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The Physiological Cost of Antibiotic Resistance

BY

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Abstract
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Becoming antibiotic resistant is often associated with fitness costs for the resistant bacteria. This is seen as a loss of competitiveness against the antibiotic-sensitive wild-type in an antibiotic-free environment. In this study, the physiological alterations associated with fitness cost of antibiotic resistance *in vitro* (in the laboratory medium), and *in vivo* (in a mouse infection model), are identified in the model system of fusidic acid resistant (Fus^R) *Salmonella enterica* serovar Typhimurium.

Fus^R mutants have mutations in *fusA*, the gene that encodes translation elongation factor G (EF-G). Fus^R EF-G has a slow rate of regeneration of active EF-G·GTP off the ribosome, resulting in a slow rate of protein synthesis. The low fitness of Fus^R mutants *in vitro*, and *in vivo*, can be explained in part by a slow rate of protein synthesis and resulting slow growth. However, some Fus^R mutants with normal rates of protein synthesis still suffer from reduced fitness *in vivo*. We observed that Fus^R mutants have perturbed levels of the global regulatory molecule ppGpp. One consequence of this is an inefficient induction of RpoS, a regulator of general stress reponse and an important virulence factor for *Salmonella*. In addition, we found that Fus^R mutants have reduced amounts of heme, a co-factor of catalases and cytochromes. As a consequence of the heme defect, Fus^R mutants have a reduced ability to withstand oxidative stress and a low rate of aerobic respiration.

The pleiotropic phenotypes of Fus^R mutants suggest that antibiotic resistance can be associated with broad changes in bacterial physiology. Knowledge of physiological alterations that reduce the fitness of antibiotic-resistant mutants can be useful in identifying novel targets for antimicrobial agents. Drugs that alter the levels of global transcriptional regulators such as ppGpp or RpoS deserve attention as potential antimicrobial agents. Finally, the observation that Fus^R mutants have increased sensitivity to several unrelated classes of antibiotics suggests that the identification of physiological cost of resistance can help in optimizing treatment of resistant bacterial populations.

Keywords: fusidic acid, fitness cost, protein synthesis, EF-G, ppGpp, RpoS, oxidative stress, heme

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Posvećeno

Umetnosti istraživanja mogućnosti,

Ljudima koje volim, koji veruju da se može i nemoguće

i jednom Nemogućem Gradu u kojem je sve moguće.

S ljubavlju

List of Papers

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

I. Macvanin, M., Johanson, U., Ehrenberg, M., Hughes, D. (2000) Fusidic acid-resistant EF-G perturbs the accumulation of ppGpp. *Molecular Microbiology*, 37(1): 98-107

II. Macvanin, M., Björkman, J., Eriksson, S., Rhen, M., Andersson, D. I., Hughes, D. (2003) Fusidic acid-resistant mutants of *Salmonella enterica* serovar Typhimurium with low fitness in vivo are defective in RpoS induction. *Antimicrobial Agents and Chemotherapy*, 47(12)

III. Macvanin, M., Ballagi, A., Hughes, D. Reduced levels of heme and respiration rate in elongation factor G mutants of *Salmonella enterica* serovar Typhimurium. (manuscript)

IV. Macvanin, M., Hughes, D. Hypersensitivity of fusidic acid-resistant mutants of *Salmonella enterica* serovar Typhimurium to several classes of antibiotics. (manuscript)

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1. INTRODUCTION

1. 1. BACTERIAL GROWTH

The study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of Microbiology. Jacque Monod, 1949

For me, encountering the bacterial growth curve was a transforming experience. As my partner and I took samples of the culture at intervals to measure optical density and plotted the results on semilogarithmic paper, we saw, after the lag period, a straight line developing…beautiful in precision and remarkable in speed. As the line extended, itself straight-edge true, I imagined what was happening in the flask - living protoplasm being made from glucose and salts as the initial cells grew and divided. The liquid in the flask progressed from having a barely discernible haze to a milky whiteness thick with the stuff of life, all within a very brief Boston winter afternoon. Frederick C. Neidhardt, 1999.

1. 1. 1. General characteristics of bacterial growth

At the beginning of 20th century, many microbiologists shared the suspicion that growth of bacterial cultures is not a random and uncontrolled phenomenon but rather, it obeys certain rules that remained yet to be discovered (Ducleaux, 1898-1901; Henrici, 1928). Experimental support to that belief came during 1940s, when Monod executed the series of experiments in which the actual rate of growth and the yield of bacterial cells cultured in media with limiting concentrations of a single carbon source was measured (Monod, 1942). He found that both growth rate and yield depended upon the substance which served as a carbon source. This finding led Monod to claim that "*the growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws, which make it possible to define certain quantitative characteristics of the growth cycle…The accuracy, the ease, the reproducibility of bacterial growth constant determinations is remarkable and probably unparalleled, so far as quantitative biological characteristics are concerned.*" (reviewed in Neidhardt, 1999). Further support of Monod's convictions regarding bacterial growth came from the seminal experiment of Schaechter, Maaløe, and Kjeldgaard in 1958, who found a systematic change in size and composition of cells of *Salmonella typhimurium* when the growth rate was varied by nutrition. Monod's experiments and findings of Schaechter, Maaløe and Kjeldgaard from half a century ago established the foundation of our present, improved and still improving understanding of bacterial growth which can be briefly summarized thorough the following statements:

(i) Size and overall chemical composition of cells are coordinated with the growth rate

Cell size and cellular content of proteins, DNA, RNA, carbohydrates and lipids are independent of the chemical nature of the medium and correlate positively with the growth rate. Except for the very lowest of growth rates, the faster the growth rate, the larger the cells, the richer they are in ribosomes

and tRNA and the greater their level of transcription and translation factors, including aminoacyl tRNA-synthetases (Neidhardt, 1963; Schaechter, Maaløe and Kjeldgaard, 1958; Neidhardt, 1999).

(ii) The rates of chain elongation of proteins, DNA and RNA vary little over a range of growth rates at a given temperature

Cellular levels of enzymes involved in replication, transcription and translation vary directly with the growth rate but the rates of their function are constant over a range of growth rates. This implies that cell's enzymatic machinery for replication, transcription and translation usually operate at near saturation level (Maaløe and Kjeldgaard, 1966; Pedersen, 1985). Bacterial cells do not generally vary the rate of protein synthesis by changing how fast ribosomes work but rather by adjusting their number. During the conditions that support slow growth rates, by reducing the number of ribosomes, bacteria reduce the rate of production of translational machinery. The advantage of this cellular strategy becomes evident if it is considered that, during fast growth, protein-synthesizing machinery comprises half or more of the cell's mass. However, there is a limit in reduction of number of ribosomes; at low growth rates, the cells maintain an irreducible, minimal amount of ribosomes, some of which are non-functioning and will spring into action upon enrichment of the environment (reviewed in Neidhardt, 1999).

(iii) Pattern of macromolecule synthesis upon change in environmental conditions that force a reduction or permit an increase in growth rate is consistent.

On enrichment, initially there is increase in synthesis of RNA, then protein, and finally DNA. Frequency of cell division is the last parameter to increase. Cells quickly adopt the size and macromolecular composition characteristic of a rapidly growing cell. Upon nutrient restriction, the order of decrease in rates of the synthesis follows the same pattern. Net accumulation of RNA ceases almost instantly, followed by declining rates of protein and DNA synthesis and of cell division. The cells respond in such a way as to assume the phenotype of a small, slow-growing cell as rapidly as possible (Neidhardt, 1999).

(iv) Interplay between macromolecular synthesis machinery and energy-supplying reactions sets the growth rate

For a given strain grown aerobically at a particular temperature, the specific growth rate and yield depend upon the carbon source. It is generally assumed that the yield of ATP controls the growth yield and that the rate of ATP production determines the growth rate. The origin of this assumption can be found in the report that there is a constant yield of 10.5g of cells of lactic acid bacteria per mol of ATP (Bauchop and Elsden, 1960). Further support that rate of ATP production sets the growth rate came from experiments in which growth of *E.coli* in batch cultures, in minimal media supplemented with various carbon sources which supported broad range of growth rates, was monitored. As expected, it was observed that the yield was positively correlated with growth rate. The calculated ATP requirements, together with the measured growth rates and growth yields on the different carbon sources were used to calculate the rate of ATP synthesis by oxidative phosphorylation. This rate was closely related to the respiration rate which suggests that aerobic growth of *E.coli* is limited by the rate of respiration and the concomitant rate of ATP generation through oxidative phosphorylation.

(v) Environmental changes are met with alterations in gene expression which result in cellular enzyme profiles unique to each particular environment

When growing at constant temperature in excess of nutrients, bacterial cells synthesize all of their protoplasmic constituents at near-constant differential rates and divide at a particular cell mass. Growth under constant conditions results in each cellular component increasing by the same proportion in each interval of time. This state, called balanced growth (Campbell, 1957), is often maintained only transitorily because of rapid utilization of nutrients, accumulation of metabolic by-products, and increasingly inadequate rates of gas exchange, all of which induce changes in the expression of many genes (reviewed in Neidhardt, 1999). In most cases, detrimental changes in the environment are met with changes in gene expression that result in enzymic makeup that permit continued growth, if only at reduced rate. This can be regarded as an adaptive pattern which is responsible for the outstanding ability of bacteria to grow under a variety of ambient conditions (Neidhardt, 1999).

(vi) During growth, specific molecular mechanisms coordinate the expression of large sets of genes.

One of the most prominent mechanisms that globally coordinate the gene expression is the stringent response ["the granddady of global control systems" (Neidhardt, 1999)]. Its central place in adjusting transitions from fast to slow growth originates from its ability to restrict the synthesis of the entire translation machinery and other major cellular components during periods of limiting availability of charged tRNA or constrained energy supply. Another important mechanism of far-reaching gene regulation is mediated by RpoS. This mechanism operates under conditions of nutrient exhaustion or various environmental stresses, with aim to switch gene expression to "protective mode" which will provide cells with means to persist under unfavourable conditions.

1. 1. 2. Growth *in vivo*

In the previous section, overview of general factors which influence bacterial growth was given. These factors were identified by studying bacterial growth under defined, *in vitro,* conditions. However, natural niches where bacteria reside do not usually provide defined and controlled growth conditions which can be maintained in laboratory setting. *Salmonella enterica* serovar *Typhimurium,* the model organism used for the studies presented in this thesis, live in vertebrate hosts as pathogen or commensal but also survive in nutrient-deficient soils and water (Matin, 1991). In both habitats it leads a "feast-and-famine existence" (Koch, 1971), mainly famine punctuated by rare periods of nutritional surplus. In this section, growth of *Salmonella enterica* serovar *Typhimurium* in infected host will be discussed.

The genus *Salmonella* consists of a single species-*Salmonella enterica*, comprising over 2000 serovars that differ in host specificity and disease symptoms. The human-adapted *S.enterica* serovar *Typhi* is responsible for typhoid fever, a systemic disease prevalent in developing countries. In contrast, *S.enterica* serovar *Typhimurium* (*Salmonella typhimurium* in the following text) causes gastroenteritis (i.e. food poisoning) in humans, but typhoid-like disease in susceptible strains of mice. The infection that *S. typhimurium* provokes in mice has many of the hallmarks of typhoid fever and has been extensively used as a model system for this human disease.

Salmonella is facultative intracellular pathogen which has the special ability to survive and replicate inside host phagocytic cells (Jones and Falkow, 1996). During infection, *Salmonella* follows a complex lifestyle full of challenges in which it faces multiple environments, including different cell types as well as extracellular fluids. It is typically acquired by the consumption of contaminated water or food and must survive the acid pH in the stomach before progressing to the small intestine. To gain access to host tissues, *Salmonella* must adhere to and enter the intestinal epithelium. Highly specialized M-cells in the lining of the gastro-intestinal tract serve as the vehicle of *Salmonella* entrance into the host tissues. *Salmonella* then must reach the gut-associated lymphoid tissue, where it invades the resident tissue macrophages. It is thought that these macrophages act as "Trojan horses" and mediate the spread of *Salmonella* to other organs such as liver and spleen (reviewed in Janssen *et al*., 2003). Additionally, serovars of *Salmonella* that cause systemic disease must survive in blood before being phagocytosed by the macrophages of liver and spleen. Phagocytosis, which constitutes the initial step for degradation of dying cells, inert particles and live infectious agents by animal cells, occurs in "professional" phagocytes (macrophages and neutrophils) but also, to a lesser extent, in non-professional phagocytes such as fibroblasts, endothelial and epithelial cells.

When *Salmonella* finally finds itself inside a host cell, it resides within membrane bound vacuole, phagosome, (also referred to as "Salmonella containing vacuole", SCV) that is poor in nutrients and rich in antimicrobial compounds. *Salmonella* will remain enclosed within phagosome for the duration of its intracellular life. This is in contrast to some facultative intracellular pathogens, such as the

Gram-positive bacterium *Listeria monocytogenes*, that lyse the phagosomal membrane and escape into the cytoplasm (Sheehan et al, 1994), which is permissive environment for bacterial growth. In order to survive within the phagosome, *Salmonella* species have developed multiple strategies to evade defensive, microbicidal action of the host cells and acquire nutrients needed for growth. Moreover, it is believed that *Salmonella* directs it own entry into host cells by entailing a sorting mechanism at the host cell surface that excludes some cytoskeleton-associated host proteins from entering the SCV or causes rapid recycling of host cell surface components back to the plasma membrane. This is achieved by "trigger mechanism" of invasion which occurs with both professional and non-professional phagocytes. The strategy *Salmonella* employs is to manipulate the host cell cytoskeleton by delivering virulence factors directly into the host cytosol, triggering host-cell signalling pathways that lead to localized membrane ruffling, macropinocytosis and bacterial uptake. *Salmonella* use type-III secretion systems, needle-like complexes spanning bacterial inner and outer membranes (Kubori *et al*., 1998), to target effectors into host cells. Cytoskeletal rearrangements and bacterial uptake are promoted by effector proteins such as SipA and SipC (Zhou *et al*., 1999; Hayward and Koronakis,1999; Hardt *et al*., 1998) which are encoded by chromosomal genes of the *Salmonella* type-III secretion system (TTSS), clustered in *Salmonella* pathogenicity island-1 (SPI-1) (Hueck, 1998). A second TTSS encoded by *Salmonella* pathogenicity island-2 (SPI-2) is only expressed after uptake of *Salmonella* by host cells, appears to function intracellularly and is required for survival of *Salmonella* within the host cells (Shea *et al*., 1996; Hensel *et al*., 1998).

