, which prevents it from prematurely re-associating.
with the large subunit. The initiation factor IF1 will position itself in the A-site of the small subunit and IF2 then recruits the initiator tRNA, fMet-tRNA$_{f}^{met}$ to the P-site. When this complex has formed, IF1 and IF3 dissociate and the remaining 30S initiation complex is free to interact with the large subunit, forming the 70S ribosome. For initiation of leaderless mRNAs (without a 5' UTR), it has been shown that a codon-anticodon interaction between the mRNA and the initiator tRNA is needed. (Reviewed in Laursen et al, 2005 and Marintchev & Wagner, 2004.)

Initiation is completed when the two ribosomal subunits are bound to each other, with the mRNA positioned with the start codon and the initiator fMet-tRNA$_{f}^{met}$ in the P-site.

**Elongation**

The next step in protein synthesis is to read and translate the mRNA and build a polypeptide according to the mRNA codon sequence (illustrated in Figure 4). This is done by the ribosome together with three elongation factors (EF-Tu, EF-Ts and EF-G) during what is termed the elongation phase of protein synthesis.

![Figure 4](image)

*Figure 4. Translation elongation and the recycling of EF-Tu. Elongation cycle: When the ribosomal A-site is unoccupied, aa-tRNA in the ternary complex can interact with the mRNA. If the codon-anticodon basepairing is correct, EF-Tu will hydrolyze GTP to GDP and leave the ribosome. Peptidyl transfer and translocation closes this cycle. EF-Tu recycling: EF-Tu-GDP dissociated from the ribosome will be bound by EF-Ts, which catalyzes the exchange of GDP to GTP. This promotes the formation of a new ternary complex.*
When an aminoacylated tRNA that matches the second codon, which is in the ribosomal A-site, docks with the ribosome, the elongation phase starts. The aa-tRNA interacts with the ribosome in the form of a ternary complex (elongation factor EF-Tu•GTP•aa-tRNA), which by hydrolysis of GTP and codon-anticodon interaction positions the aa-tRNA in the A-site, bringing it in close contact with the fMet in the P-site. The close contact and the molecular environment, created by the 23S rRNA in the peptidyl transferase center, facilitate the transfer of fMet to the amino acid on the incoming aa-tRNA and create a peptide bond between them. After peptidyl transfer, the elongation factor EF-G catalyzes an inter-subunit movement that shifts the ribosome by a distance equivalent to three bases along the mRNA. The deacylated initiator tRNA is thereby shifted into the ribosomal E-site, where it leaves the ribosome and the second tRNA (now carrying the dipeptide) is shifted to the P-site. The A-site is now empty and ready for a new round of the elongation cycle. Off the ribosome, the elongation factor EF-Ts catalyzes the exchange of GDP to GTP on EF-Tu, thereby recycling it. (Reviewed in Ramakrishnan, 2002.)

Termination
When the ribosome eventually reaches the end of a coding sequence on an mRNA and a stop codon becomes exposed in the A-site, one of the release factors, RF1 or RF2, interacts with it and initiates the process leading to release of the polypeptide from the tRNA in the P-site. Polypeptide release induces a conformational change in the ribosome that allows the binding of release factor RF3 bound to GTP. This, in turn, releases RF1 or RF2, and RF3 is itself released upon the hydrolysis of its GTP to GDP. The ribosome recycling factor RRF, together with EF-G are responsible for making the 70S ribosome dissociate into separate 30S and the 50S subunits, and allowing IF3 to bind to the small subunit preventing re-association until a new round of translation begins. (Reviewed in Ramakrishnan, 2002 and Kisselev & Buckingham, 2000.)

Elongation factor EF-Tu
The protein elongation factor EF-Tu plays a central role in protein synthesis, delivering aa-tRNA to the ribosome as part of the ternary complex. Thus, EF-Tu function has a strong influence on bacterial growth rate. Once mRNA translation has initiated, the rate limiting step of protein synthesis is the delivery of aminoacylated tRNAs to the ribosome, a process dependent of EF-Tu (Tubulekas & Hughes, 1993a). The ternary complex, aa-tRNA•EF-Tu•GTP, has a much higher affinity for the ribosome than free aminoacylated tRNA. This ensures rate efficiency in the elongation phase, while the codon-anticodon interaction gives specificity. Later proofreading steps also

EF-Tu is built up from 393 amino acids (394 counting the start codon-encoded fMet) and consists of three globular domains. Domain I binds the GTP/GDP molecule (Kjeldgaard & Nyborg, 1992). Binding of the aminoaerylated tRNA involves all three domains and domain II has been demonstrated to be important for the interaction between the ternary complex and the ribosome (Tubulekas & Hughes, 1993b). In Gram-negative bacteria, EF-Tu is encoded by two almost identical genes, tufA and tufB, located in separate operons. In Salmonella the sequence similarity is 99% on the nucleotide level and the protein sequences are identical (Jaskunas et al, 1975; Hughes, 1986). The tufA gene is the final gene in an operon that also contains the genes for the ribosomal protein S12 (rpsL), S7 (rpsG), and the elongation factor EF-G (fusA) (Jaskunas et al, 1975). tufB is the final gene of an operon that also carries four tRNA genes, thrU, tyrU, glyT and thrT (van Delft et al, 1987; Lee et al, 1981).

In a fast-growing cell of S. enterica, about 9% of the protein in the cytoplasm is EF-Tu, which makes it the most abundant cytoplasmic protein in the cell (van der Meide et al, 1983b; Tubulekas & Hughes, 1993a). In wild-type E. coli and S. enterica about two thirds of the EF-Tu is expressed from tufA and one third from tufB (Pedersen et al, 1976; van der Meide et al, 1982; van der Meide et al, 1983b; Hughes, 1990). In S. enterica, when either one of the tuf-genes is deleted, the cell remains viable but the amount of EF-Tu is reduced to approximately two thirds of the wild-type level, and the growth rate goes down similarly to about two thirds of the wild-type rate (Hughes, 1990; Tubulekas & Hughes, 1993a). Since a knock-out of tufA doubles the amount of EF-Tu protein expressed from the tufB locus, it has been suggested in a series of papers that EF-Tu protein could take part in the regulation of tufB transcription, but no mechanism for this suggested regulation was discovered (van der Meide et al, 1982; van der Meide et al, 1983a; van der Meide et al, 1983b; van Delft et al, 1988a; van7 Delft et al, 1988b). This is discussed and expanded on in Paper III.

The antibiotic kirromycin targets EF-Tu specifically and kirromycin-resistant bacteria have mutations in EF-Tu (Wolf et al, 1974; Wolf et al, 1977; Hughes, 1986). This has proven to be a useful tool for the genetic and molecular studies of the protein. A kirromycin resistant mutant was studied in Paper I.

Translational control of gene expression

It has been known for a long time that the structure and sequence of the mRNA as well as particular patterns of codon usage can regulate the translation of an mRNA.
Role of the 5’ UTR in regulation of translation initiation

Regulation of gene expression can take place at the stage of translation initiation. As discussed above, translation initiation involves an interaction between the ribosomal protein S1 and the 5’ untranslated region of the mRNA. The 5’ monophosphorylated end of the mRNA is also where RNase E usually binds to initiate degradation, which makes the fate of mRNAs into a competition between translation and degradation. This is discussed more in detail in Paper II.

Also other proteins can regulate the initiation of translation. A good example of this type of regulation is illustrated by the thrS gene, encoding threonyl tRNA synthetase, ThrS (Springer et al, 1986). The 5’ UTR of thrS can fold into two stem-loops that structurally mimic the acceptor stem of the threonyl tRNA. These stem-loops contain the Shine-Dalgarno sequence and mask it from interaction with the ribosome. Because of the molecular mimicry, the ThrS protein can, when it is highly abundant, bind to the stem-loop structures in its own leader mRNA. This binding stabilizes the stem-loops and occludes the ribosomal binding site and the start codon, thereby repressing translation of ThrS, when it is present in sufficient amounts (Moine et al, 1988; Romby et al, 1990).