Once the membrane-bound vacuole (the nascent phagosome) is formed, its composition and pH change over time as it undergoes a maturation process into a hydrolase-rich phagolysosome. It is reported that acidification of the SCV to pH 4.5 is usually complete within 30 min of *Salmonella* entry into macrophages (Rathman, Sjaastad and Falkow, 1996). Vacuolar acidification appears to be required for *Salmonella* replication (Rathman, Sjaastad and Falkow, 1996), probably because secretion of SPI-2 encoded proteins require acidic environment (Beuzon *et al*., 1999). It is established that SpiC protein, encoded by SPI-2, is exported through the Spi/Ssa secretion system into the host-cell cytosol and inhibits phagosome-lysosome fusion (Uchiya *et al*., 1999). Also, it was shown that at the onset of bacterial replication (3-6 h after uptake, depending on bacterial strain and host cell type), *Salmonella* manipulates the host cell cytoskeleton for the second time. This second round of actin polymerisation occurs in a variety of host cell types including macrophages, and requires the SPI-2 TTSS (Méresse *et al*., 2001). Polymerisation of actin appears to take place on the SCV membrane itself, and leads to the formation of a meshwork or coat of F-actin around the bacterial microcolony (Méresse *et al*., 2001). Actin meshwork formation was implicated to be the mechanism of maintenance of vacuolar membrane integrity (Holden, 2002).

The SPI-2 TTSS also has an important role in avoiding microbicidal host action by preventing trafficking of the macrophage NADPH-oxidase to the SCV. NADPH-oxidase is composed of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$, and four cytosolic components, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and RacGTPase. The active NADPH-oxidase is formed after recruitment and assembly of these components, resulting in the formation of cytochrome *b558* that accepts electrons from NADPH and donates them to molecular oxygen (reviewed in Babior, 1999). Upon stimulation of the phagocyte

with opsonized microorganisms or any other activating agent, the oxygen consumption increases dramatically ("respiratory burst") and a large amount of superoxide is produced. Superoxide is believed not to pass over membranes, but it can diffuse through anion selective pores and will in this manner reach the periplasmic space of Gram-negative bacteria like *Salmonella*. Spontaneous or enzymatic dismutation of superoxide results in the generation of hydrogen peroxide, which is more reactive than superoxide and unlike this compound, can diffuse readily across cell membranes (reviewed in Janssen *et al*., 2003). Hydrogen peroxide can cause damage to membranes, enzymes and DNA directly and it is considered that to be one of the main determinants for killing of *Salmonella* by macrophages (Vasquez-Torres *et al*., 2000a). Interestingly, when macrophages are infected with wild-type *Salmonella*, there is hardly any superoxide or hydrogen peroxide production in the vicinity of internalized bacteria, indicating that *Salmonella* can prevent production of reactive oxidative species (ROS) by phagocytes (reviewed in Vasquez-Torres and Fang, 2001). The evidence that mechanism is dependent on SPI-2 TTSS comes from the finding that the SPI-2 mutants are unable to inhibit superoxide production by phagocytes (Vasquez-Torres, 2000b). The virulence of SPI-2 mutants is highly attenuated in wild-type mice and is restored to a considerable degree in $gp91^{phox}$ knock-out animals, which lack NADPH oxidase activity (Vazquez-Torres *et al*., 2000). It was reported that the translocation of $gp91^{phox}$ and $gp47^{phox}$ subunits of the NADPH-oxidase complex to SCV is inhibited in phagosomes containing wild-type *Salmonella*. Components of the NADPH oxidase complex appear to be excluded from the SCV membrane in an SPI-2 dependent manner (Vazquez-Torres, 2000; Gallois *et al.*, 2001).

SPI-2 TTSS seems to be important for survival of *Salmonella* in nonphagocytotic cells. *Salmonella* species have the capacity to multiply within vacuoles in nonphagocytic cells after an initial lag of approximately 4 hours (Leung and Finlay, 1991). The lag period that precedes initiation of bacterial replication in nonphagocytic cells indicates that specific bacterial genes may be required for replication in this unique niche. At 4 to 6 hours after invasion, intracellular *Salmonella* induce the formation of stable filamentous structures within epithelial cells (Garcia-del Portillo et al, 1993). These tubules, also called Sifs, appear to be extensions of the SCV membrane, and are enriched in proteins characteristic of the SCV membrane. The kinetics of formation of these filaments parallels the rate of intracellular replication, including the initial lag period. Sifs formation requires viable intracellular bacteria, since addition of antibiotics blocks the formation of these novel structures. The formation of these tubules requires the *S. typhimurium sifA* gene, which is required to maintain the integrity of the SCV, and the SPI-2 TTSS (Beuzón *et al.*, 2000; Stein *et al*., 1996; Brumell *et al*., 2001). The exact physiological significance of Sif formation is unknown; one proposed function of these structures is that they provide access to nutrients for the intracellular bacteria, possibly by intersection with endocytic or exocytic vesicular transport pathways (Finlay and Falkow, 1997).

Considering the complexity of *Salmonella* survival *in vivo*, the multitude of challenges to which it is exposed and constant requirement for dynamic interaction with the infected host, it is not surprising that the actual growth rates of *Salmonella* in infected tissues differ considerably from the generation times that are observed *in vitro*. When given abundant nutrient supplies, such as those present in enriched culture media, *Salmonella* can grow and divide rapidly, with the generation time which can

be as low as approximately 20 minutes. However, monitoring of the segregation of a nonreplicating marker revealed that the doubling time of *Salmonella typhimurium* in infected mice is 5-10 hours (Maw and Meynell, 1968). Also, measurements of the growth rates of *Salmonella* within eukaryotic cells in culture indicate that the majority of the intracellular bacteria are either quiescent or slowly growing compared with their growth in culture media (Lowrie *et al*., 1979; Buchmeier and Heffron, 1989; Abshire and Neidhardt, 1993). There is evidence that different populations of *Salmonella*, in respect to their growth rate, exist within macrophages during infection (Abshire and Neidhardt, 1993). If the extent to which ribosomal protein L12 is acetylated to produce ribosomal protein L7 is taken as a measure of growth rate, it appears that the intracellular bacteria are growing rapidly. However, measurements of viable bacteria indicated that the bacteria were growing slowly within macrophages (Abshire and Neidhardt, 1993). A solution of this apparent growth rate paradox was brought by treating macrophage cells infected with *S. typhimurium* with antibiotics ampicillin or chloramphenicol to determine the number of bacteria that were actively growing and dividing in the intracellular condition. Use of these antibiotics showed that by 2 h after invasion, the intracellular bacteria consisted of at least two populations, one static and the other rapidly dividing (Abshire and Neidhardt, 1993). These findings suggest that, although *Salmonell*a can survive for prolonged periods in the intracellular environment, bacterial growth may be restricted by interplay of limited supply of essential nutrients and bacterial ability to circumvent microbicidal action of host cells.

1. 2. PROTEIN SYNTHESIS

Protein synthesis (translation) is fundamental to all life forms and of critical importance for growth in any environment that will support it. A huge leap forward in our understanding of translation process occurred during the past few years when the structure of the complete bacterial ribosome was solved with resolution at atomic level. Here, the overview of translation, with emphasis on elongation step and role of elongation factor-G (EF-G) in it will be given.

1. 2. 1. The ribosome

The ribosome is a translator which provides communication between the worlds of nucleic acids and proteins. It does so by using the information contained in messenger RNA (mRNA) to produce the corresponding sequence of amino acids. Each codon of mRNA is matched with the amino acid it encodes. The physical connection between the worlds of RNA and protein is the pool of transfer RNAs (tRNAs). One end of each tRNA, the anticodon, is complementary to the codon of mRNA, while the other end, termed the CCA end, is covalently attached to the amino acid specific for that codon. The correct aminoacylation of tRNAs is important for ensuring the fidelity of translation. This task is performed by synthetases, such that for each of the 20 amino acids there is a corresponding synthetase. Synthetase recognizes the amino acid as well as the tRNA which binds this amino acid. The duty of every ribosome is to ensure that the mRNA is read in the correct frame and that each tRNA faithfully follows the code. The ribosomes perform this task with amazing accuracy and at high speed: 10-20 amino acids are incorporated per second into the growing polypeptide chain, with only one error for every 3000 codons deciphered (reviewed in Wilson and Nierhaus, 2003).

Ribosomes are ribonucleoprotein particles composed of two subunits of unequal size. Bacterial ribosomes have a relative sedimentation rate of 70S and can be separated into a large 50S and a small 30S subunit. In *Escherichia coli* one third of the mass of a ribosome consists of protein and other two thirds of ribosomal RNA (rRNA): the 50S subunit contains 5S (120 nucleotides) and a 23S RNA (about 2900 nucleotides), while the 30S subunit contains 16S RNA (approximately 1500 nucleotides). The protein fraction consists of 21 different proteins in the small subunit and 33 proteins in the large subunit.

The overall three-dimensional shapes of the 70S ribosome and its component subunits have been characterized by a variety of electron microscopy techniques. The small subunit is described in anthropomorphical terms, with a head, connected by a neck to a body with a shoulder and a platform. The large subunit presents a more compact structure consisting of a rounded base with three protuberances termed L1, central protuberance, and L7/L12 stalk. Important feature of ribosome is a tunnel that traverses the large subunit. The tunnel starts at the peptidyltransferase (PTF) center at the interface and exits at the base of the large subunit. The growing polypeptide chain is believed to travel through the tunnel before exiting into the cytosol of the cell. The tunnel has a length of approximately 100Å and can house between 30 and 50 amino acid residues of the growing chain. It has been proposed that the tunnel provides en environment suitable for the early stages of protein folding or

simply it protects the nascent chain from proteases until sufficient amino acids have been synthesized to enable secondary structure formation (reviewed in Wilson and Nierhaus, 2003).

1. 2. 2. The movement of the tRNA through the ribosome

The place where the codons of the mRNA are recognized and deciphered by the complementary anticodon of the tRNA is located on the small subunit and is called the decoding site. There are three sites on the ribosome that tRNA can occupy (A, P, and E site). At the A site, aminoacyl tRNA (aa-tRNA) which brings in the new amino acid to extend the growing polypeptide chain, binds according to the codon displayed at this site. The P site is where the peptidyl-tRNA is bound before formation of the peptide bond. Peptidyl-tRNA is the tRNA carrying the nascent polypeptide chain. The E site is the exit site for the deacylated or uncharged tRNA. The tRNA move through each of three sites sequentially during translation, starting at the A site and passing through the P site to the E site, before leaving the ribosome. The exception to this rule is the binding of the very first tRNA (the initiator tRNA), which binds directly to the P site. Initiator tRNA decodes the start codon, usually AUG, and carries the amino acid formylmethionine in bacteria. The codon following the start codon is displayed at the A site and dictates which aa-tRNA will now bind. The aa-tRNA are delivered to the A site in the form of a ternary complex consisting of elongation factor (EF-Tu in bacteria, EF1α in the eukaryotes), GTP and the aa-tRNA (Figure 1). After GTP hydrolysis, EF-Tu·GDP is released from the ribosome and the aa-tRNA docks into the A site. The formation of a peptide bond involves the transfer of the peptidyl moiety of the P-tRNA to the aminoacyl-moiety of the A-tRNA. The ester bond which links peptidyl chain to the tRNA is hydrolysed and a peptide bond with the amino group of the aa-tRNA forms (peptidyl transfer) in a reaction catalyzed by the ribosome. The whole polypeptide chain is added to the new amino acid rather than the addition of the new amino acid to the chain. Formation of peptide bonds occurs on the large subunit at the PTF center. The formation of the peptide bond has no significant change in the positions of the two tRNAs although the P site now contains an uncharged tRNA and the A site contains a peptidyl-tRNA.

The role of elongation factors is to accelerate the elongation cycle to the rate of 50 msec per elongation cycle *in vivo*. The rate without the elongation factors is about four orders of magnitude slower (Gavrilova *et al*., 1976) because of the high energy barrier (120kJ/mol) that separates the pre- and posttranslocational states in *Escherichia coli* ribosomes (Schilling-Bartetzko, Bartetzko and Nierhaus, 1992). Transfer of the A- and P-tRNAs to the P and E sites respectively, is termed translocation and is mediated by second elongation factor, EF-G, in GTP bound state (Figure 1). Translocation places the deacylated tRNA at the E site and peptidyl-tRNA at the P site, thus freeing the A site for the binding of the next aa-tRNA. During translocation, GTP is hydrolysed and EF-G·GDP complex leaves the ribosome. Exchange of GDP for GTP bound to EF-G occurs off the ribosome and ensures that the new round of translocation can occur. Binding of the next A-tRNA releases the E-tRNA and the cycle repeats until the stop codon appears at the A site. At this point, release factors (RF) 1 or 2 release the completed polypeptide from tRNA in the P site, resulting in termination of the elongation cycle. The release is stimulated by RF3, which is also a guanine nucleotide binding protein (Grentzmann *et al*., 1994; Mikuni *et al*., 1994). Release factors together with EF-G dissociate the ribosome into subunits in preparation for the next round of translation.