Ribosome-mediated transcription attenuation

The attenuation mechanisms of the trp and his operons are classical examples of how codon usage can affect transcription and translation. These operons encode proteins for the biosynthesis of tryptophan or histidine, respectively. In a leader sequence close to the beginning of these operons, there is a short stretch of codons for tryptophan (for the trp operon) or histidine (for the his operon). These codons work as nutritional sensors for the particular amino acids. The speed of translation of the leader sequence open reading frames for these biosynthetic operons is very much governed by the availability of tryptophan or histidine.

When the cell experiences nutritionally good conditions there is a sufficient supply of the amino acid in question and plenty of the particular tRNA isoacceptor to carry it. In such a case, ribosome movement across the stretch of tryptophan or histidine codons in the leader sequence of the mRNA proceeds rapidly. When the ribosome translates the mRNA rapidly, a stretch of mRNA that can interact with itself is generated, leading to the formation of a transcriptional termination hairpin structure. This results in that the RNA polymerase terminates transcription close to the end of the leader sequence, preventing transcription of the whole operon. This way the cell avoids making the amino acid under nutritionally favourable conditions.

If, on the other hand, the cell is being starved for the amino acid in question, there will be a shortage of the particular tRNA isoacceptor charged with that amino acid. This results in ribosome pausing during translation of the
specific run of codons in the leader sequence, which in turn results in a different stretch of naked mRNA being available to fold and the termination hairpin does not form. Consequently, transcription continues through the operon and the proteins will be produced, ensuring biosynthesis of the amino acid. (Yanofsky, 1981; Johnston & Roth, 1981.)

**Codon usage bias**
The genetic code is redundant, meaning that the same amino acid can be encoded by more than one codon. Different codons for the same amino acid can be read by different tRNA isoacceptors. Some codons are more frequently used than others, a phenomenon called codon usage bias. Different organisms have different biases and within an organism different genes can have different codon usage bias. Genes that are expressed at very high levels (e.g. genes for ribosomal proteins and other proteins directly associated with translation) tend have a stronger codon usage bias. Genes that are not highly expressed during fast growth (e.g. biosynthetic genes) tend to have a more even distribution of all codons, and consequently a higher relative frequency of rare codons (Karlin et al, 2001). The origin and possible selection for a codon usage bias is not known in detail, but the different biases in different organisms has been suggested to originate from slightly different mutational biases. A codon usage bias could increase the efficiency and accuracy of translation and also play a part in folding of newly translated polypeptides (Reviewed and discussed in Sharp et al, 2010 and Novoa & Ribas de Pouplana, 2012.)

**Post-translational modifications**
Once a protein has been made, its properties, affinities and stability can be modified by the addition of different chemical groups. These kinds of post-translational modifications have long been known in eukaryotes, but only recently has their importance in bacteria been appreciated. The two best-studied types of protein modification in bacteria are phosphorylation (mostly on serine, threonine and tyrosine residues) and acetylation (which mainly occurs on lysine residues, or on the amino-terminus of the protein). Interestingly, the translation elongation factors EF-G, EF-Tu and EF-Ts as well as the degradosome-associated enzymes enolase and PNPase have been shown to be both phosphorylated and acetylated (reviewed in Soufi et al, 2012).

**Phosphorylation**
Phosphorylation of bacterial proteins was first studied for signal transduction proteins in two-component systems. When a high-accuracy mass spectrometry study on the *E. coli* proteome was done, 79 proteins were found to be
phosphorylated on at least one position each, and termed phospho-proteins (Macek et al, 2008). This and other studies have showed that phospho-proteins are involved in various cellular processes such as transcription, translation, metabolism of carbon and amino acids, cell growth, protein secretion and virulence. It was also found that the phospho-proteome is more conserved among bacteria than the general proteome (Macek et al, 2008; Soufi et al, 2012).

### Acetylation

Lysine-acetylation of a protein is believed to be a reversible and dynamic modification (in contrast to the irreversible amino-terminus acetylation), which makes it a good tool for regulation. In eukaryotes acetylation of lysine residues is known to alter the properties of a protein for e.g. DNA binding, stability, localization and protein-protein interactions (reviewed in Hu et al, 2010). In a pioneering study, the bacterial protein acetyl transferase Pka (also known as Pat or YfiQ) was identified and found to inactivate the protein acetyl-CoA-synthetase, Acs, by acetylation of a lysine residue (Starai & Escalante-Semerena, 2004). Since then, the field of protein acetylation in bacteria has expanded. Studies on the whole proteome from *E. coli* and *S. enterica* have revealed that the vast majority of the enzymes in central metabolism can be acetylated and that the acetylation pattern differs depending on what carbon source the bacteria have access to. Also, proteins involved in transcription, translation and stress responses were found to be acetylated. These findings have been interpreted to mean that acetylation is used as a tool to guide the cellular metabolism into pathways optimal for the available carbon source, as a way to fine-tune growth regulation (Zhang et al, 2009; Wang et al, 2010).

The molecule acetyl-coenzyme A (acetyl-CoA) is an important node in acetate metabolism and acetylation, as it can feed the TCA cycle with acetyl groups (leading to NADH and ATP production) or be used as an acetyl donor in acetylation reactions. The acetyl-CoA to CoA ratio has been found to determine the amount of acetylation in *E. coli* (Lima et al, 2011). The α, β and β’ subunits of RNA polymerase can be acetylated, and in the case of the α subunit, also phosphorylated (Zhang et al, 2009; Lima et al, 2011). The acetylation state of the RNA polymerase can up- or down-regulate transcription from at least one promoter, depending on which lysine residue is acetylated (Lima et al, 2012).

### Bacterial metabolism

As described and discussed above the expression and function of proteins can be regulated at several different levels. All of these regulatory steps will
shape the cellular contents of the bacterial cells to be well adapted to the growth environment. As the cells grow, there is a continuous need to adapt to a changing environment. This is done by regulation of protein expression, regulation of metabolic pathways and connection of the many different signals received from the environment. In the following paragraphs, some examples of this is described and discussed.

Aging colonies, acetate and metabolism

A schematic view of glycolysis, the tricarboxylic acid (TCA) cycle and acetate metabolism is outlined in Figure 5. Acetate, a two-carbon compound, can be produced during glycolysis and can also be taken up from the environment. Since it is a small lipophilic molecule, acetate can freely diffuse over the cell membrane (Repaske & Adler, 1981; Kihara & Macnab, 1981; Salmond et al, 1984; Gimenez et al, 2003). Salmonella has an acetate transporter, ActP, but actP deletion mutants in the close relative E. coli still take up acetate, presumably by diffusion (Gimenez et al, 2003). Once inside the cell, acetate needs to be modified not to diffuse out again. This can be done by either of two pathways. At high acetate concentration, the low-affinity enzyme acetate kinase, AckA, phosphorylates acetate to acetyl-P (Kumari et al, 1995). The enzyme phosphotransacetylase, Pta, can then convert acetyl-P into acetyl-CoA (Rose et al, 1954). The AckA-Pta pathway is reversible. When acetate concentrations are low, the high-affinity enzyme AMP-forming acetyl-CoA synthetase, Acs, can convert acetate to acetyl-CoA in an irreversible reaction with acetyladenylate as an intermediate (Kumari et al, 1995; Berg, 1956). Cells that lack both the AckA-Pta and the Acs pathways cannot grow on acetate as a sole carbon source (Kumari et al, 1995).

When bacterial cells grow exponentially in a medium containing glucose, the glycolytic pathway will be utilized, catabolizing the glucose into acetyl-CoA and feeding into a branched version of the TCA cycle, thus inhibiting oxidative phosphorylation, a phenomenon called “the bacterial Crabtree effect” (Crabtree, 1929; Mustea & Muresian, 1967; Doelle et al, 1982). Products from the branched TCA will be used for biosynthesis and ATP will be produced from glycolysis by substrate phosphorylation (Wolfe, 2005). In order to regenerate coenzyme A, exponentially growing bacteria using glucose as the primary carbon source are excreting acetate (El-Mansi, 2004). This is done by the Pta-AckA pathway, re-gaining ATP and coenzyme A (El-Mansi & Holms, 1989).
Eventually the glucose will be consumed and the cells have to switch to other carbon sources. In rich laboratory media, these can be short peptides or amino acids (Prüss et al, 1994). The lower concentration of glucose will induce a catabolite repression system where the transcriptional regulator complex cAMP-CRP increases transcription of acs (Kumari et al, 2000a). Since the Acs conversion of acetate to acetyl-CoA is irreversible, this will limit the extracellular accumulation of acetate in response to the glucose availability, thereby increasing the level of intracellular acetate (Renilla et al, 2012). Transcription of acs is also regulated by sigma factor utilization, as it is stimulated by $\sigma^{70}$ and inhibited by RpoS, which tunes this induction to take place before the cells enter stationary phase (Kumari et al, 2000b). The excretion – import cycle of acetate is called the acetate shift and has been thoroughly reviewed in (Wolfe, 2005).

Acetyl-CoA is a central molecule in metabolism, where the product from the glycolysis and acetate import meet and can be used in several pathways. Acetyl-CoA can be used for synthesis of fatty acids in an essential pathway involving the multi-subunit enzyme acetyl-CoA carboxylase (encoded by the
genes *accA, accB, accC* and *accD*) catalyzing the first committed reaction (Cronan & Waldrop, 2002). For acetylation of proteins, acetyl-CoA acts as the donor of the acetyl group (Starai & Escalante-Semerena, 2004; Lima et al, 2011) and acetyl-CoA is also the molecule that drives the TCA cycle by being added to oxaloacetate to complete the cycle to citrate. If bacterial cells are growing on acetate as a sole carbon source, it is futile to run the full TCA cycle, as two CO₂ molecules are being dissimilated for every round of the cycle. To prevent carbon depletion, an alternative cycle is run, called the glyoxylate shunt. This bypass cycle converts isocitrate to malate, via glyoxylate (Kornberg, 1966). These two reactions are dependent on the enzymes isocitrate lyase, AceA, and malate synthase, AceB. These proteins are encoded together with the regulatory protein AceK in the *aceBAK* operon (Maloy & Nunn, 1982; LaPorte et al, 1985; Chung et al, 1988). The TCA cycle and the glyoxylate shunt branch at isocitrate, which can be metabolized either into glyoxylate, by AceA, or into α-ketoglutarate, by isocitrate dehydrogenase, Idh. During growth on glucose, Idh is active and the TCA cycle is run, in a split version if glucose is in excess. When the glucose is depleted and the cells re-assimilate acetate, the enzyme isocitrate kinase/dehydrogenase, AceK, will phosphorylate Idh and inactivate it. This increases the availability for AceA to act on isocitrate and the glyoxylate shunt is run (LaPorte & Koshland, 1982; LaPorte & Chung, 1985; LaPorte, 1993).

This complex regulation ensures an efficient use of the carbon compounds available in the growth environment, for synthesis of fatty acids and cell membranes, for protein modification by acetylation and for the production of reducing agents and energy metabolism.

The stringent response

During exponential growth the bacterial cell grows and divides as fast as possible for its genotype in the available growth medium. To achieve this maximal growth rate, fast and efficient translation is a central requirement. The vast majority of the RNA transcripts produced in an exponentially growing bacterial cell are ribosomal and transfer RNAs and mRNAs that code for ribosomal proteins and other proteins associated with the translation machinery. An exponentially growing bacterial cell in a rich medium can have tens of thousands of ribosomes per cell (Bremer & Dennis, 1996).

Already in the 1960s it was discovered that bacterial cells that experience starvation for one or several amino acids respond by rapidly changing their pattern of transcription. This effect was initially observed as a decrease in the total amount of RNA per cell (Sands & Roberts, 1952; Pardee & Prestidge, 1956; Neidhardt, 1966). This normal switch in transcriptional pattern upon amino acid starvation was called the stringent response (Stent & Brenner, 1961). When the phenotypes and cellular contents of these starved cells
were further investigated, the nucleotides guanosine pentaphosphate and guanosine tetraphosphate (pppGpp and ppGpp, hereafter referred to as ppGpp) were discovered to be present in unusually high concentrations (Cashel & Gallant, 1969).

The nucleotide ppGpp functions as a signal molecule that “transduces” the stringent response from the ribosome, where the amino acid starvation is detected, to the RNA polymerase where the response is regulated. It does so by binding to the interface of the β’ and the ω subunits (Mechold et al, 2013; Ross et al, 2013) which changes the promoter affinity of the RNA polymerase (Chatterji & Fujita, 1998; Barker et al, 2001). This results in a reduced level of transcription from the seven ribosomal RNA operons, and from the operons that contain genes for ribosomal proteins. Instead, in the presence of ppGpp, RNA polymerase has an increased affinity for promoters of biosynthetic operons, encoding genes for synthesis of e.g. amino acids (Traxler et al, 2008). Thus, by the ppGpp-mediated stringent response, bacterial cells respond to amino acid starvation by biogenesis of amino acids. That is, when cells grow in poor medium they respond to this by producing more of the building blocks needed and less of the machinery for synthesizing proteins and for fast growth.

The RpoS-dependent regulation is related to the stringent response and ppGpp levels. It has been shown that increased levels of ppGpp help RpoS to outcompete σ70 for binding to the core RNA polymerase (Jishage et al, 2002). Also the RNA polymerase-binding protein DksA, a suggested functional partner of ppGpp, is important for the synthesis of RpoS (Brown et al, 2002). Thus ppGpp is the central signal that is produced in response to stress or starvation. It then carries out its effect on transcription by changing open complex half-lives and the sigma factor preferences of core RNA polymerase (Magnusson et al, 2005).

Synthesis and hydrolysis of ppGpp

Starvation for amino acids leads to ribosome stalling with the A-site of the ribosome unoccupied, without aa-tRNA. Amino acid starvation will also lead to an increased concentration of uncharged transfer RNA isoacceptors. In S. enterica, E. coli and other closely related species, ppGpp can be produced by two different enzymes, RelA and SpoT (Magnusson et al, 2005; Potrykus & Cashel, 2008). When an uncharged tRNA binds in a codon-specific manner to the mRNA in an empty A-site during starvation for single amino acids, the ribosome-associated protein RelA is triggered to synthesize ppGpp (Stent & Brenner, 1961; English et al, 2011). The second ppGpp-producing enzyme, SpoT, has a dynamic level of activity that depends on nutritional availability. SpoT also has a ppGpp hydrolysis activity that is low during fast growth and gets inhibited by starvation for multiple amino acids or carbon. The hydrolytic form of SpoT seems to be much more stable than
the ppGpp-synthesizing form but it is not known what mechanism determines whether SpoT has synthetic or hydrolytic activity (Murray & Bremer, 1996).

Under nutritionally favourable conditions during exponential growth, RelA is inactive and the SpoT synthetase and hydrolase activities vary with fine changes in nutrient concentrations. Under such conditions the cellular concentration of ppGpp is low, RNA polymerase is mainly free of ppGpp and transcriptional activity is focused on ribosome-related genes. When nutritional conditions change and the cell begins to experience starvation, RelA is activated to produce ppGpp and the hydrolytic activity of SpoT is inhibited. These changes lead to an accumulation of ppGpp and an associated switch in transcriptional pattern. Under starvation conditions, when ppGpp levels are high, RNA polymerase is more likely to be in complex with ppGpp and thus have a promoter preference shifted towards biosynthetic operons.

An interconnected response to starvation

One way for bacterial cells to connect starvation signals to a physiological response is via a pathway that involves a toxin-antitoxin system, ppGpp and ribosome rescue by trans-translation.

Toxin-antitoxin (TA) systems were first discovered for plasmids, as a way to avoid loss of the plasmids and have also been described as “addiction systems” (Ogura & Hiraga, 1983; Gerdes et al, 1986). There are five types of TA systems described, depending on the antitoxin mode of action (Cook et al, 2013). They all have in common that the toxin and the antitoxin are encoded together in an operon, that the antitoxin is unstable and that the toxin is stable and can carry out an activity that, at least under some circumstances, can be toxic for the cell (Gerdes & Maisonneuve, 2012; Fineran et al, 2009; Masuda et al, 2012; Wang et al, 2012). Since the antitoxin is unstable but is needed to inhibit or antagonize the action of the toxin, there will be selection to keep the gene for the antitoxin. As the two genes always come in a pair, the selection will effectively act to keep the full operon (Gerdes et al, 2005; Van Melderen & De Bast, 2009; Van Melderen, 2010).