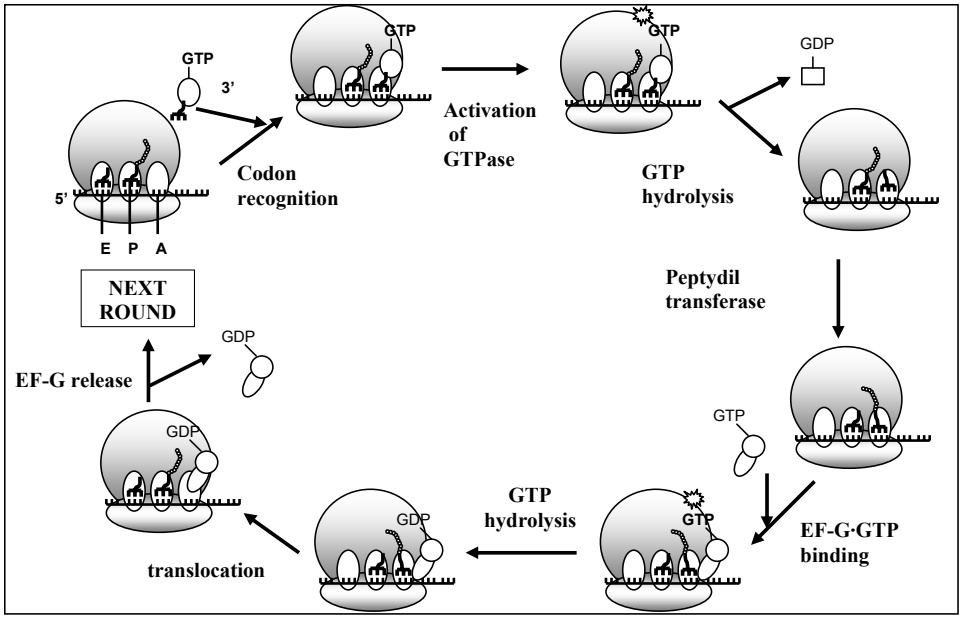
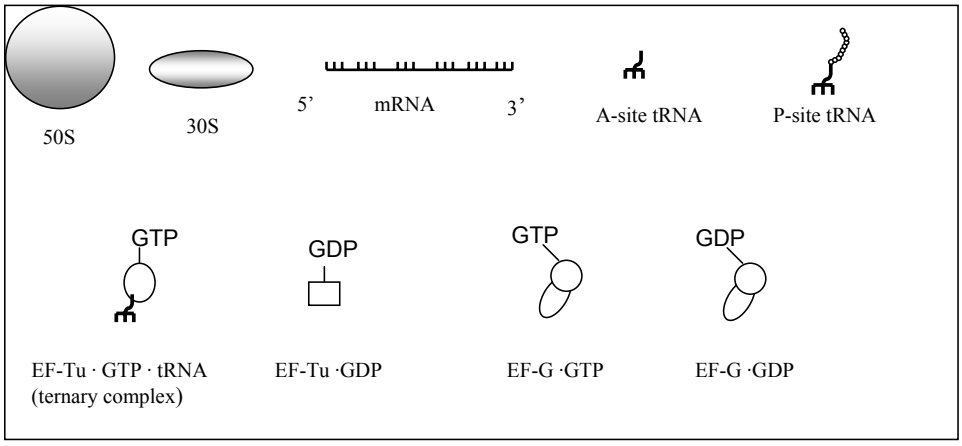


Figure 1. Overview of elongation step of translation (adapted from Ramakrishnan, 2002).

Figure legend:



1. 2. 3. Elongation factor G

EF-G is a member of the GTPase superfamily of proteins which possess a guanine binding site (Bourne *et al*., 1990; Bourne *et al*., 1991). Together with EF-Tu, IF-2 and RF-3, EF-G is included in the translation factor subfamily of the GTPase proteins (t-GTPases) (Bourne *et al*., 1991; Ævarsson, 1995) whose members share consensus motifs that are involved in GTP binding and hydrolysis. When GTP is bound, these proteins are activated and can interact with an effector, which is specific to the GTPase. The intrinsic GTPase activity is triggered by a GTPase activating protein (GAP) which can be part of the effector or a separate protein. The result of this interaction is the hydrolysis of GTP to GDP, which converts GTPase protein into GDP-bound, inactive state. In the case of EF-G, the GAP is provided by components of the ribosome. Perhaps the best candidates for the GAP role are a region of the 23S rRNA termed the sarcin-ricin loop (SRL) which contains the longest (12 nucleotides) universally conserved stretch of rRNA and the pentameric stalk complex of the ribosomal proteins L10-$(L7/L12)_4$. The ribosomal protein L11 (and associated L11 binding site on the 23S rRNA) is often considered as a candidate for taking over the function of the GAP. This is because mutations in both L11 and its binding site on the 23S rRNA can confer resistance against the antibiotic thiostrepton, a potent inhibitor of EF-G- and EF-Tu-dependent GTPase activities. However, the direct involvement of L11 in the factor-dependent GTPase is not very likely, since mutants lacking L11 are viable although extremely compromised. The role of L11 is unclear but, in any case, L11 is in the proximity to the elongation factors. Thus it seems likely that EF-G binding stimulates conformational changes in the ribosome, probably through the L10-$(L7/L12)_4$ complex which triggers translocation of the A- and P-tRNAs (Wilson and Nierhaus, 1994).

The crystal structure of EF-G from *T. thermophilus* has been solved in the absence of the nucleotides and in the complex with GDP (Ævarson *et al*., 1994; Czworkowski *et al*., 1994). EF-G has five domains and shows elongated, flat form (Figure 2). The first domain, named the G-domain, is approximately 200 amino acids long and contains the guanine binding site. The other four domains are about half the size of the G-domain, i.e. around 100 amino acids. The G-domain and domain II are structurally very similar to the corresponding domains of EF-Tu (Ævarson *et al*., 1994; Czworkowski *et al*., 1994). This observation, as well as the finding of the conserved sequence named E2 motif in domain II of all t-GTPases, led to proposal that the G-domain together with domain II was present in an ancestral translation factor (Ævarson *et al*., 1994). Domain III and V are suggested to be RNA-binding domains, based on the folding of secondary structures (Ævarson *et al*., 1994). Domain IV was proposed to be ribosome-binding domain (Nygård and Nilsson, 1985; Kohno *et al*., 1986) which is strengthened by observation that mutants with the amino acid replacement in domain IV are defective in the interaction with the ribosome (Hou *et al*., 1994).

Structure for the ternary complex (Nissen *et al*., 1995) is elongated and very similar to the EF-G structure. It was proposed that domains III and IV as well as parts of domain V are protein homologues of tRNA in the ternary complex. The implication of this suggestion was that EF-G might bind to the A-site during the translocation. Recently, it was shown that EF-G occupies A site (Stark *et al*., 2000). EF-G could be trapped on the 70S ribosome using antibiotic fusidic acid. This fixation allows translocation and GTP hydrolysis but blocks EF-G in GDP bound state, thus preventing its

dissociation from the ribosome. The complex was formed in pretranslational state, with A- and P-tRNAs present. As expected, the tRNAs were translocated to the P and E sites, but of special interest is that the tip of EF-G, domain IV, was shown to occupy the position of the A site. EF-G mediated translocation is also possible in the presence on nonhydrolyzable GTP analogues, such as GDPNP, which suggest that binding of EF-G alone is sufficient for translocation and that hydrolysis is necessary for the conformational change and release of EF-G·GDP (Kaziro, 1978).

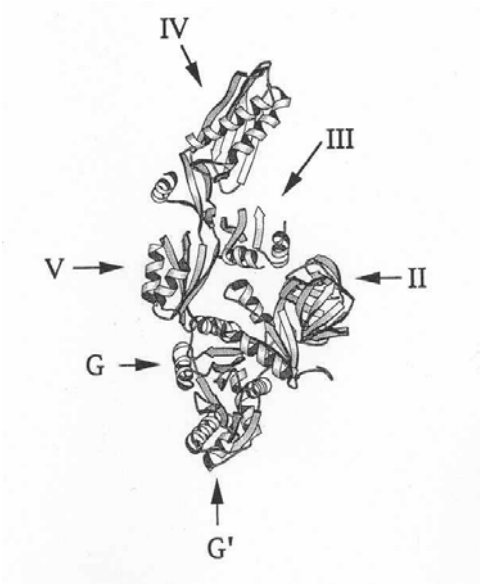


Figure 2. The structure of EF-G in GDP conformation

1. 3. GLOBAL REGULATORS

A common motif in bacterial adaptive responses is the use of a single master regulator which coordinates the expression of large number of genes in response to environmental signals. In *Salmonella typhimurium* (and related bacteria) one of the top-level master regulators during growth is ppGpp. RpoS (σ^S) is another such master regulator for general stress response. Master regulators take the central place in a regulatory network which exhibits hierarchical and modular structure. A network can be subdivided into lower-level modules that are under the control of secondary regulators which also allow specific signal input at such lower and more confined levels (Hengge-Aronis, 2002). One predictable consequence of global regulation is the commitment of the cell to a certain growth/developmental program with a profile which is determined by products of genes whose expression are modulated through the action of the master regulator.

1. 3. 1. ppGpp

1. 3. 1. A. ppGpp synthesis

Bacteria adapt to nutritional stress by adopting a physiological state characterised by the transcriptional repression of genes associated with the translational apparatus (Lazzarini and Dahlberg, 1971; Dennis and Nomura, 1974) and the simultaneous upregulation of genes encoding metabolic enzymes, especially those involved in amino acid biosynthesis (Cashel *et al*., 1996). This change in cellular metabolism, named the "stringent response", is the result of a global network that operates in response to nutrient limitation. Initially, it was found that the loss of stable RNA (rRNA and tRNA) accumulation was the first regulatory response to amino acid starvation (Sand and Roberts, 1952). The suppression of this phenotype, characterized by the continuation of stable RNA synthesis, was termed the "relaxed response" and the mutation was genetically mapped to a single locus called *relA*. In pursuit of the molecular effectors involved in this regulatory circuit, Cashel and Gallant discovered that pppGpp (guanosine 3'-diphosphate, 5'-triphosphate) and ppGpp (guanosine 3'-diphosphate, 5'-diphosphate) accumulate in response to starvation (Cashel and Gallant, 1969). These nucleotides, initially called "magic spots", are synthesised by enzymatic phosphorylation of GTP or GDP, using ATP as a phosphate donor. In enteric bacteria, there are two enzymes which synthesize (p)ppGpp, RelA and SpoT (Figure 3). RelA (also known as ppGpp-synthase I, PSI) is active during amino acid starvation, whereas SpoT (PSII) has both hydrolytic and synthetic activity and is active during exponential growth (reviewed in Cashel *et al*., 1996).

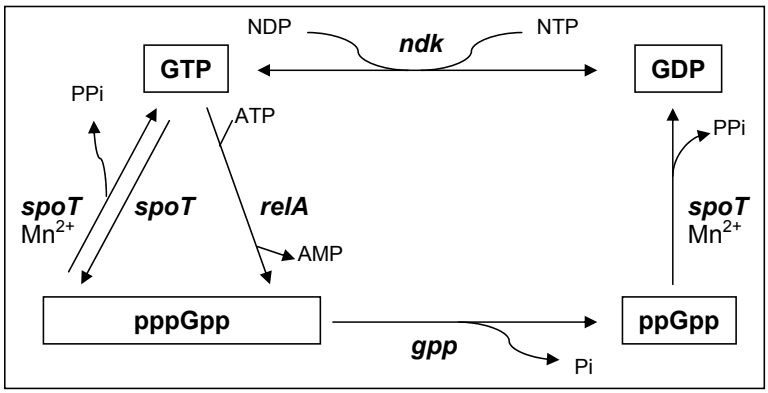


Figure 3. (p)ppGpp metabolism (adapted from Cashel *et al*., 1996). The enzymes involved in (p)ppGpp metabolism are represented by their respective structural genes: PSI (*relA*), PSII (3'-pyrophosphotransferase activity) (*spoT*), (p)ppGpp 3'-pyrophosphohydrolase (*spoT*), (p)ppGpp 5'-phosphohydrolase (*gpp*), and nucleoside 5'-diphosphate kinase (*ndk*).

Activation of stringent response initially stems from the shortage of one (or more) amino acid(s), which in turn produces a significant increase of uncharged, deacylated tRNA, for the corresponding amino acid(s). In log phase bacterial cells, deacylated tRNA constitutes approximately 15% of the total tRNA, the majority of which is present in a ribosome- or synthetase- bound state. Under conditions of amino acid starvation, the deacylated tRNA fraction can increase to over 80% of the total tRNA (Yegian, Stent and Martin, 1966). The scarcity of the aminoacylated tRNA, compounded by the large pools of free deacylated tRNA, enables deacylated tRNA to bind an empty ribosomal A site, conditional to presence of a cognate codon. When deacylated tRNA is encountered at the A-site of the 50S ribosome, protein synthesis is stalled, resulting in an idling reaction in which ribosome-bound RelA is activated to synthesize (p)ppGpp (Haseltine and Block, 1973).

The exact mechanism of RelA-mediated (p)ppGpp synthesis is still not fully understood. Early studies demonstrated that RelA binding to 70S ribosomes is essential for the production of (p)ppGpp (Ramagopal and Davies, 1974; Richter, 1976; Richter *et al*., 1975) and that RelA binding is enhanced by the presence of a poly(U)-mRNA (Wagner and Kurland, 1980). Apart from the presence of deacylated tRNA at the A site, the synthesis of (p)ppGpp has been shown to be dependent on presence of ribosomal protein L11 *in vivo* (Friesen *et al*., 1974, Wendrich *et al*., 2002). Recently, interesting model was proposed in which binding of RelA to the ribosome is predominantly influenced by mRNA and not by deacylated tRNA or L11 (Wendrich *et al*., 2002). It was proposed that binding of deacylated tRNA to the ribosomal A site blocks ribosome in such way that 3' end of the mRNA protrudes from the ribosome. Recognition of blocked ribosome with extended 3' mRNA by RelA activates ppGpp synthesis. RelA mediated (p)ppGpp synthesis occurs simultaneously with the release of RelA from the ribosome.The model predicts that RelA then "hops" to the next blocked ribosome, and the synthesis of (p)ppGpp is repeated.