The RelBE toxin-antitoxin system

One of the most studied TA systems in *E. coli* and *S. enterica* is the RelBE system. The only known function, *in vitro* and *in vivo*, of the toxin RelE is to cleave mRNA in the ribosomal A-site (Pedersen et al, 2003; Christensen & Gerdes; 2003; Neubauer et al, 2009; Hurley et al, 2011). The antitoxin RelB is mainly unstructured and is believed to wrap around the RelE protein, thus making it too bulky to fit into the ribosome, where RelE by having a similar structure to domain IV of EF-G otherwise could fit into the A-site decoding center (Takagi et al, 2005, also discussed in Wilson & Nierhaus, 2005).
Transcription from the relBE promoter is normally repressed by a feedback binding of free RelB or the RelBE complex to the operator sequences. If the antitoxin level is lowered by proteolysis, transcription will be de-repressed (Gotfredsen & Gerdes, 1998; Bøggild et al, 2012).

The chromosomal relBE locus in E. coli was first identified by mutations in relB. These were responsible for a delayed relaxed response, similar to the relaxed response of a relA knockout, where amino acid starvation does not lead to a rapid shift in the transcription pattern as it would in a wild-type cell. This was later attributed to an increased activity of RelE, due to less active mutant RelB antitoxin (Diderichsen et al, 1977; Christensen & Gerdes, 2004).

When a bacterial cell experiences amino acid starvation, the level of the global regulator molecule ppGpp will increase, either by RelA activation, or by inactivation of the SpoT hydrolysis function. ppGpp will inactivate the enzyme exopolyphosphatase, which in turn will lead to increased levels of inorganic polyphosphate (PolyP) (Kuroda et al, 1997; Kuroda et al, 1999). Binding of PolyP to the Lon protease promotes complex formation between Lon and its protein substrates, thereby stimulating proteolysis (Kuroda et al, 2001). The RelB antitoxin is one of the targets of Lon proteolysis (Christensen & Gerdes, 2004; Maisonneuve et al, 2013).

Proteolysis of RelB will lead to an elevated amount of free RelE toxin that can associate with mRNA in the ribosome. This will be even more pronounced since the increased production of ppGpp will deplete the cellular pool of GTP, which leaves the elongation factors EF-Tu and EF-G without their source of energy and thereby keep them in an inactive state. This makes the translating ribosomes more prone to stalling and the RelE toxin has a higher probability to reach and cleave mRNA in the A-site (Wilson & Nierhaus, 2007).

**Trans-translation**

The resulting ribosomes, stuck on mRNAs without stop codons, can then be “rescued” by transfer-messenger RNA (tmRNA) (Roche & Sauer, 1999; Yamamoto et al, 2003; Christensen & Gerdes, 2003). The 5’ and 3’ ends of the tmRNA molecule fold into a tRNA-like structure with an acceptor arm. The middle part of the molecule forms an unstructured mRNA-like moiety. tmRNA can interact with the adenyl-tRNA synthetase to be charged with an adenine amino acid, and with EF-Tu•GTP to form a ternary complex (Rudinger-Thirion et al, 1999; Barends et al, 2000). Together with the protein partner SmpB, the tRNA-like moiety will interact with the ribosomal A-site and translation of the mRNA-like sequence will tag the unfinished polypeptide to be recognised for degradation (Keiler & Waller, 1996; Haebel et al, 2004). At the end of the mRNA part of the tmRNA there is a stop codon, and the ribosome will be released and recycled as normal.
By this cascade of signals and events, ppGpp – RelE – tmRNA, the cells very rapidly interconnect signals for starvation into release of ribosomes that can then be used for translation of biosynthetic proteins (Wilson & Nierhaus, 2007). Together with the effects of ppGpp on the bacterial transcription pattern, this cascade ensures a fast and efficient response to starvation.

**Salmonella as a model organism**

The work in this thesis has been done using the Gram negative, rod-shaped bacterium *Salmonella enterica* serovar Typhimurium. *Salmonella* is a genus within the γ-proteobacteria Enterobacteriaceae family, which also contains the *Escherichia* genus with the common model organism *E. coli*. Some serovars of *S. enterica* are pathogenic to humans, causing typhoid fever (serovar Typhi) or gastroenteritis (serovar Typhimurium).

Within *S. enterica* serovar Typhimurium, there are several different laboratory strains, of which work in this thesis have been done using the strains LT2 (Paper I, II and III) and ATCC 14028s (Paper IV). Both of these strains have been whole genome sequenced (McClelland et al, 2001; Jarvik et al, 2010) and the sequences are publically available. The ATCC 14028s strain is fully pathogenic whereas the LT2 strain is non-virulent, which has been ascribed to the less active TTG start codon in the LT2 rpoS gene (Wilmes-Riesenberg et al, 1997; Swords et al, 1997).

*S. enterica* is a good model organism for laboratory work, as it is facultative aerobe, grows well at 37°C and is easy to modify genetically. Wild-type *S. enterica* has a generation time of approximately 20 minutes in LB medium and can be grown in liquid cultures and as colonies on solid media. Selection for antibiotic resistance as well as screening for phenotypes such as fast or slow growth, temperature sensitivity or suppression thereof can be done directly in cultures or on plates. Since bacteria are single-celled, haploid organisms, the deduction of the genotype behind a studied phenotype is straightforward.

For this thesis-work, genetic modifications in the *S. enterica* strains were made by generalized transduction with the high-frequency transducing P22 phage (Schmieger & Backhaus, 1973) and with lambda red-based linear transformation and recombineering (Datsenko & Wanner, 2000; Yu et al, 2000). Double-stranded genetic cassettes have been used to recombine in selectable markers linked to interesting mutations, or to knock out genes. Recombineering with single-stranded oligonucleotides was used for counter selecting the loss of *cat-sacB* cassettes (Ellis et al, 2001). The genetic constructions on transformable plasmids have been done both with restriction enzyme-based cloning and with a recombineering system (Sharan et al, 2009). In Paper I, conjugation of an F-plasmid was used as a means of genetic modification.
Present investigations

Paper I

For protein translation to function, the ribosome needs to be supplied with aminoacylated tRNAs. This is done in the form of a ternary complex, which consists of the elongation factor EF-Tu, a GTP molecule and the aa-tRNA. EF-Tu is the most abundant protein in the cell, making up approximately 9% of the cytosolic proteins in exponentially growing *Salmonella* (Tubulekas & Hughes, 1993a) and it is encoded by two widely separated but near-identical genes, *tufA* and *tufB* (Jaskunas et al, 1975; Hughes, 1986). A cell dependent on a single mutant *tufA* allele, *tufA499* encoding a Glu125Arg mutant of EF-Tu, grows extremely slowly. This mutant was isolated as resistant to the antibiotic kirromycin and the slow growth phenotype has previously been shown to partly be due to an increased degradation of nascent mRNA transcripts (Abdulkarim et al, 1994; Hammarlöf & Hughes, 2008). The mutant EF-Tu Glu125Arg has also been shown to have a lowered affinity to aminoacylated tRNAs and therefore be impaired in the ability to form ternary complexes (Abdulkarim et al, 1996).

We set out to study if the extreme slow-growth phenotype of this EF-Tu mutant could be compensated for by overexpression of a wild-type gene, other than *tuf* itself. If such a gene, or genes, could be identified, we were interested to see if their identity could reveal more detail on the mechanism for the extreme slow growth of this mutant.