The levels of ppGpp during exponential growth (basal levels) vary in dependence of composition of the growth medium. Basal levels are high during slow growth in nutritionally poor media, and low during fast growth in rich media (Ryals, Little and Bremer, 1982) Since both *relA*$^+$ and *relA* strains produce similar low levels of ppGpp during exponential growth, it is believed that the balance between hydrolytic and synthetic activities of cytoplasmatic enzyme SpoT determines basal ppGpp levels (Fehr and Richter, 1981; Friesen, Fiil and von Meyenburg, 1975; Lagosky and Chang, 1980; Metzger et al, 1989; Richter, Fehr and Harder, 1979). The greater the number of different amino acids in the medium, the lower the SpoT synthetic activity (Murray and Bremer, 1996). Thus both RelA and SpoT activities are controlled by amino acids, albeit in different ways.

1. 3. 1. B. ppGpp and growth rate control

Regulation of the transcription of rRNA (*rrn*) operons is thought to be a result of an active interaction between ppGpp and RNA polymerase (RNAP) that alters the promoter selectivity, presumably through a ppGpp-induced conformational change in the enzyme. All seven *rrn* genes of *E.coli* have similar dual promoters, P1 and P2, which are separated by about 120bp (Gilbert, DeBoer and Nomura, 1979). Transcription from P1 is preferentially inhibited by ppGpp (Sarmientos and Cashel, 1983; Kajitani and Ishihama, 1984). The inhibition depends on the presence of the discriminator sequence GCGC bordering the -10 hexamer (TATAAT) (Zacharias, Göringer and Wagner, 1989; Zacharias *et al*., 1991). The P2 promoters lack this discriminator; nonetheless, during the stringent response, transcription from P2 also decreases because the total RNA polymerase activity is greatly reduced (Ryals and Bremer, 1982), presumably due to ppGpp-dependent transcriptional pausing. Since the synthesis of stable RNA directly correlates with the growth rate of an organism, it is perceived that ppGpp affects the control of growth rate in bacteria, exerting growth rate-dependent transcriptional control. Over the past fifteen years, several models for ppGpp control of stable RNA synthesis were proposed, but it seems that the consensus on how ppGpp controls growth rate is still not reached (reviewed in Zhang *et al*., 2002). First proposal was that that ppGpp determines the "partitioning of RNA polymerase" into stable RNA and mRNA synthesizing fractions (Ryals, Little and Bremer, 1982; Bremer *et al*., 1987). This model is based on the finding that the fraction of the total rate of RNA synthesis that is stable rRNA and tRNA, defined as r_s/r_t decreases with increasing ppGpp levels. At near zero levels of ppGpp during growth in rich media or during relaxed response, r_s/r_t has a value close to 1.0, which means that almost all of the RNA being made in the bacteria is stable rRNA and tRNA and very little is mRNA. In contrast, at increasingly higher levels of ppGpp during growth in poor media or during the stringent response, r_s/r_t approaches a minimum value of 0.25; i.e. 25% of all RNA synthesized at any instant is stable RNA and 75% is mRNA (Ryals, Little and Bremer, 1982). The residual rRNA synthesized under the latter conditions originates almost exclusively from the P2 promoters of *rrn* operons (Zhang and Bremer, 1995). Jensen and Pedersen proposed that (i) that stable RNA promoters are constitutive and have low V_{max}/K_m ratios, but high V_{max} and K_m values. In contrast, mRNA promoters have high V_{max}/K_m ratios and low values of V_{max} and K_m (ii) elevated levels of ppGpp induce frequent pausing during the transcription of both mRNA and stable RNA genes, which decreases the free RNAP concentration (Jensen and Pedersen, 1990). This model suggests that mRNA promoters are favoured when free RNAP concentration is low (high ppGpp levels) and that stable RNA promoters are favored when concentration of free RNAP is high (low

ppGpp levels). However, an alternative model proposes that the intracellular concentration of the NTP pool regulates growth-rate-dependent gene expression. The model is based on measurement of relative *in vivo* NTP concentrations and *in vitro rrn* transcription rates at different NTP concentrations, which suggested that initiation at *rrn* promoters is controlled by changes in the concentrations of the initiating NTPs (Gaal *et al*., 1997). However, it was recently demonstrated that maintenance of the NTP pool is independent of growth rate (Peterson and Moller, 2000), which argues against this model. The argument is further complicated by the possibility that ppGpp regulates the intracellular NTP pool by interfering with the biosynthesis of purine nucleotide (Hou *et al*., 1999).

Amid this unsettled debate, the evidence of a physical interaction between ppGpp and RNAP is emerging that may contribute to understanding of ppGpp-mediated growth rate control. Using aminonaphthalenesulfonate (AmNS)-ppGpp as a fluorescent substrate it was showed that ppGpp binds to the β-subunit of RNAP (Reddy *et al*., 1995). Consistent with these *in vitro* observations, mutations in the β -subunit of RNAP that controlled the stringent promoters even in the absence of ppGpp were identified (Zhou and Jin, 1998). The term "stringent RNAP" for the ppGpp-bound enzyme was introduced (Zhou and Jin, 1998). Chemical cross linking of azido-ppGpp to the carboxy-terminal region of the RNAP β-subunit unambiguously indicated that the nucleotide binds to the enzyme (Chatterji *et al*., 1998). Recently, a thio-derivative of ppGpp with a spacer length of zero has been found to crosslink to the amino-terminal domain of the β'-subunit of RNAP (Toulokhonov *et al*., 2001). Thus, it appears that the amino-terminal domain of the β'-subunit and the carboxy-terminal domain of the β-subunit of RNAP may constitute the ppGpp-binding site. However, mutations in region 3 of the 70 subunit of RNAP has also been shown to release ppGpp requirement (Hernandez and Cashel, 1995), indicating that ppGpp affects the structure of the enzyme at the interface of the three subunits.

1. 3. 1. C. Examples of ppGpp-mediated transcriptional regulation

The first observation of positive regulation during the stringent response was reported for the *his* operon. This observation was met with surprise, as synthesis of all cellular RNA was considered previously to be coordinately inhibited by ppGpp. Subsequently, more examples of genes positively-regulated by ppGpp have been reported. An important example, which gives insight into mechanism of ppGpp-mediated transcriptional regulation, is the positive regulation of *rpo*S (Gentry *et al*., 1993). It was shown that ppGpp is mandatory for RpoS-dependent promoters, even in the presence of excess RpoS (Kvint *et al.*, 2000). Furthermore, the requirement for ppGpp was bypassed by a mutation in *rpoB* (the gene encoding the β-subunit of RNAP). This observation suggests that a ppGpp-induced structural change in RNAP facilitates RpoS to compete more successfully for the enzyme, thereby providing selectivity for RpoS-dependent promoters. The model proposed presumes that the rate-limiting step for positively-regulated, weaker promoters is the recruitment of RNAP. Efficient enzyme recruitment on weaker promoters requires a high concentration of free RNAP, which is achieved by early dissociation of the open complex from negatively-regulated, stronger promoters.

Recently, the dual control mechanism in which both nucleoid proteins (HNS/StpA) and ppGpp participate in regulation of activity of the stringent promoters, was uncovered (Johansson *et al*., 2000).

The presented results correlate the topological state of stringent promoters with their sensitivity to ppGpp. It was proposed that, when the stringent promoter is in a reduced, negative-supercoiled state, transcription initiation is hypersensitive to ppGpp. It was shown that reducing basal levels of ppGpp restores the expression of stringent promoters in a *hns/stpA* mutant strain. Interestingly, one of the promoters of the genes studied encodes the cyclic-AMP receptor protein (CRP), a global activator that regulates various operons responsible for the utilization of a poor carbon source. This is an anomalous relationship between ppGpp and the utilization of alternate carbon source, and requires more detailed studies to understand the intriguing features of the stringent response.

1. 3. 1. D. Role of ppGpp in protein abundance and DNA maintenance

The growth-rate-dependent transcriptional control of gene expression by ppGpp indicates that other growth-related processes in the cell, such as turnover of cellular proteins, DNA replication and repair are also affected by the stringent pathway (reviewed in Chatterji and Ojha, 2001). However, the involvement of ppGpp in these processes has not been extensively investigated so far. Here, the finding of several studies that were carried out to address these issues will be summarized.

The rate of protein synthesis and degradation depends on the cellular growth requirements. A report by Bremer and his group argues that intracellular abundance of a protein is maintained by the distribution of translating ribosome, which remains constant throughout the growth phase, between the encoding mRNA and bulk mRNA (Liang *et al*., 2000). Hence, qualitative changes in the population of bulk mRNA as a consequence of the stringent response would passively regulate the translation of specific mRNA. This argument is supported by the observation that a high intracellular level of ppGpp has a positive effect on the abundance of LacZ from various relatively unrelated weak promoters, a model that suggests the passive mode of positive regulation of gene expression by ppGpp.

A possible link between ppGpp and protein degradation has been provided by Kuroda and collaborators who reported that the rate of protein degradation is severely impaired in *ppk ppx* double mutants that lack the ability to accumulate polyphosphate (PolyP) during amino acid starvation (Kuroda *et al*., 1999). The accumulation of PolyP has been genetically shown to be under the positive control of ppGpp (Kuroda *et al*., 1997).

In cells undergoing the stringent response, there is evidence that DNA replication is also inhibited in order to cope with decreased cellular growth rate. In *E. coli*, the slow rate of initiation of replication at *oriC* and the negative regulation of *dnaA* during nutritional stress are positively related to increased ppGpp concentration (Cashel *et al*., 1996). Arrest of DNA synthesis in *B. subtilis* during the stringent response, however, is a result of regulated termination of replication (Autret *et al*., 1999). It was shown that the termination occurs always at a specific stop site, called the stringent terminator (STer), which maps to a location between 100 and 200 kilobases on either side of *oriC*, and that the termination of DNA synthesis during stringency essentially required ppGpp and replication termination protein, RTP, which is required for normal termination.

Recently, it was found that ppGpp participates in the DNA repair pathway in the cell (McGlynn and Lloyd, 2000). Ultraviolet-induced DNA lesions stall the elongation complex of RNAP at the point of lesion, leading to an impasse for the replication fork. The ppGpp-mediated early dissociation of the stalled elongation complex facilitates the regression of the replication fork, efficient repair of lesions and re-initiation of replication by a RecG-dependent pathway. As *recG* and *spoT* exist in the same operon, a study on the regulation of their expression may provide interesting clues.

1. 3. 1. E. Cell cycle inhibition and morphology

Microscopic observations of (p)ppGpp^0 strains show that the bacterial population is heterogeneous in size, including small cells, elongated cells and long filaments (Xiao *et al*., 1991). This finding is suggestive of involvement of ppGpp in cell division which could be indirect, caused by perturbation of DNA replication or partitioning process. Alternatively, this phenotype could be due to a requirement of ppGpp for expression of one or more genes essential for cell division.

Most of the results attributing a role for ppGpp in cell division come from studies of the effect of mecillinam (Lund and Tybring, 1972) an antibiotic of the penicillin family which specifically binds and inactivates penicillin-binding protein 2 (PBP2) (Spratt, 1977; Spratt and Pardee, 1975). It is believed that PBP2 is required for the maintenance of the rod shape since PBP2 inactivation gives phenotype of spherical cell shape. PBP2 seems also to be essential for cell survival, since deletion of *pbpA* which encodes PBP2 is lethal for wild-type strains (Ogura *et al*., 1989). The same conclusion comes from the inability to transduce a deletion of *pbpA* into wild-type strains (Ogura *et al*., 1989). The lethality of PBP2 inactivation has been attributed to cell division inhibition (Vinella *et al*., 1993). However, mecillinam-resistant (Mec^R) mutants in which the *pbpA* deletion is no longer lethal were isolated (Vinella *et al*., 1992). These mutant cells divide and survive as spherical cells. Two such mutants, called *lov*-1 and l*ovB*, have been shown to possess partially defective aminoacyl-tRNA synthetases, and their Mec^R phenotype was shown to be *relA*-dependent (Vinella *et al*., 1992). The link between Mec^R phenotype and ppGpp levels is further supported by findings that: (i) in a wild-type strain, decrease of the growth rate or IPTG induction of ppGpp results in Mec^R phenotype (Vinella *et al*., 1992); (ii) elevated ppGpp levels in a Δ*relA* strain carrying a *spoT* mutation result in Mec^R phenotype (reviewed in Cashel *et al*., 1996). In addition, an *rpoB* mutation which failed to exhibit mecillinam resistance on minimal media but was resistant when plated on media which caused mild amino acid starvation was discovered (Vinella *et al*., 1992). The mutation was designated *rpoB*$_{(Fts)}$ because it caused filamentation at high temperatures (Vinella and D'Ari, 1994). Both phenotypes of *rpoB*$_{(Fts)}$ strain are completely suppressed by increased ppGpp pools.