The slow-growing EF-Tu mutant has an increased level of ppGpp

By screening a genetic library of wild-type *S. enterica* we found that overexpression of the gene *spoT* increased the growth rate and growth yield of the EF-Tu mutant strain. *spoT* encodes a bi-functional protein with both ppGpp hydrolase and synthase activities and was found to decrease the ppGpp levels when overexpressed in the slow-growing mutant strain. Inactivation of *relA*, a ribosome-associated ppGpp synthase, had a similar effect. By thin layer chromatography, the slow-growing EF-Tu mutant strain was found to have a 2- to 3-fold increased ppGpp concentration during exponential phase, compared to the wild-type.
Decreased proline tRNA aminoacylation in the *tufA499* mutant

Since ppGpp is synthesized by RelA as a response to de-acylated tRNAs in the ribosomal A-site, and the slow-growing mutant previously has been shown to have lower levels of several mRNAs, we assayed the expression of the aminoacyl tRNA synthetases ThrS, CysS, ValS and ProS by qRT-PCR. In the *tufA499* mutant these were all found to be down-regulated to 40 – 75% of the isogenic wild-type. To study the effect of this on the respective tRNAs, we measured the levels of aminoacylation for the corresponding isoacceptors by Northern blotting. All three isoacceptors for proline were acylated at a lower level in the mutant than in the wild-type, which explains the elevated levels of ppGpp and part of the slow-growth phenotype.

A molecular model for the extremely slow growth phenotype

A previous model for the slow growth of the *tufA499* mutant suggested that the decreased affinity between the mutant EF-Tu and aminoacylated tRNAs would cause a slow-down of translation leading to ribosome pausing and exposure of naked mRNA, susceptible to RNase E cleavage (Hammarlöf & Hughes, 2008). In this study we confirm that the mutant EF-Tu will lead to a lower gene expression for at least some genes, including the tested aminoacyl tRNA synthetases. This in turn will result in a decrease in charged proline tRNAs and a corresponding increase in de-acylated tRNAs that, if they interact codon-specifically with the A-site mRNA, act as the induction signal for ppGpp-producing RelA.

By this series of events the EF-Tu mutant cells are trapped in a futile starvation response. The vicious circle can be broken either by mutations in *rne*, since a mutant RNase E can be less efficient in cleaving mRNA, or by decreasing the cellular level of ppGpp, either by overexpressing the hydrolysis function of SpoT or by deleting the gene for the ppGpp-synthesizing enzyme RelA.
The endoribonuclease RNase E is involved in processing and maturation of rRNAs and tRNAs and is rate-limiting for the degradation of several mRNA species (Luciano et al, 2012). The enzyme is essential for viability in S. enterica and related species. The processing of tRNAs has been proposed to be the essential function of RNase E, but it is unknown if this is the only essential function (Li & Deutscher, 2002; Ow & Kushner, 2002).

To study the essentiality of RNase E by genetic means, we used two temperature sensitive (ts) mutants of rne, the gene encoding RNase E, which had been isolated and partly characterized during previous projects (Hammarlöf & Hughes, 2008; Hammarlöf et al, 2011). Since the ts phenotype indicates that at least one essential function is non-functional at the non-permissive temperature, our aim was to select extragenic suppressors of the ts phenotypes. If this previously untested approach would work, we hoped that the identity of these potential suppressors would help us to rationalize essential function(s) of RNase E and let us set up hypotheses to test our predictions.

Three classes of extragenic suppressors isolated

We isolated and identified 15 different external suppressor mutants of the ts rne phenotype. These fell into three classes: one was a nonsense mutation in the ribosomal protein S1; four were point mutations or small in-frame deletions in the exoribonuclease RNase R; ten external suppressors were found in and around a RelBE-like toxin-antitoxin gene pair.

The S1 mutation isolated would lead to a truncation of the protein by removal of the last of four repeated RNA binding motifs. The ribosomal protein S1 is important for initiation of translation and similar S1 mutants studied in E. coli showed that cells with a small C-terminal truncation of S1 are viable (Skorski et al, 2007).

RNase R is a 3’→5’ exoribonuclease that is involved in degradation of tRNAs, rRNAs and structured mRNAs (Arraiano et al, 2013). Two of the mutations isolated in RNase R would lead to amino acid substitutions, Ile444Ser and Arg446Pro. The other two RNase R mutants were in-frame deletions of amino acids 435 – 438 or 524 – 535. All four RNase R mutations map closely to the nuclease site of the enzyme (Vincent & Deutscher, 2009).

The only known function for the RelE toxin is to cleave mRNA in the ribosome, if translation pauses (Pedersen et al, 2003; Christensen & Gerdes, 2003; Hurley et al, 2011). When the relBE operon is expressed under exponential, non-stressed growth, RelB is binding to and inactivating RelE. Of the ten relBE-associated suppressors isolated during this study, six were deletions upstream of relB ranging between 900 and 2400 nucleotides. One of the mutations also deleted most of the relB gene. None of them deleted
any part of the toxin relE gene. We also isolated three point mutations in relB, Ala33Thr, Ala33Ser and Val45Met, and one in-frame deletion of the last seven amino acids of RelB. By subcloning the RelE toxin on an inducible plasmid, we confirmed that overexpression of RelE gives a growth advantage to the ts rne mutant at higher temperatures. We therefore suggest that all 10 relBE extragenic suppressor mutations confer an increase in RelE activity.

Suppressor mutations specifically restore mRNA degradation

Since the all three classes of extragenic suppressors are connected to mRNA, either by translation initiation (S1) or degradation (RNase R and the RelE toxin), we hypothesized that mRNA degradation is an essential function of RNase E. In a ts rne strain, the loss or decrease of this function could be suppressed by less efficient translation initiation (conferred by the mutant S1 protein), which would restore the competition for the 5’ end of the mRNAs between S1 and RNase E. Suppression could also occur by increased mRNA degradation, by a slightly changed or increased RNase R function or by an increased RelE activity.

To test this, the processing of some stable RNAs and the half-lives of a set of mRNAs were measured by qRT-PCR. It was shown that all these processes were disturbed in the ts RNase E mutants, but that only the rapid degradation of some mRNAs was restored in the double mutants carrying the ts and suppressor mutations. From these data, and the fact that mRNA recognition is the only common denominator for the isolated ts suppressors, we suggest that the degradation of a subset of mRNA species is an essential function of RNase E, albeit possibly not the only one.
Paper III

As described previously in this thesis, in *S. enterica* and related species, the translation elongation factor EF-Tu is encoded by the two widely separated but near-identical genes *tufA* and *tufB* (Jaskunas et al, 1975; Hughes, 1986). The expression level of *tufB* is known to be influenced by the state of the *tufA* gene, where an inactivation of *tufA* can double the expression from *tufB* (Hughes, 1990). During the 1980s, this was studied extensively by a research group in Leiden, the Netherlands. They used strains of *E. coli* that carried *tuf* genes encoding electrophoretically distinguishable versions of EF-Tu and measured the effects on EF-TuA and EF-TuB levels as response to increased or decreased *tuf* gene dosage. Since they found that translation of EF-TuB, but not EF-TuA, was sensitive to the cellular level of EF-Tu they proposed that the translation of *tufB* mRNA was regulated directly by the level of EF-Tu (van der Meide et al, 1983a; van Delft et al, 1988a; van Delft et al, 1988b). Despite much work, a mechanism for such a regulation was never worked out.

In this project, we aimed to study the regulation of *tufB* expression and describe it in terms of regulation on the transcriptional or translational level and any repressors/activators that may be involved.

Bioinformatic clues to a regulatory region upstream of *tufB*

When a strain of *S. enterica* with an inactive *tufA* gene was evolved for fitness compensation, three different non-coding or synonymous point mutations were selected around the *tufB* start codon. By computational methods, we identified the region just upstream of and in the early parts of *tufB* coding sequence to be highly conserved between *Salmonella* and other Enterobacteriaceae genera. This mRNA region was modelled to fold into a structure of stems and loops, which would cover the *tufB* start codon and the ribosomal binding site (RBS) upstream of the *tufB* coding sequence.

Three models for an EF-Tu-dependent regulation of *tufB*

Based on previous data on *tufB* regulation and our bioinformatic findings of the highly conserved stem-loop structure, we suggest three different models for how regulation of *tufB* could occur. All the models have in common that the cellular level of EF-Tu is detected by a molecular signal that is converted into a response in *tufB* translation.