PBP2 inactivation may cause a defect in some element needed for cell division and ppGpp can compensate for this defect, possibly at the level of transcription. The *ftsZ* gene seems a likely candidate (Cashel *et al*., 1996). The FtsZ protein appears to be the positive regulator of cell division and has been reported to be limiting for the process (Garrido *et al*., 1993). The *ftsZ* gene is in a complex transcription unit along with *ftsQ* and *ftsA* gene. The overproduction of FtsZ, FtsA and FtsQ proteins confer mecillinam resistance (Vinella *et al*., 1993). The transcription of *ftsZ* is driven from at least five promoters (Robinson *et al*., 1996). At least two promoters are activated by slow growth, as

judged by *lacZ* fusion behaviour and immunoblot analysis. (Robin, Joseleau-Petit and D'Ari, 1990). At least one of the promoters is probably σ^s-dependent Since expression of *rpoS* encoding σ^s is induced by ppGpp (Gentry et al., 1993), it is possible that *ftsZ* expression is induced by ppGpp through induction of σ^s. This is supported by the finding that overproduction of the RelA protein gives partial suppression of the phenotype of an *ftsZ*84 mutation (Gervais, Phoenix and Drapeau, 1992).

1. 3. 1. F. Virulence and long-term persistence of pathogenic bacteria

Although the mechanisms of infection and evasion of the host immune response are species-specific, the possibility that common global regulatory pathways affect these properties cannot be ruled out. So far, it has been established that high ppGpp levels induce virulent properties in the opportunistic pathogen, *Legionella pneumophila* (Hammer and Swanson, 1999). In this case, the stringent response to environmental stress is an important factor in modulating the virulent attributes that help the pathogen's survival in the host. This has been strengthened by observations made in *Mycobacterium tuberculosis*-a pathogen that persists in the host for years. *M. tuberculosis* has one dual-function enzyme, Rel_{Mtb} for both (p)ppGpp synthesis and hydrolysis (Avarbock *et al*., 1999). *In vitro* study on regulation of (p)ppGpp production in *M.tuberculosis* show that uncharged tRNA induces synthase activity of Rel_{Mtb}, whereas charged tRNA induces hydrolase activity (Avarbock *et al*., 2000). Rel_{Mtb} was shown to be essential for survival of this organism under conditions prolonged starvation (Primm et al., 2000). Microarray analysis demonstrated that *M.tuberculosis* strain with deletion of rel_{Mtb} gene suffers from a generalized alteration of the transcriptional apparatus, as well as specific changes in the expression of virulence factors, cell-wall biosynthetic enzymes, heat-shock proteins and secreted antigens (Dahl *et al*., 2003). This data suggest that Rel_{Mtb} is critical for successful establishment of persistent infection *in vivo.* Overexpression of *rel* gene in *Mycobacterium smegmatis* (which is genetically similar to its pathogenic counterpart, *M. tuberculosis*) results in a spherical morphology similar to that of mycobacterial persistors (Ojha *et al*., 2000). These observations suggest that RelA-dependent pathways may be required by mycobacteria to shuttle between the replicative and persistent state, two physiologically distinct phases of bacteria.

Recently, the list of organisms in which ppGpp mediates establishment of virulent properties was expanded. It was shown that stringent response plays role in biofilm growth and adherence in *Listeria monocytogenes* (Taylor *et al*., 2002) and quorum sensing and cell density-dependent gene expression in *Pseudomonas aeruginosa* (van Delden *et al*., 2001). Although the functions of RelA and SpoT have been extensively characterized in pathogens such as *Salmonella typhimurium* and *Vibrio spp*., it is rather suprising that its involvement in the virulence of these organisms is yet not fully understood.

1. 3. 2. RpoS

1. 3. 2. A. The role of RpoS in general stress response

RpoS (σ^s) is a sigma subunit of RNA polymerase that is induced upon entrance into stationary phase or under various stress conditions and can partially replace the vegetative sigma factor RpoD (σ^{70}). As a consequence, transcription of many σ^s-dependent genes is activated (reviewed in Hengge-Aronis, 2002). Consistent with the multiple functions of the σ^s regulon, the *rpoS* gene was discovered independently and named differently by several groups. It was identified as a gene involved in near-UV resistance (*nuv*) (Tuveson and Jonas, 1979), as a regulator for the *katE*-encoded catalase HPII (*katF*), (Loewen and Triggs, 1984; Sak, Eisenstark and Touati, 1989), as a exonuclease III (*xthA*), (Sak, Eisenstark and Touati, 1989), an acidic phosphatase (*appR*), (Touati, Dassa and Boquet, 1986), and finally, as a starvation-inducible gene encoding a central regulator for stationary-phase inducible genes (*csi*-2) (Lange and Hengge-Aronis, 1991). All the previous studies had described alleles of the same gene which codes for this sigma factor. Because of its crucial role in stationary phase or under stress conditions, the name σ^s was proposed (Lange and Hengge-Aronis, 1991). The term σ^{38} is sometimes used although the molecular mass of σ^s deviates from 38kDa in various species and even in some *E.coli* strains. The *rpoS* gene has been identified in other enteric and related bacteria. At present, it seems that σ^s occurs in the γ branch of the proteobacteria, i.e., in a group of gram-negative bacteria that includes many species with special importance for humans because of their pathogenic or beneficial potential. With minor variations, the general function of σ^s in these bacteria appears to be similar to that in *Escherichia coli* (Henge-Aronis, 2002).

RpoS is now seen as the master regulator of the general stress response which provides the cells with the ability to survive the actual stress as well as additional stresses not yet encountered (cross-protection). While specific stress responses tend to eliminate the stress agent and/or to mediate repair of cellular damage that has already occurred, the general stress response renders cells broadly stress-resistant in such way that damage is avoided rather than needing to be repaired (Hengge-Aronis, 2002). Thus, the major function of the general stress response is preventive, which is reflected in the RpoS-dependent multiple stress resistance observed with starved or otherwise stressed cells (Hengge-Aronis, 2000a). Accordingly, the majority of the more than 70 σ^s-dependent genes known so far, confer resistance against oxidative stress, near-UV irradiation, potentially lethal heat shocks, hyperosmolarity, acidic pH, ethanol and probably other stresses yet to be identified. RpoS-controlled gene products generate changes in the cell envelope and overall morphology since stressed *Escherichia coli* cells tend to become smaller and ovoid. Metabolism is also affected by σ^s-controlled genes, consistent with σ^s being important under conditions where cells switch from a metabolism directed toward maximal growth to a maintenance metabolism. Finally, a number of virulence genes in pathogenic enteric bacteria have been found to be under σ^s control, consistent with the notion that host organisms provide stressful environments for invading pathogens (Hengge-Aronis, 2000b). For example, it was shown that RpoS regulates *Salmonella* virulence and is essential during infection (Fang *et al*., 1992).

Cellular concentration of RpoS is the decisive parameter in general stress response. RpoS levels increase in response to starvation for carbon, nitrogen, phosphate sources or amino acids. This leads to entry into stationary phase, i.e., cessation of growth, but RpoS can be also induced by a partial reduction of the growth rate (Gentry *et al*., 1993; Lange and Hengge-Aronis, 1994a; Notley and Ferenci, 1996; Teich *et al*., 1999). Additional inducing conditions are oxidative stress, hyperosmolarity, nonoptimally high or low temperature, acidic pH and high cell density (Hengge-Aronis, 2002).

1. 3. 2. B. Regulation of RpoS production

Regulation of RpoS production occurs at the level of transcription, translation and protein degradation. Transcription of *rpoS* is stimulated by controlled downshifts in growth rate in a chemostat (Notley and Ferenci, 1996; Teich *et al*., 1999) as well as by continuous reduction in growth rate which occurs when cells grown in rich medium enter stationary phase. Under these conditions, *rpoS* transcription is activated approximately 5- to 10-fold (Lange and Hengge-Aronis, 1991; Lange and Hengge-Aronis, 1994a; Mulvey *et al*., 1990). In contrast, abrupt cessation of growth in response to sudden glucose starvation, only weakly increases *rpoS* transcription (less than twofold).

Several promoters are involved in *rpoS* transcription. Two transcripts can be detected by Northern analysis (Arnqvist, Olsén and Normark, 1994). Polycistronic *nlpD-rpoS* mRNA originates from two closely spaced promoters (*nlpD*p1 and *nlpD*p2) upstream of the *nlpD* gene which encodes a lipoprotein of unknown function. Another promoter (*rpoS*p) is located within the *nlpD* gene and produces monocistronic *rpoS* mRNA with an unusually long untranslated 5` region of 567 nucleotides (Lange, Fischer and Hengge-Aronis, 1995; Takayanagi, Tanaka and Takahashi, 1994). Studies with transcriptional fusions that included a 5` deletion analysis indicated that this transcript is the major *rpoS* mRNA (Lange, Fischer and Hengge-Aronis, 1995) and that the leader sequence is functionally important since 5` deletions in it reduce *rpoS* expression (Cunning, Brown and Elliott, 1998). Moreover, *rpoS*p accounts for activation of transcription during entry into stationary phase (Lange, Fischer and Hengge-Aronis, 1995). The NlpD protein is not stationary phase induced which indicated that the *nlpD* promoters are not growth phase regulated (Lange and Hengge-Aronis, 1994b).

Even under conditions where σ^s protein is hardly detectable, cells produce fair amounts of *rpoS* mRNA. The rate of translation of already existing *rpoS* mRNA is stimulated by high osmolarity, during growth on moderately low temperatures (20^oC), upon reaching a certain cell density (approximately 1 - $2x10^8$ cells/ml), during growth in minimal glucose medium and in response to a pH downshift from pH 7 to 5 in rich medium (reviewed in Hengge-Aronis, 2002). Model for the control of the rate of *rpoS* mRNA translation is based on mRNA secondary structure in which the translation initiation region (TIR) is base-paired and therefore not sufficiently accessible to the ribosomes under noninducing conditions. It is hypothesised that certain stress signals trigger changes in this mRNA secondary structure that allow more frequent translational initiation (Hengge-Aronis, 2002). However, the actual appearance of this structure is still a matter of speculation.

Also, it was suggested that binding of proteins HU and Hfq to *rpoS* mRNA could facilitate translational initiation. HU is a major protein component of the bacterial nucleoid which affects the overall nucleoid structure and topology. HU also participates in regulation of gene expression, DNA recombination and DNA repair (Nash, 1996) and is required for survival during the prolonged starvation (Claret and Rouvière-Yaniv, 1997). Possible involvement of HU in *rpoS* translation control is suggested by findings that HU-defficient mutants exhibit strongly reduced RpoS levels because of reduced *rpoS* translation (Balandina *et al*., 2001). *In vitro*, HU binds with high affinity to a small *rpoS* mRNA fragment (150 nucleotides covering the TIR and the upstream antisense region probably base paired with TIR) (Balandina *et al*., 2001) as well as to larger fragment (covering more than 700 nucleotides starting from the original mRNA 5` end) that also binds Hfq. It was suggested that, HU preferentially recognizes and binds to secondary-structure elements in *rpoS* mRNA and that the result of the binding is that TIR becomes more accessible to ribosomes.

Hfq is an accessory component of phage Qβ replicase that binds to several sites in Qβ RNA including the 3`end (Senear and Steitz, 1976; Barrera *et al*., 1993; Miranda *et al*., 1996). The *hfq* mutant was observed to have pleiotropic phenotype (Tsui *et al*., 1994), which resembles the phenotype of an *rpoS* mutant. This led to the discovery that Hfq is required for efficient *rpoS* translation (Brown and Elliott, 1996). It is proposed that, by binding to a few crucial positions of *rpoS* mRNA, Hfq may affect the equilibrium between possible alternative secondary structures that are productive for translational initiation. Another possibility is that Hfq does not affect *rpoS* mRNA secondary structure but, when bound to *rpoS* mRNA, acts as a platform that recruits additional factors involved in *rpoS* translational control. Hfq can bind to DsrA (Sledjeski, Whitman and Zhang, 2001) which is small regulatory RNA partially complementary to *rpoS* mRNA that stimulates *rpoS* translation at low temperatures. A hypothetical model consistent with data available is that Hfq bound to *rpoS* mRNA recruits DsrA and facilitates the translational stimulation by DsrA (Hengge-Aronis, 2002).

There is also control of RpoS degradation. In cells growing on minimal medium, RpoS (which is produced at low but measurable rate) has a half-life that range between 1 minute and few minutes, depending on carbon source. However, in response to stresses such as carbon starvation, osmotic upshift or shift to acidic pH, RpoS proteolysis is reduced or completely inhibited, and as a consequence, σ^s rapidly accumulates in the cell (Hengge-Aronis, 2002).

1. 4. Oxidative stress

The consequence of aerobic growth is exposure of bacterial cells to endogenously formed reactive oxygen intermediates (ROI) such as hydrogen peroxide (H_2O_2). This is because respiratory activity generates superoxide anions (O_2^-) which are converted into hydrogen peroxide (H_2O_2) by enzyme superoxide dismutase. In particular, the exponential phase of aerobic growth is associated with risk of endogenous oxidative stress in which cells need to cope with about 10-fold increase in the rate of H_2O_2 generation. In *E.coli*, intracellular H_2O_2 concentration is kept at an almost constant steady-state value of around 0.2μM over a broad range of cell densities in the rich medium (Gonzales-Flecha and Demple, 1995). This regulation is achieved by activation of mechanisms such as SoxR/S, OxyR and RpoS, aimed to decrease intracellular H_2O_2 concentration.

Exposure of *E. coli* or *Salmonella* to elevated levels of intracellular superoxide results in activation of the SoxR/S regulon. SoxR is a constitutively expressed transcription factor whose activity is regulated by reduction or oxidation of its iron-sulfur cluster. Oxidation of the iron-sulfur cluster in conditions of oxidative stress results in a conformational change of the protein, leading to its activation. Activated SoxR is a transcription factor whose only known target gene is *soxS*, which in turn will activate the whole regulon. SoxR/S regulon is composed of at least ten genes with diverse functions (reviewed in Jenssen *et al*., 2003). For instance, the cytoplasmic superoxide dismutase which neutralizes superoxide is regulated by the SoxR/S system. Other genes regulated by this system include those involved in uptake of superoxide or oxidizing compounds (e.g., *micF* which regulates the expression of pore protein OmpF), and those involved in the repair of DNA damage, (e.g., *nfo*, encoding an endonuclease).