Model 1: Unbound aa-tRNA as the signal

If the level of EF-Tu were too low, the level of unbound aa-tRNA would increase. This could be a signal to increase *tufB* translation. There are known examples where uncharged tRNAs regulate translation initiation by affecting
the structure of the 5’ UTR and the availability to the RBS (reviewed in Raina & Ibba, 2014). We do not know of any example where an aa-tRNA has been shown to regulate translation by interfering with mRNA structure.

Model 2: Unbound EF-Tu as the signal
A related scenario to Model 1 would be a case where an excess of free EF-Tu could act as a signal that the cellular levels of EF-Tu were too high and that translation of tufB should be down-regulated. We suggest that it is possible that EF-Tu could bind to the 5’ UTR of its own mRNA and stabilize the stem-loop structure that we have identified, thereby masking the ribosomal binding site and start codon. This sort of autoregulation is well known for other proteins, including the ribosomal protein L20 and the threonyl tRNA synthetase ThrS (Lesage et al, 1990; Springer et al, 1986). Both these proteins have a primary RNA-binding function, L20 in binding to ribosomal RNA and ThrS in binding to threonyl tRNA, and their mRNA binding sites are structural mimics of the primary binding partner (Chiaruttini et al, 1996; Guillier et al, 2002; Moine et al, 1988; Romby et al, 1990). Since EF-Tu binds aa-tRNAs in the ternary complex, it is possible that it can recognize part of the proposed tufB stem-loop structure as a tRNA mimic.

A possible interaction between in vitro transcribed tufB operon mRNA and pure EF-Tu will be addressed by a native gel-shift assay. Detection of a mobility shift in the gel would indicate that there is a direct interaction between tufB mRNA and EF-Tu and would support this model.

Model 3: Ribosome pausing as the signal
A third alternative for an EF-Tu-dependent tufB regulation is that the elongation rate of the translating ribosomes could influence the tufB mRNA structure. A closer look at the tufB operon mRNA revealed that it can be folded into two different structures, where one is hiding the RBS and one is exposing it. We suggest that these two forms are in equilibrium in vivo and that the open form allows initiation of translation. For translational regulation, we suggest a mechanism where, at high levels of EF-Tu, ribosomes would translate rapidly, which would lead to low ribosome density on the mRNA and a maintained equilibrium between the two RNA structures. At a lower level of EF-Tu the ribosomes would be more prone to pausing on the mRNA, thereby blocking the formation of an RBS-occluding structure, which would facilitate further initiation of tufB translation.

This model is currently being tested by construction of specific mutations predicted to stabilize or destabilize the stem structures of the tufB mRNA. We have developed an in vivo fluorescence-based system to monitor the translation of the chromosomal tufB locus and are using this as a reporter to measure if the changes in the RNA structure have any impact on the tufB translation level. To date, we have introduced 18 synonymous mutations in the early part of tufB and assayed their effect on translation by the YFP flu-
sion. We noted that all mutations that were predicted to destabilize either the closed structure or both the closed and open structure increased \textit{tufB} translation significantly. Five of the six mutations that were modeled to destabilize the open structure resulted in decreased \textit{tufB} translation.

Model number three also implies that mRNA sites prone to induce ribosome stalling, \textit{e.g.} by rare codons, could sensitize this regulatory system. We suggest that this could be the case for \textit{tufB} regulation. \textit{tufB} is one of the most highly translated genes in \textit{S. enterica} and is very biased in codon usage (Karlin et al, 1998). The gene has 30 threonine codons and all but three of them are either of the common ACC or ACT codons. The three rare Thr codons, codon 9 (ACA) and codons 26 and 27 (ACA, ACG), are all very early in the gene. There are also other relatively rare codons in this region that could conceivably act to cause ribosome pausing. As codon usage and tRNA abundance co-varies, the probability for a ribosome to detect a decreasing concentration of EF-Tu would be highest on a rare codon. We suggest that translation of the first rare Thr codon could deplete the pool of rare Thr-tRNAs and that the pair of rare Thr codons at positions 26 / 27 consecutively acts as a sensor of EF-Tu availability and functions as a potential ribosomal pause site. Interestingly, we have showed that altering the rare Thr codons to more common ones decreases translation of \textit{tufB} significantly.

The data from the computational modeling of the two \textit{tufB} mRNA structures, the measurements of \textit{tufB} translation in strains with altered RNA structures and the impact of the rare Thr codons all fit into Model 3 for an EF-Tu-dependent translational regulation of \textit{tufB}. In the near future, we are also planning to perform ribosomal footprinting studies of the wild-type and a set of mutants to examine the \textit{tufB} gene for ribosomal pause sites.
Paper IV

It has been known for more than 20 years that mutant subpopulations of bacteria can continue to grow even during stationary and long-term stationary phase (Zambrano et al., 1993). Accumulating mutants have often been found to be mutated in the rpoS gene, encoding the stationary phase sigma factor RpoS (Zambrano et al., 1993; Notley-McRobb et al., 2002; Saint-Ruf et al., 2004). Also mutations in rpoB, encoding the RNA polymerase β-subunit, have been shown to accumulate in aging liquid cultures in and colonies on solid medium (Taddei et al., 1995; Bjedov et al., 2003; Wrande et al., 2008). This has been shown to be due to an ability of some pre-existing mutants to continue growing when the background wild-type population goes into stationary phase (Wrande et al., 2008).

Here, we aimed to see if there is a specific nutrient that is important for the continued growth of mutant subpopulations with altered RNAP genes.

Proteomic analysis of aging colonies

To examine this, we constructed an rpoB P564L mutant and a deletion mutant of rpoS, ΔrpoS. Both of these were confirmed to have a growth advantage as subpopulations on aging colonies of wild-type S. enterica, compared to a labelled wild-type added as a subpopulation on a wild-type background colony.

A mass spectrometry screening of the full proteomes of the wild-type and the rpoB and rpoS mutants revealed that genes for carbon scavenging and utilization were up-regulated in 7 day-old colonies, compared to 1 day-old colonies. This suggested that an increased and possibly altered carbon metabolism was important to support the growth in aging colonies. This is interesting, since bacteria entering stationary phase are known to undergo a metabolic switch where acetate is excreted during exponential growth on glucose and then re-assimilated intracellularly and used as a carbon source later in the growth cycle (Wolfe, 2005).

To analyze this in more detail, a quantitative mass spectrometry analysis was done on a subset of the proteins, known to be important for acetate uptake and utilization, from 1, 3, 5 and 7 day-old colonies. We found that the levels of acetyl-CoA synthase, Acs, and the glyoxylate shunt enzymes AceA and AceB were more up-regulated in the rpoB and rpoS mutants than in the wild-type.

Genetic and biochemical evidence that acetate utilization is key to continued aging

As a genetic test of this, we constructed double mutant strains that, in addition to rpoB P564L or ΔrpoS, also carried a Δacs mutation. When these
strains were aged on wild-type background colonies they were found to have a decreased advantage compared to the single mutant rpoB or rpoS parental strains. In similar studies where the rpoB or rpoS mutations were combined with either an aceBAK deletion or a deletion of the acetyl transferase gene pka, no difference could be detected. We concluded that conversion of acetate to acetyl-CoA, but neither the glyoxylate shunt nor protein acetylation were critical for the continued growth of the rpoB P564L and ΔrpoS mutants.

To measure the acetate accumulation in the cells directly, we used a biochemical assay where cells from 1 day- and 7 day-old colonies were exposed to 14C-labelled acetate for one minute. Every 10 seconds, an aliquot was taken and chased in a solution with excess non-radioactive acetate. When the accumulation rates were compared, we noticed that it decreased from day 1 to day 7 for the wild-type cells, remained constant for the rpoB P564L mutant and increased almost five-fold for the ΔrpoS mutant.

As a final test of the model that acetate uptake and utilization is important for the continued growth of the rpoB and rpoS subpopulations on aging wild-type colonies, an experiment was set up where mutant or wild-type subpopulations were added to Δacs background colonies. We reasoned that these background colonies should be unable to re-assimilate the available acetate, thereby leaving more to the subpopulations that were wild-type for acs. The predictions proved to be true, as the growth of all three subpopulations was increased when grown on a Δacs background, compared to when aged on a wild-type background.