The OxyR system is activated upon exposure to hydrogen peroxide, and the activation of this transcription factor also involves oxidation of the tetrameric protein (reviewed in Jenssen *et al*., 2003). In this case, oxidation of the cysteine residues in this complex results in the formation of disulfide bridges. Only this oxidized form of OxyR is active as a transcription factor. OxyR transcriptionally induces expression of *katG* gene during exponential growth or upon exposure to H_2O_2 (Storz, Tartaglia and Ames, 1990). The *katG* gene encodes a bifunctional catalase hydroperoxidase I (HPI) (Claiborne and Fridovich, 1979) which is present in the periplasm (Heimberger and Eisenstark, 1988) and active as tetramer of 81-kDa subunits (Claiborne and Fridovich, 1979). Another species of catalase present in enteric bacteria is encoded by *katE* gene. The *katE* codes for the monofunctional HPII, which is a tetramer of 78-kDa subunits (Claiborne, Malinowski and Fridovich, 1979), present in the cytoplasm (Heimberger and Eisenstark, 1988) and is under control of RpoS (Sak, Eisenstark and Touati, 1989). RpoS also seems to exert control of *katG*-encoded HPI production since Oxy-R independent regulation of HPI by RpoS is observed in stationary-phase cultures (Ivanova *et al*., 1994).

Hydrogen peroxide can directly cause damage to membranes, enzymes, and DNA; however, in conjunction with iron, hydroxyl radicals are formed. The reaction in which hydroxyl radicals are generated by transfers of an electron from ferrous ion to hydrogen peroxide is called Fenton reaction. Hydroxyl radicals are highly reactive and will not diffuse over long distance but cause damage at the

site of production. Fe (II) is present in the backbone of DNA and it is likely that most of the cell death that occurs after exposure to hydrogen peroxide is caused by DNA damage via hydroxyl radicals (reviewed in Jenssen *et al*., 2003). DNA repair mechanisms are therefore crucial for *Salmonella* in order to cope with ROI. Their relative importance is exemplified by the fact that *recA* mutants are attenuated in mouse infection model, whereas catalase mutants are not (Buchmeier *et al*., 1995).

Because of its role in oxidative damage, intracellular iron levels must be tightly controlled. Genes involved in the uptake of iron are regulated by *fur*, the ferric iron uptake repressor, which is regulated by both SoxR/S and OxyR. The *fur* regulon will only be expressed when the amount of iron is limiting and Fe(III) will be taken up from the environment. Therefore, under high-iron conditions, *fur* repression not only leads to decreased expression of genes involved in iron uptake, but also to increased expression of proteins involved in binding iron in the cytoplasm of bacteria.

One iron binding protein is heme, which is, interestingly, cofactor of catalases HPI and HPII and cytochromes of respiratory chain. The first step in heme biosynthesis is the formation of 5-aminolevulinic acid (ALA). In the enteric bacteria, this occurs by a C_5 mechanism. Glutamate, which has first been activated by esterification to $tRNA^{Glu}$, is reduced by the *hemA*-encoded glutamyl-tRNA reductase (HemA) to form glutamate-1-semialdehyde, which is then converted to ALA by the *hemL*-encoded enzyme, glutamate-semialdehyde aminotransferase (HemL) (reviewed in Beale 1996). The final step of heme biosynthesis is linked to iron homeostasis. This step is catalysed by *hemH* gene product, and involves the insertion of ferrous iron into protoporphyrin IX. Defective heme production is associated with generation of ROI. The accumulation of porphyrins or iron is toxic to cells because both compounds stimulate the generation of highly reactive oxygen species (Nakahigashi *et al*., 1991; Storz and Imlay, 1999). Also, reduced production of heme results in respiratory defect which is associated with increased generation of hydroxyl radicals. Inhibition of respiration results in an increased amount of cytosolic reductants, such as NADH, available to reduce free ferric iron. In the presence of hydrogen peroxide, ferrous ion thus formed will donate electron and generate hydroxyl radicals (Elgrably-Weis *et al*., 2002). Taken together, there is a complex interplay between products of genes involved in respiration, defense against oxidative stress and control of intracellular iron levels.

Exposure of bacteria to ROI results in extensive alterations in protein expression patterns. Exposure of *Escherichia coli* to superoxide stress has shown that the expression of a total of 112 genes was modulated by exposure to the redox-cycling agent paraquat (Pomposiello, Bennik and Demple, 2001). Approximately 60% of the genes were up-regulated and 40% down-regulated under these conditions. Similar experiments performed with hydrogen peroxide have shown that under these conditions, 140 genes are induced in *Escherichia coli* (Zheng *et al*., 2001). These data indicate that the defense against ROI involves complex mechanisms and, although insight into ROI defense mechanisms has increased extensively over the past decade, the exact function of many of the genes whose expression is modulated under oxidative stress is still unknown.

1. 5. ANTIBIOTIC RESISTANCE

There is probably no chemo-therapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring "fastness" (resistance). Sir Alexander Fleming, 1946

Human intelligence, culture and technology have left all other plant and animal species out of the competition…But we have too many illusions that we can govern…the microbes that remain our competitors of last resort for domination of the planet. In natural evolutionary competition, there is no guarantee that we will find ourselves the survivor(s). Joshua Lederberg, 1994

The introduction of antibiotics for the treatment of bacterial diseases in the late 1940s was heralded as one of the great innovations of the modern world, "on a par with radio and the internal combustion engine" (Mazel and Davies, 1998) and led many to believe that infectious diseases would be conquered once and for all. This optimistic belief is supported by finding that antibiotics are reckoned to have extended the life span of the average US citizen by 10 years, whereas curing all cancer would prolong life by 3 years (McDermott, 1982). However, recent evidence points to an increase in the prevalence of microbial drug resistance apparently paralleling the expansion of the use (and misuse) of antimicrobial agents. Thus, antibiotic resistance has become an increasingly important problem with serious implications for the prevention and treatment of infectious diseases (reviewed in Burman and Olsson-Liljequist, 2001). As time goes by and new reports of increase of antibiotic resistance are emerging, the previously stated optimistic outlook is slowly being replaced with a pessimistic concern that "post-antimicrobial era" is approaching-a return to the bad old days when a simple infection could turn life-threatening for lack of effective treatment.

Resistance to antimicrobial agents has existed since before they were introduced into human and veterinary medicine, probably because most of the classes of compound used clinically are produced also by microorganisms in the environment. Evidence for this is the presence of drug resistant strains in collections of bacteria dating from before the antibiotic era (Datta and Hughes, 1983; Murray and Moellering, 1978) and in wild rodents with no apparent contact with man-made drugs (Gilliver *et al*., 1999). The introduction and increasing clinical use of each antimicrobial agent has been followed sooner or later by an increasing isolation rate of resistant bacteria. Shortly upon the introduction of penicillin after World War II, penicilinase production was discovered in *Staphylococcus aureus* and *Escherichia coli* and in the early 1950s the use of the first broad spectrum agents such as chloramphenicol, streptomycin and tetracycline was rapidly followed by resistance in *Staphylococci* and Gram negative bacteria. Also, resistance was quick to develop by mutational target alteration against quinolones, rifampicin, fusidic acid and mupirocin after their introduction into clinical use (reviewed in Burman and Olsson-Liljequist, 2001). In some cases, however, resistance turned out to be a complicated task that took three decades to develop and included a series of genetic events until levels of resistance of clinical relevance were obtained (penicillin beta-lactam resistant *pneumococci*, vancomycin resistant *enterococci*). Resistance development markedly increased the risk of therapeutic

failure which could be seen from the case of resurgence of tuberculosis in 1980s. Resurgence was accompanied by increasing rate of appearance of multi-resistant *Mycobacterium tuberculosis* isolates, defined as those resistant to both isoniazid and rifampicin, with or without resistance to other agents.

The resistance problem has traditionally been approached by development of new antimicrobials. In recent years, however, there has been a slowing down in the introduction of such agents and only one truly novel antibacterial drug, linezolid, has been marketed in the past 25 years. This could be partly due to the realization that surveillance system of usage of presently existing antibiotics could be and needs to be improved. Numerous worldwide debates at academic, industrial and governmental levels are taking place in order to improve surveillance of antibiotic usage and antibiotic resistance development and to analyze their relationship. This is accompanied with an effort to establish and make public general recommendations on strategy how to better control resistance problem in the future (The Copenhagen Recommendations, 1998; WHO, 1998; European Commission, 1999).

1. 5. 1. Mechanisms of antibiotic action

At present, five classes of antibiotics which inhibit different bacterial cellular processes exist. Classification of antibiotics is based on their mechanism of action (Table 1). This list of effective antibiotics could expand since new strategies based on bacterial genome sequence data, bioinformatics and functional genomics could help in identifying molecular targets for design of novel classes of antibiotics (Hughes, 2003).

The effectiveness of an antibiotic against a bacterial strain is described in terms of the minimal inhibitory concentration (MIC). This is the lowest concentration of antibiotic that prevents visible growth of an inoculum incubated under defined conditions (reviewed in Andrews, 2001). In almost all cases the inhibitory or killing effect of given antibiotic is a continuously varying function of its concentration. The relationship between drug concentration and apparent growth rate (the difference between the cell division rate and cell death rate) has been measured for a number of antibiotics. For each antibiotic, there is a concentration range in which net rate of growth declines continuously.

Growth in the presence of sub-MIC of various antibiotics has been shown to modify the surface of bacteria and change their ability to adhere to host cells (Shibl, 1984), affect bacterial toxin production (Hinton and Orr, 1960) and inhibit the synthesis of soluble virulence factors (Molinari et al, 1993). Recent study demonstrated that growth in the presence of sub-MIC of erythromycin and rifampicin results in alterations of global transcriptional patterns of *Salmonella typhimurium* (Goh *et al*., 2002). Thus, it appears that many antibiotic inhibitors, when used at low concentrations, have in common the ability to modulate gene transcription, which is distinct from their inhibitory effect. Also, some antibiotics can indirectly or directly modify the humoral and cellular immune response of host to microorganisms. The drugs spiramycin, sparfloxacin and ciprofloxacin can have direct effects on phagocytic cell functions such as chemotaxis (Frank *et al.*, 1992), phagocytosis (Desnottes *et al.*, 1988) and superoxide production (Aoki *et al.*, 1994).

Table 1. Classification of antibiotics according to their mechanism of action

Mechanims of action	Antibiotic
(i) Inhibition of synthesis or damage to cell wall	Penicillins Bacitracin Cephalosporins Monobactams Carbapenems Fosfomycin Cycloserine Vancomycin, Teichoplanin
(ii) Inhibition of synthesis or metabolism of cytoplasmic membrane	Polymyxins
(iii) Inhibition of synthesis or metabolism of nucleic acids	
	Nitrofurantoins Nitroimidazoles
DNA gyrase inhibition:	Quinolones
RNA polymerase inhibition:	Rifampin
(iv) Inhibition of protein synthesis	
50S inhibitors:	Erythromycin (macrolides) Chloramphenicol Clindamycin
30S inhibitors:	Tetracycline Spectinomycin Streptomycin Aminoglycosides
Inhibitors of elongation factors:	
EF-G	Fusidic acid
EF-Tu	Kirromycin
tRNA binding:	Mupirocin
(v) Modification of energy metabolism	
Folic acid metabolism	Trimethoprim Sulfonamides
Mycolic acid metabolism	Isoniazid

1. 5. 2. Genetic determinants and mechanisms of resistance

Most of the early studies of antibiotic resistance involved laboratory experiments with *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella typhimurium* and streptomycin, which was the most studied antibiotic at that time. Spontaneous streptomycin resistant (Sm^R) mutants with chromosomal mutations in *rpsL* gene which encodes ribosomal protein L12 were isolated, and were found to occur at frequencies of 10^{-9} or less per bacterial generation. The conclusion of these experiments was that the emergence of antibiotic-resistant strains during therapy was unlikely to be a serious clinical problem (reviewed in Davies, 1994). These studies could not have anticipated the wide variety of resistance mechanisms, which are summarized in the Table 2, as well as the possibility that genetic determinants encoding resistance could be present on mobile genetic elements called resistance plasmids. Today, it is known that resistance to antibiotics can be caused by mutations in chromosomal genes, acquisition of accessory elements or point mutations in acquired accessory elements (Trnobranski, 1998). Mutated chromosomal genes conferring resistance are often

housekeeping genes associated with essential cellular functions. Accessory elements encoding resistance are plasmids, phages or transposons. Plasmids can exist free or be integrated into the chromosome; transposons can be integrated in a plasmid or in the chromosome (Bryan, 1988).

Antimicrobial agents are inactivated by three major mechanisms (Table 2): (i) modification of drug accumulation, usually by altering uptake or efflux of an antibiotic; (ii) modification of the target, which prevents the target from binding the antibiotic; (iii) drug detoxification, i. e., inactivation of the antibiotic by destruction or modification.

Table 2. Mechanisms and genetic determinants of resistance.