Acetate utilization and the limitations of the wild-type

By these genetic and biochemical experiments, we conclude that availability of acetate and the possibility to use it is key to the continued growth of the rpoB and rpoS mutant subpopulations on an aging colony. The experiment in which we showed that also wild-type cells grow better on a Δacs colony than on a wild-type background colony emphasizes the importance of carbon limitation for bacterial cells to enter stationary phase.

Interestingly, neither the activity of the glyoxylate shunt nor the Pka-dependent protein acetylation seems to be critical for the growth advantage of rpoB and rpoS mutants on aging colonies. Acetyl-CoA can also be used for fatty acid synthesis, which raises the possibility that this could be a growth advantage-determining pathway, potentially by a continued ability to synthesize the phospholipid cell membrane.
Concluding remarks

The aim of this thesis work has been to study growth and growth regulation at various levels in the bacterium *S. enterica*. Since most free-living bacteria will encounter different environments with various conditions for nutrient and oxygen availability, temperature and pH, it is critical to be able to rapidly adjust to new growth conditions in order to be evolutionary successful. Using wild-type cells and various mutants, I have studied these processes, their interconnections and impact on cellular metabolism.

An extremely slow-growing mutant, depending on a Glu125Arg mutant version of EF-Tu, was shown to be trapped in a futile stringent response. The perceived starvation was demonstrated to be due to down-regulation of aminoacyl tRNA synthetase genes leading to lower prolyl-tRNA levels. This reflects the uncoupling of transcription and translation occurring in this mutant together with the importance of a balanced turnover of RNA.

In the study of two ts mutants of RNase E, we selected and mapped three different classes of extragenic suppressors of the ts phenotype. Since all classes specifically suppress the defect in mRNA degradation found in the ts RNase E mutants, we suggest that the degradation of at least a subset of cellular mRNAs is an essential function of RNase E. One of the suppressor classes confers up-regulation of the RelE toxin function, which is very interesting, as RelE is known to play an important part in an extended stringent response by mRNA cleavage, thus contributing to a rapid response to a changed environment.

Previous studies have suggested that the level of EF-Tu protein can affect the translation of the EF-Tu-encoding *tufB* gene. We are examining this and have suggested three different models for how such a regulation could occur. Regulation of translation initiation also connects to RNA turnover, as many mRNAs have a 5’ UTR that is the binding site for ribosomal protein S1 as well as for RNase E. The outcome of the competition between these proteins will determine whether the mRNA is translated or degraded and, over time, how highly translated the mRNA will be.

Examination of the growth advantage phenotype exhibited by two RNA polymerase mutants, *rpoB* P564L and Δ*rpoS*, when grown as subpopulations on aging wild-type colonies, revealed that this phenotype depended on the utilization of acetate. Increased growth of wild-type cells aged as a subpopulation on a colony unable to re-assimilate acetate demonstrates that in aging colonies, acetate is available in levels enough to sustain growth of at least a small subpopulation. The ability to use this acetate depends on expression of the protein Acs, converting acetate to acetyl-CoA. The up-regulation of Acs in the growth advantage mutants illustrates the importance of RNAP status and sigma factor utilization, as a mutation in the RNAP β-subunit and deletion of the stationary phase sigma factor RpoS could confer a similar change in transcription pattern. Bacteria isolated from natural sources often display a
genetic polymorphism in the \textit{rpoS} gene, highlighting the need for a dynamic regulation of gene expression.

As discussed throughout this thesis, bacteria constantly need to adapt their physiology to changes in the surrounding environment. This is largely determined by the set of proteins that is being produced during growth (regulated at all levels of the central genetic dogma, DNA–RNA–protein) and fine-tuning of protein function (by post-translational modifications). Taken together, the work of this thesis illustrates connections between the different levels of gene expression regulation and highlights the importance of this for an efficient growth regulation.

Tillverkning av protein sker i flera steg, vilket illustreras i Figur 6. All information en cell behöver ligger lagrad i dess arvsmassa i form av en dubbelsträngad molekyl, DNA, som bildar en kromosom. En bit DNA som innehåller information om ett protein kallas för en gen. För att informationen i DNA ska kunna användas för att bygga proteiner behöver den kopieras över till RNA, en enkelsträngad molekyl som liknar DNA och fungerar som cellens ”arbetskopia” av den genetiska informationen i DNA. RNA läses i sin tur av stora molekylkomplex som kallas ribosomer.

RNA kan ha olika funktioner i cellen. Det RNA som används som arbetskopia för att översättas till protein kallas meddelar-RNA, mRNA. En annan sorts RNA fungerar som adapter och hjälper ribosomen att läsa av mRNA:t genom att leverera en aminosyra som matchar en kort sekvens på mRNA:t. En sådan molekyl kallas transport-RNA, tRNA. En tredje sorts RNA bygger upp ribosomerna, tillsammans med en mängd olika proteiner. Detta RNA kallas ribosomalt RNA, rRNA.

Alla dessa steg mellan DNA och färdigt protein måste regleras för att bakterien ska fungera så bra och effektivt som möjligt. I min avhandling har jag undersökt några av dessa regleringsmekanismer. I Artikel I och IV studerade vi kopieringen av DNA till mRNA, nedbrytning av RNA var huvudämnet för Artikel II, och i Artikel I och III undersökte vi regleringen av översättningen från mRNA till protein. I Artikel IV studerade vi även hur bakteriers förmåga att använda näring påverkas av vilka proteiner som tillverkas.

I arbetet med min avhandling har jag arbetat med Salmonella enterica, en bakterie som kan orsaka magsjuka och andra infektioner som kan påverka hela kroppen. Orsaken till att vi studerar just Salmonella är att denna bakterie har en kort generationstid, är enkel att förändra genetiskt och växer bra vid 37°C, antingen i flytande näringsmedium eller som kolonier (grupper av
Figur 6. Schematisk översikt av den centrala genetiska dogmen: Lagring av genetisk information i DNA, avskrivning till ”arbetskopian” mRNA och översättning av mRNA till protein i ribosomerna.

bakterier som härstammar från samma cell) på fast näringsmedium. Dessutom finns det varianter som inte kan infektera människor.

Jag har studerat olika mutanter, bakterier som är förändrade i olika gener, och jämfört dessa med en vildtyp av Salmonella. Genom att se hur en mutant skiljer sig från den oförändrade vildtypen kan vi dra slutsatser om vad den muterade genen har för funktion och lära oss mer om hur bakterier fungerar.

Artikel I: En långsamtväxande mutant har fastnat i svältrespons

En vildtypvariant av Salmonella har en generationstid på 20 minuter om den får god tillgång till näring. Sedan tidigare har en mutant Salmonella-stam med en generationstid på 80 minuter (fyra gånger långsammare än normalt) isolerats. När detta projekt började visste vi redan att den här mutanten hade problem med översättningen från mRNA till protein eftersom den hade en förändring i proteinet EF-Tu, som levererar aminosyra-laddade
tRNA:n till ribosomerna. Det var också känt att detta förmodligen leder till att ribosomerna pauser på mRNA-molekylen och att RNA då kan brytas ner. Vi ville studera ifall den extremt långsamma tillväxten hos denna mutant kunde kompenseras för genom överproduktion av något protein. Om det var möjligt skulle detta kunna ge fler ledtrådar till vad som händer i dessa celler och varför de växer så långsamt.

Vi upptäckte att förmodligen leder till att ribosomerna pausar på mRNA-molekylen och att RNA då kan brytas ner.

Vi vill studera ifall den extremt långsamma tillväxten hos denna mutant kunde kompenseras för genom överproduktion av något protein. Om det var möjligt skulle detta kunna ge fler ledtrådar till vad som händer i dessa celler och varför de växer så långsamt.


Men varför har EF-Tu-mutanten en svältrespons trots att den inte svälter? Eftersom det var känt att denna mutant hade ökad nedbrytning av vissa mRNA, med minskad översättning till protein som följd, undersökte vi om några av de proteiner som ansvarar för att ladda tRNA-molekyler med aminosyror var påverkade. Vi fann att fyra sådana protein fanns i lägre koncentration i den långsamma mutanten än i vildtypen. Detta resulterade i att de tRNA:n som kan bära aminosyran prolin var laddade med prolin i lägre grad i EF-Tu-mutanten än i vildtypen.


Artikel II: Nedbrytning av mRNA gör RNas E livsnödvändigt

För att en bakteriecell ska fungera så bra som möjligt och kunna svara på förändringar i miljön behöver den reglera stabiliteten hos olika mRNA:n, modifiera tRNA och rRNA för ökad funktion och bryta ned dem när de inte behövs. De protein som bryter ner RNA kallas ribonukleaser, RNaser.

RNas E är ett viktigt ribonukleas som kan bryta ner mRNA samt klipta i tRNA- och rRNA-molekyler för att de ska få sin aktiva form. Utan RNas E
kan celler av *Salmonella* och närbesläktade arter inte leva, vilket gör att RNas E kallas för ett *essentiellt*, livsnödvändigt, protein. Flera forskargruppar har försökt ta reda på vilken funktion hos RNas E som gör proteinet essentiellt, men frågan är fortfarande öppen.

För att undersöka detta använde vi en hittills oprövad metod. Vi använde två RNas E-mutanter där vi kan styra hur stor effekt mutationen får genom ändra temperaturen eftersom åtminstone någon essentiell funktion hos det mutanta RNas E inte fungerar vid högre temperaturer. För att kunna växa vid den högre temperaturen måste dessa RNas E-mutanta bakterier ha ytterligare en mutation, någonstans på kromosomen, som kan kompensera för den funktion som saknas i det mutanta RNas E.

På detta sätt isolerade vi 15 olika kompensatoriska mutationer som totalt föll inom tre klasser. En av kompensations-mutationerna var en förändring av ett protein i ribosomen som är viktigt för att påbörja översättningen av mRNA till protein. Fyra av de kompensatoriska mutationerna var olika små förändringar i ett annat ribonukleas, RNas R, som är känt för att kunna bryta ner de flesta sorters RNA. Den sista klassen var 10 olika förändringar som alla aktiverar proteinet RelE, vars enda kända funktion är att klippa i mRNA.

Vi mätte hur mycket klippting och nedbrytning av tRNA, tRNA och mRNA som sker i vildtypsceller, RNas E-mutanter och i celler som har både en RNas E-mutation och en kompensatorisk mutation. Vi såg att allt detta fungerade sämre i RNas E-mutanten än i vildtypen och att enbart nedbrytning av vissa mRNA:n kunde återställas med en kompensatorisk mutation.

Baserat på dessa observationer har vi ställt upp en modell där vi föreslår att mutationen i det ribosomala proteinet kompenserar för ett mutant RNas E genom att återställa balansen mellan nedbrytning av mRNA och översättning till protein. De båda RNA-nedbrytande proteiner som också isolerades ökar troligen nedbrytningen av mRNA i cellerna där RNas E inte fungerar. Vi föreslår därför att nedbrytning av mRNA är en livsnödvändig funktion hos RNas E, även om det är möjligt att det finns fler essentiella funktioner.

**Artikel III: Reglering av uttrycket av EF-Tu**

I Artikel I studerade vi effekterna av en mutation i proteinet EF-Tu. I vildtypsceller finns den genetiska informationen om detta protein på två ställen på DNA-kromosomen; i generna *tufA* och *tufB*. När *Salmonella* eller närbesläktade arter av bakterier växer under optimala förhållanden kommer det mesta av EF-Tu från *tufA*. Det är också känt att inaktivering av *tufA* kan dubbla mängden EF-Tu som uttrycks från *tufB*.

Detta är intressant eftersom det betyder att en minskad mängd EF-Tu-protein ökar uttrycket från *tufB*-genen. Uttryck av en gen kan regleras på flera nivåer och trots mycket forskning är det ännu inte helt klarlagt hur en EF-Tu-beroende kontroll av *tufB*-genuttrycket sker.


Den andra modellen för reglering av tufB-översättning liknar modell ett, men här föreslår vi i stället att EF-Tu-proteiner kan binda till tufB-mRNA-strukturen och stabilisera den. Om nivån av EF-Tu sjunker skulle detta leda till att den stabila RNA-strukturen som döljer RBS skulle destabiliseras och sannolikheten för en ribosom att börja översätta tufB till EF-Tu att öka.

För modell nummer tre föreslår vi att mängden EF-Tu känns av genom ribosomernas översättningshastighet. Vid höga koncentrationer av EF-Tu är sannolikheten hög att en ribosom kommer åt aminosyra-tRNA genom bärarmolekylen EF-Tu, och översättningen kommer att gå snabbt. Vid lägre nivåer av EF-Tu kommer ribosomerna att behöva vänta längre på aminosyra-tRNA buret av EF-Tu, vilket saktar ned översättningen. Vi föreslår att när att ribosomerna tar längre tid på sig för att översätta tufB-mRNA blockerar de en bit av det mRNA som kan interagera med sig själv, vilket förhindrar uppkomsten av en stabil RNA-struktur som döljer RBS. På så vis ökar sannolikheten för att fler ribosomer kommer åt att påbörja översättningen av tufB.

Projektet som Artikel III bygger på pågår fortfarande. Vi testar nu de upptäckta modellerna med genetiska och biokemiska metoder.

Artikel IV: Mutanter som växer i gamla kolonier är bättre på att använda acetat

När en koloni med Salmonella åldras på fast näringsmedium kommer de flesta cellerna att växa och dela sig i ungefär ett dygn. Efter detta kommer bakterierna att gå in i en stationär fas, där ingen nettoförändring av mängden bakterier sker. Vissa grupper av celler kommer att dö under den stationära
fasen och vissa kommer att fortsätta att växa. Det har tidigare visats att de bakterier som kan fortsätta att växa har mutationer som gör dem mer anpassade för miljön i en gammal koloni. Två sådana mutanter som tidigare isolerats har varsin förändring i RNA-polymeraset (RNAP), det proteinkomplex som skriver av DNA till RNA. Vi ville ta reda på om dessa mutanter var beroende av någon näringskälla för sin tillväxt i gamla kolonier.

För att undersöka detta läste vi av hela proteininnehållet i unga och gamla kolonier av vildtypsbakterier och de två RNAP-mutanterna. Vi fann att alla gamla kolonier hade ökat mängden protein för att bryta ner olika kolkällor. Mer specifikt fann vi att RNAP-mutanterna, men inte vildtypen, hade ett ökat uttryck av de proteiner som utför det första steget i den kemiska processen för att använda den lilla kolbaserade molekylen acetat i cellen.

Bakterier som växer snabbt på rika näringskällor, till exempel sockerarten glukos, utsöndrar acetat för att återbilda molekyler som behövs för metabolismen av socker. När glukosen börjar ta slut kommer cellerna i stället att samla in den tidigare utsöndrade acetaten och använda den som näringskälla tills populationen går in i stationärfas.


Allt detta tyder på att det fria acetat som finns kvar i en koloni där majoriteten av cellerna är i stationärfas räcker för att understödja en fortsatt tillväxt för en liten grupp bakterier. För att en sådan grupp med celler ska ha möjlighet att använda det acetat som finns behöver dessa celler uttrycka de proteiner som behövs för att kunna använda acetat. Vi föreslår också att de två studerade mutationerna i RNA-polymeraset ger ett förändrat mönster in vilka gener som skrivs om till mRNA och därmed vilka protein som uttrycks.

Sammanfattningsvis har jag under arbetet med denna avhandling studerat olika mekanismer för hur bakterier kan anpassa sig till förändringar i sin omgivning och vad som händer om denna anpassning inte fungerar. Anpassning till miljön sker ofta genom reglering av vilka gener som ska skrivas av och översättas till protein. Denna reglering kan ske på olika nivåer; i kopieringen av DNA till RNA (Artikel I och IV), i stabilitet hos RNA-molekyler (Artikel II), och i effektivitet av översättningen till protein (Artikel I och III). Allt detta leder till ett sammanlagt metaboliskt svar på miljön (Artikel IV).
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