Mechanism	Antibiotic	Genetic determinants	
		Mutation	Gene acquisition
Modification of drug accumulation			
Reduced uptake into cell	Chloramphenicol		+
	Cycloserin	+	
	Aminoglycosides	+	+
Active efflux from cell	Tetracycline		+
	Fluoroquinolones	+	
Modification of drug target to eliminate/reduce antibiotic binding	Erythromycin		+
	Lincosamides		+
	Marolides		+
	Tetracycline		+
	Sulfonamides		+
	Trimethoprim		+
	β-lactams	+	
	Fluoroquinolones	+	
	Rifampicin	+	
	Fusidic acid	+	
	Streptomycin	+	
Drug detoxification			
Inactivation of antibiotic by enzymic modifications (hydrolysis or derivatization)	β-lactams	+	+
	Aminoglycosides		+
	Chloramphenicol		+
Sequestration of antibiotic by protein binding	β-lactams	+	+
	fusidic acid		+
Overproduction of antibiotic target (titration)	Sulfonamides	+	
	Trimethoprim	+	
Binding of specific immunity protein to antibiotic	Bleomycin		+

Target modifications can be acquired in three ways: (i) by accumulation of point mutations in the chromosomal gene encoding the target (e.g. streptomycin and fusidic acid resistance); (ii) by recruitment of a new gene encoding an alternative, antibiotic resistant molecule (e.g. sulphonamide and methicillin resistance) (iii) by intragenic recombination of the chromosomal gene encoding the sensitive target with related genes which encode resistant proteins (e.g. β-lactam and sulphonamide resistance). The latter can occur as a result of transformation event and generates novel alleles that are mosaic genes (reviewed in Hughes and Andersson, 2001). β-lactam resistance in *Streptococcus pneumoniae* is an example of resistance originating from of mosaic genes.

1. 5. 3. Fusidic acid resistance

Fusidic acid is a steroid-like antibiotic isolated form the fungus *Fusidium coccineum* (Godtfredsen *et al*., 1962). This antibiotic blocks the release of EF-G·GDP from the ribosome thus inhibiting further protein synthesis. The drug is mainly active against Gram positive bacteria although the translation apparatus is sensitive in Gram negative bacteria (Godtfredsen et al, 1962; von Daehne, Godtfredsen and Rasmussen, 1979). This difference is probably due to low levels of uptake through the outer membrane in the gram negative species (Plesiat and Nikaido, 1992). *E.coli* and *S.typhimurium* cells can be made more sensitive to fusidic acid, either by mutations which affect the function of the outer membrane (Vaara, 1993), or by the addition of EDTA in the medium (Tanaka, Kavano and Kinoshita, 1971). The most important elements in the molecular structure of fusidic acid are acetoxyl group at C-16 and the carboxyl group at C-20. Any change at these positions, or a reduction of the double bond between C-17 and C-20, abolishes the activity of the drug (von Daene et al, 1979; Willie *et al*., 1975).

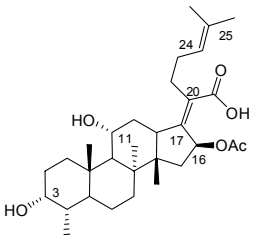


Figure 3. The structure of fusidic acid

Mechanism of modification of drug accumulation resulting in fusidic acid resistant (Fus^R) phenotype seems to be plasmid-mediated. Resistance to fusidic acid in *S.aureus* was described soon after the drug's introduction and appears to be due to alterations in cell wall/membrane permeability, as studies of cell free extract show that protein synthesis is inhibited normally in these strains, and there is no evidence of altered target site or drug inactivation (Chopra, 1976). Also, it was suggested that fusidic acid resistance may sometimes be conferred by multi-drug resistant efflux pumps. *In vitro* selection of low-level resistance to ciprofloxacin has been shown to increase the rate of mutants in methicillin-resistant *S.aureus* (MRSA) with reduced susceptibility to fusidic acid as well as a number of other antibiotics unrelated to fluoroquinolones (Fung-Tomc, Kolek and Bonner, 1993).

Drug detoxification mechanism is mediated by R-plasmids or transposons related to Tn9 which carry a gene encoding chloramphenicol acetyltransferase type I (CAT_I). CAT_I is also present in plasmids pBR325 and pBR328 and confers resistance to both chloramphenicol and fusidic acid (Volker *et al*., 1982). Studies of CAT_I have shown that this protein can bind fusidic acid and a K_i value of 1.7μM for fusidic acid was calculated from the inhibition of the CAT_I activity (Bennett and Shaw, 1983). This K_i value is comparable with the K_d value of 0.4uM for fusidic acid from the ribosome-EF-G-GDP complex (Willie *et al*., 1975). No degradation or acetylation of fusidic acid is detected in the presence of CAT_I , indicating that the mechanism is binding which results in a reduced concentration of the free drug in the cell (Benett and Shaw, 1983).

In several *Streptomyces* species and in *Rhodococcus erythropolis*, resistance due to extracellular inactivating enzymes has been reported (von der Haar and Schrempf, 1995; Dabbs, 1987). One of these enzymes acts as a specific esterase and hydrolyses the acetoxyl group at C-16, which results in spontaneous lactonization between the carboxyl group at C-20 and the remaining hydroxyl group at C-16 thus forming the inactive product (von der Haar and Schrempf, 1995).

An important mechanism of resistance is target modification, i.e. alteration of EF-G, which is presumed to be chromosomally mediated. It was shown that resistant mutants of *Staphylococcus aureus* selected on fusidic acid-containing media have altered elongation factor (Nagaev *et al*., 2001). Where mechanism of resistance is altered EF-G, the organisms generally were initially considered less virulent and less metabolically active (Shanson, 1990). Although not clinically relevant, altered elongation factor has also been demonstrated in Gram-negative bacteria which have MIC much higher than wild type strains (Richter Dahlfors and Kurland, 1990; Tanaka, Kawano and Kinoshita, 1971). In *Salmonella typhimurium* and *Staphylococcus aureus* altered EF-G has been shown to result from mutations in three specific regions of the *fusA* gene which codes for elongation factor G, the target of fusidic acid action (Johansson and Hughes, 1994; Nagaev *et al*., 2001).

In addition to described examples, there are several reports of resistance to fusidic acid for which actual mechanisms are not yet know. In *E. coli* there is a report of a mutant L7/L12 conferring resistance to fusidic acid (Kirsebom, 1985). This is of interest since this protein has been suggested to interact with EF-G. Beside *fusA* there is also *fusB* locus described in *E.coli* which maps to the 14 min region on the chromosome (Isaksson and Takata, 1978). A temperature sensitive mutation in *fusB* gene has a very pleiotropic phenotype including ten-fold higher resistance to fusidic acid. Recent study demonstrated that *fusB* is an allele of *nadD*, encoding nicotinate mononucleotide adenylyltransferase in *E. coli* (Stancek, Isaksson and Rydén-Aulin, 2003). It was proposed that NAD^+ deficiency associated with temperature sensitive *fusB* mutant could affect membrane proteins and alter uptake of fusidic acid (Stancek, Isaksson and Rydén-Aulin, 2003).

1. 5. 4. Fitness cost of antibiotic resistance

The fitness of an antibiotic resistant pathogen is determined by relative rates at which resistant and sensitive bacteria: (i) grow and die in host and environment, (ii) are transmitted between hosts, and

(iii) are cleared from infected host (Andersson and Levin, 1999). The overall fitness (F) of a pathogen can be described according to the following equation:

$F = T(\alpha, \beta, \gamma) / D1+D2+C$

Where F= fitness of pathogen, T= transmission rate, α=fitness in host, β=fitness outside host, γ=infectivity, D1=death rated due to pathogen, D2= death rate due to other causes, C= curing rate.

In majority of cases, antibiotic resistance is associated with fitness cost. This cost can be defined as the reduction of fitness (essentially reproductive rate) of the resistant population with the respect to the ancestor population, in the absence of the selection pressure (i. e. antibiotics). The biological cost of resistance can be measured in three ways: (i) retrospectively, by fitting quantitative models to the changes in frequencies of humans infected with sensitive and resistant bacteria following known changes in the volume of antibiotic use in human populations, (ii) prospectively, by measuring the rates with which individuals become infected with and cleared of sensitive and resistant bacteria, and (iii) experimentally, by estimating the rates of growth, transmission and clearance of sensitive and resistant bacteria in laboratory media and experimental animals (Andersson, Björkman and Hughes, 2001).

Experimental studies of the costs of resistance are usually aimed at determining the rates of growth, survival and competitive performance of sensitive and resistant bacteria, usually by pairwise competition experiments (Andersson and Levin, 1999). Mixtures of isogenic sensitive and resistant strains are grown *in vitro* (in chemostats or batch cultures) or *in vivo* (laboratory animals) and changes in their relative frequencies are followed by selective plating, using either neutral genetic tag (e.g. transposon) or the resistance itself as a selective marker. These pairwise competitions experiments estimate several components of the competitive fitness of sensitive and mutant bacteria: their lag periods, rates of exponential growth, resource utilization efficiencies and their mortality in the presence or absence of host defences (Andersson, Björkman and Hughes, 2001). By repeated measurements these pairwise competitions allow detection of fitness differences that are <1% (Lenski, 1991).

In most studies, resistance caused by target alterations has been found to confer some costs (Table 3) (reviewed in Andersson, Björkman and Hughes, 2001). Costs, measured by competitions between sensitive and resistant bacteria *in vitro* or *in vivo*, are considerable and not easily predictable, neither from the type of target molecule nor from the nature of specific mutation. From these studies it is clear that the fitness cost associated with resistance strongly depends on growth conditions. For example, resistant mutants that show no cost in laboratory medium may have large costs in mice and conversely, mutants that show no cost in mice may have substantial costs *in vitro* (Bjorkman et al., 2000).

1. 5. 5. Compensatory evolution and restoration of the fitness cost of resistant bacteria

In the absence of antibiotics, evolution to reduce the cost of resistance by second-site mutations is more common than reversion to drug sensitivity (Table 3) (Andersson and Levin, 1999). This is mainly due to two processes: (i) compensatory mutations are more common than true reversion which

requires a specific single nucleotide substitution; (ii) serial passages are sometimes associated with severe population bottlenecks resulting in the selection of the most frequently occurring clones of mutants rather than the best (Levin, Perrot and Walker, 2000). Thus, low frequency revertants are rarely sufficiently common to be transferred in a serial passage experiment. In contrast, compensated mutants, which may be generally less fit than the true revertants, have a higher probability of being transferred in each passage simply because they are more common (Andersson, Björkman and Hughes, 2001). The physiological mechanisms by which compensatory mutations restore fitness have been determined for few cases. Compensation of the cost of streptomycin resistance in *E.coli* and *S.typhimurium rpsL* mutants can be achieved by extragenic mutations that restore the efficiency and rate of translation to nearly wild-type level (Björkman *et al*., 1999). Compensation of the rifampicin resistance cost in *rpoB* mutants is through intragenic compensatory mutants which restore the rate of transcription to wild type level (reviewed in Andersson, Björkman and Hughes, 2001). The degree of fitness restoration by the compensatory mutations varies greatly and in some cases restoration appears complete, whereas in others it is only partial. It seems that selection conditions affect the spectrum of mutations. One example is streptomycin resistant *rpsL* mutants in *S. typhimurium*. When selected in mice, compensatory mutations are only intragenic. In contrast, when selection for restored fitness is done in laboratory medium, compensation occurs by extragenic suppressor mutations (Björkman *et al*., 1998; Björkman *et al*., 1999, Björkman *et al*., 2000). Also, for fusidic acid resistant *fusA* mutants compensation occurs by different mutations *in vitro* and in experimental animals. In laboratory medium, compensation is almost exclusively by intragenic compensatory mutations whereas in mice almost only true revertants are found. When the relative fitness of the bacteria was determined by pairwise competitions in mice, only the true revertans were fully compensated whereas the intragenic second-site compensated mutants showed only partial compensation. In laboratory medium, both the intragenically compensated mutants and the true revertants grew as well as the wild type (Johansson *et al*., 1996). Thus, even though the revertant mutants were expected to be rarer than the second-site suppressors because of their smaller genetic target size, they were predominantly selected in mice because they were the only mutants that had a fully restored fitness.

1. 5. 6. The link between antibiotic resistance, fitness and virulence

In most cases, it is unclear how resistance affects virulence, the ability of a pathogen to survive, replicate and cause symptoms of disease in the infected host. In cases where both fitness (assessed by competition assays) and virulence (assessed by LD_{50} tests of similar assays) were measured, it is generally observed that resistant mutants show both decreased fitness and virulence. In the case of target-altering chromosomal mutations, this is not surprising if the spectrum of resistance genes is considered (Table 3). These resistance mutations are usually in the housekeeping genes that encode proteins involved in essential cellular processes, such as DNA replication, transcription and translation, which determine the growth rate. Consequently, resistance phenotype is often associated with reduced rate of process in which modified target encoded by mutated gene participates and, therefore, reduced fitness *in vitro* and *in vivo*. The question that can be posed here is whether products of housekeeping genes can be considered as virulence factors. An argument against this consideration might be that fitness-decrease and avirulence of bacteria with mutation in housekeeping genes stem from defects in essential cellular processes which generally affect the growth but might not at all

influence invasion, persistence or the development of disease symptoms. One way to answer this question is to assess the contribution of products of housekeeping genes at each stage of infection process for a given organism and identify whether they play role in invasion, persistence, reproduction within and interaction with the host.

At present, only one study addressed the question of actual mechanism by which resistance affects virulence properties of a pathogen. *M.bovis/tuberculosis* are resistant to isoniazid due to knock-out *katG* mutations which cause loss of catalase activity. This results in slower growth in experimental animals and avirulence as measured by competition experiments, LD_{50} tests and histopathology (i.e. granuloma formation) (Wilson *et al*., 1995; Zhonming *et al*., 1997). This is because *katG*-encoded catalase is the virulence factor for these organisms, mandatory for their survival and multiplication in macrophages and formation of granulomas. The majority of clinical isolates with the *katG* mutation also contain a promoter-up mutation in the *ahpC* gene which causes an increase in the level of alkyl hydroperoxidase reductase (AhpC). It is likely that overproduction of AhpC compensates for the lack of catalase in the isoniazid resistant *katG* mutants and restores virulence (Heym *et al*., 1997).

Table 3. The cost of resistance associated with target-altering chromosomal mutations and spectrum of compensatory mutations that restore fitness (Andersson, Björkman and Hughes, 2001)

Bacteria	Resistance	Mutation	Cost	Assay system	Compensatory mutation	Selection for compensation
Salmonella typhimurium	Streptomycin	*rpsL*	Yes/no	Mice, *in vitro*	Intragenic, *rpsL* Extragenic, *rpsD/E*	Mice *in vitro*
	Rifampicin	*rpoS*	Yes	Mice, *in vitro*	Intragenic, *rpoB*	Mice
	Fusidic acid	*fusA*	Yes/no	Mice, *in vitro*	Reversion to wild type Intragenic, *fusA*	Mice *in vitro*
E.coli	Streptomycin	*rpsL*	Yes/no	*in vitro*	Extragenic, *rpsD/E*	*in vitro*
	Rifampicin	*rpoB*	Yes/no	*in vitro*	Intragenic, *rpoB*	*in vitro*
S. aureus	Fusidic acid	*fus*	Yes/no	Rats, *in vitro*	Intragenic, *fus*	*in vitro*
M. tuberculosis	Isoniazid	*katG*	Yes	Mice	Extragenic, *ahpC*	Humans

2. PRESENT INVESTIGATION

The present study represents an attempt to address the question of the nature of fitness cost of antibiotic resistant bacteria and analyze the resistance phenotypes from physiological perspective. The rationale behind this approach stems from the fact that, so far, most of the studies aimed to answer whether a particular resistance mutation is associated with a cost, and does that cost vary under different environmental conditions. It is not always clear what makes the cost, i. e. why is a resistance phenotype associated with a fitness defect and loss of virulence. Also, we wanted to address the question whether resistance phenotypes are associated with alterations in bacterial physiological processes apart from the ones affected by the particular antibiotic to which resistance occurs.

In the present study, using model system of fusidic acid resistant (Fus^R) *Salmonella typhimurium*, the physiological alterations associated with resistance were identified and characterized. Explanation for decreased fitness of Fus^R mutants in different environments (in laboratory medium and in mouse infection model) is offered by:
-characterization of the translational defect of Fus^R mutant
-measurements of changes in the status of global regulators (ppGpp and RpoS)
-identification of condition of *in vivo* growth that impose fitness cost on Fus^R mutants

We have identified additional physiological changes associated with Fus^R mutants that support the concept of association of resistance with global physiological alterations. We have measured:
-Sensitivity to ROI
-Respiration rate
-Catalase activity and heme production
-Sensitivity to different classes of antibiotics

2. 1. The details

If you wish to gain knowledge of the forms of things, begin with the detail and only move from one detail to another when you have fixed the first firmly in your memory and become well acquainted with it.
Leonardo da Vinci, Codex Trivulzianus

2. 1. 1. Fus^R mutants are less fit than the wild type *in vitro* (Paper I)

For a set of eighteen Fus^R mutants with a single mutation in *fusA* gene encoding EF-G, exponential growth rates in glucose minimal medium were measured. It was observed that generally, these Fus^R mutants grow more slowly than the wild-type. The measured doubling times ranged from 48 minutes, which is close to the doubling time of the wild-type (47 minutes), to 91 minutes. Thus, the slowest mutant *fusA*1 grows at half the rate of the wild- type.

2. 1. 2. Fus^R mutants are defective in translation (Paper I)

For the given set of *fusA* mutants, translation elongation rates *in vivo* were calculated from the step time for the synthesis of β-galactosidase. Translation elongation rate of the wild type was 13.1 aa/s. Elongation rates of Fus^R mutants ranged from 12.7 aa/s measured for *fusA*8 mutant with wild-type-like doubling time to 8 aa/s for the slowest growing mutant *fusA*1. When elongation rate is plotted in function of growth rate, a strong positive correlation is observed.

Translational properties of the mutant EF-G protein isolated from the strain carrying *fusA*1 mutation were further investigated. The choice of this EF-G mutant was based on conclusion that, in respect to growth and elongation rate, *fusA*1 mutation encodes the most extreme EF-G form. The kinetics of the interaction between wild-type and mutant EF-G with the elongating ribosome during steady-state poly(Phe) synthesis was compared. An EF-G titration with limiting amounts of ribosome was performed to monitor the ribosome cycle. When ribosome is saturated with EF-G, there is no significant difference in k_{cat} for the wild-type (8.5 s^{-1}) and mutant (7.9 s^{-1}). However, $K_{cat}/k_{m,}$ which is parameter that describes the effective association rate between the ribosome and EF-G differ between wild-type and mutant EF-G. K_{cat}/k_m for the wild –type (74 μM^{-1} s^{-1}) is 3 fold higher than for mutant (21 μM^{-1} s^{-1}).
To explain this defect, EF-G cycle was studied. A ribosome titration with limiting amounts of EF-G was carried out. In this type of titration, the k_{cat}/K_m values for both wild –type (72 μM^{-1} s^{-1}) and mutant EF-G (22 μM^{-1} s^{-1}) are almost identical to the values determined in the EF-G titration. However, k_{cat} of mutant EF-G cycle (34 s^{-1}) is less than half of the wild-type EF-G (87 s^{-1}). Since no significant difference in the ribosome cycle time was observed when there is excess of EF-G, this difference in k_{cat} is suggestive of defect in regeneration of active EF-G·GTP from EF-G·GDP.

To investigate the interaction between EF-G and GTP, a GTP titration was carried out with limiting amounts of EF-G. In this assay, the translation rate is proportional to the concentration of EF-G·GTP, which enables determination of K_m for interaction between EF-G and GTP. Difference of more than 15 fold was observed between K_m for mutant (1045 μM) and wild-type EF-G (62μM), while k_{cat} for

both mutant and EF-G were similar. This means that when EF-G saturated with GTP, there is no difference in the rate of wild-type and mutant EF-G cycle. This finding confirms that defect of mutant EF-G in translation is defect in binding of GTP which results in reduced rate of exchange of EF-G·GDP to EF-G·GTP of the ribosome.

2. 1. 3. Fus^R mutants have unusual cell morphology (Paper I)

A microscopic examination of cell cultures of Fus^R mutants revealed that many cultures contained elongated cells. The large proportion of long filamentous cells was observed in cultures of *fusA*1. Clumps of cells were observed in cultures of *fusA*13, which explained observation that the cells in cultures of this mutant sediment much faster than wild-type cells when the test tubes were left on the bench. Cell size of Fus^R mutants from exponentially growing cultures was quantified by light scattering recorded by flow cytometry. This gave an estimate of distribution of cell sizes in each culture. The largest average cell size is found in cultures of the slowest growing mutant, *fusA*1. In general, for the set of eighteen Fus^R mutants, a strong negative correlation between cell size and growth rate was observed. This is an interesting finding since positive correlation between cell size and growth rate is usually observed (reviewed in the section 1. 1. 1.)

2. 1. 4. ppGpp binds to EF-G and inhibits translation (Paper I)

Unusual phenotype of negative correlation between the cell size and growth rate suggested a potential involvement of ppGpp in the morphology of Fus^R cells (discussed in the section 1.3.1.E). In order to establish whether ppGpp has effect on translation, elongation rate *in vitro* as a function of ppGpp concentration was measured for wild-type and mutant EF-G proteins from *fusA*1, *fusA*2 and *fusA*4. In *in vivo* growth and translation assays, f*usA*2 is very close to the wild-type, while *fusA*4 is intermediate between the wild-type and *fusA*1. In each case, translation rate was reduced by increasing ppGpp concentration, but the effect is most extreme for the mutant *fusA*1 EF-G. The concentration of ppGpp needed to reduce translation rate to half for the wild-type was 2.5-fold higher than the ppGpp concentration required to exert the same effect on mutant *fusA*1 EF-G. Mutant *fusA*4 EF-G is inhibited slightly more than wild-type, while *fusA*2 EF-G is less inhibited than wild-type. These results show that EF-G binds ppGpp and is inhibited in translation by that binding. Also, the effect of ppGpp on inhibition of translation (with the exception of *fusA*2) is more pronounced in the presence of mutant EF-G forms.

2. 1. 5. Fus^R mutants are less fit than the wild type *in vivo* (Paper II)

To determine fitness of Fus^R mutants *in vivo*, competition against a fusidic acid sensitive (Fus^S) wild-type in BALB/c mouse infection model using a peritoneal challenge was carried out. We found that *in vitro* unfit Fus^R mutants also show reduced fitness *in vivo*. In the case of the most extreme Fus^R phenotype encoded by *fusA*1, no viable cells were recovered from livers or spleens of animals three days after infection. Decreased fitness of Fus^R mutants *in vitro* correlates with their reduced translation elongation rates, and translational defect also may explain decreased fitness of these mutants *in vivo*.

To determine whether factors other than translation rate are relevant for fitness *in vivo*, we studied a collection of Fus^R mutants where the rate of protein synthesis was restored to the wild-type level.

These Fus^R mutants were selected for the fast growth *in vitro* from a strain carrying unfit *fusA*1 mutation. A set of mutants that are intragenically compensated was obtained, i.e. mutants carry secondary mutations within EF-G that restore fitness *in vitro*, measured as exponential growth rate in glucose minimal media. The alleles are referred to as *fusA*1-1 to *fusA*1-15 since these growth-rate-compensated, "GRC" mutants, in most cases, retained the original *fusA*1 mutation and resistance to fusidic acid. The fitness of strains carrying these mutations *in vivo* was assessed as described above. No correlation between the degree of fitness restoration *in vitro* versus *in vivo* was observed. Fitness of GRC mutant *in vitro* is almost fully restored, to within a few percent of the wild-type growth rate. *In vivo*, however, these same strains, although improved relative to the parental *fusA*1 strain, have generally very slow growth rates. This finding suggests that there are other factors than translation rate alone that influence fitness of Fus^R mutants *in vivo*. Moreover, it seems that mechanism by which EF-G mutations affect in vivo fitness may be independent from their effects on the growth rate measured *in vitro*.

2. 1. 6. ppGpp levels are perturbed in Fus^R mutants (Papers I and II)

The inhibition of EF-G function in translation by ppGpp suggested that levels of ppGpp might be perturbed in Fus^R mutants. One consequence of perturbed levels of global transcriptional regulator ppGpp might be alteration in gene expression. We hypothesised that such changes in transcriptional patterns could affect functions needed for the fast growth in mice. In order to assess whether ppGpp production is altered in Fus^R mutants, we measured basal and starvation-induced levels of ppGpp. Within the set of mutants with single mutation in *fusA* gene and reduced growth rates *in vitro*, we observed that both basal and starvation-induced ppGpp levels were different from the one measured for the wild-type. During exponential growth, ppGpp level in wild-type is 15 pmol/OD_{460}. In slow growing strain carrying *fusA*1 mutation, basal level is only 5 pmol/OD_{460}, whereas in fast growing *fusA*2 it is 25 pmol/OD_{460}. The exception is the strain with *fusA*4 mutation in which wild-type-like basal levels of ppGpp were measured. Under starvation induced by addition of α-methylglucoside, in the wild type 29% of GTP is converted into ppGpp. For the mutant strains, conversion of GTP into ppGpp is 10% for *fusA*1, 32% for *fusA*2 and 15% *fusA*4. In general, there seem to be a correlation between the growth rate *in vitro*, fitness *in vivo* and basal and starvation-induced levels of ppGpp.

To determine whether ppGpp levels in Fus^R mutants are perturbed via PSI or PSII pathway (section 1. 3. 1. A.), we measured basal ppGpp levels in wild-type and *fusA*1 strain in which ppGpp production is altered by functional knockouts in *relA* or *spoT*. In the *spoT*::*Tn*10 strain, both synthetic and hydrolytic activities of PSII are expected to be absent, and the measured 50 pmol/OD_{460} represents synthesis of ppGpp by PSI (the RelA protein). In the *relA*21::*Tn*10 strain, the only source of ppGpp synthesis is PSII, which also has hydrolysis activity, and we measured 5 pmol per OD_{460}. In the *spoT*::*Tn*10 strain, *fusA*1 reduces the level from 50 pmol /D_{460} unit down to 15 pmol/OD_{460} unit. In the *relA*21::Tn10 strain, the *fusA*1 mutation has only a marginal effect on the amount of ppGpp. Thus, *fusA*1 apparently influences the ppGpp level by altering the amount synthesized via the PSI (RelA) pathway.

Basal ppGpp levels in GRC Fus^R mutants show tendency towards full restoration. In the case of *fusA*1-8 and *fusA*1-14 they are fully restored to the wild-type level. In other GRC mutants, basal levels were ranging between 13 and 26 pmol/OD_{460}. No apparent correlation between fitness of GRC mutants *in vivo* and basal ppGpp levels was observed. Conversion of GTP into ppGpp upon starvation was restored to the wild-type level in the most in vivo fit GRC Fus^R mutants. However, the most unfit GRC mutants *fusA*1-14 and *fusA*1-15 show reduced production of ppGpp under starvation, converting only 21% and 19% of GTP into ppGpp, respectively.

2. 1. 7. Fus^R mutants have reduced fitness in macrophage infection model (Paper II)

During infection, *Salmonella* resides within macrophages which represent nutrient-restrictive environment rich in antimicrobial compounds (reviewed in the section 1. 1. 2.). The capacity to survive within macrophages is an absolute requirement for *Salmonella* virulence and fitness *in vivo*. We tested the ability of three GRC mutants (*fusA*1-14, *fusA*1-15 and *fusA*1-7) and parental *fusA*1 strain to compete with wild-type in a macrophage infection model. The order in which these four Fus^R mutants were ranked in fitness under macrophage growth conditions was the same as that observed in the BALB/c *in vivo* model. Three Fus^R mutants (*fusA*1, *fusA*1-14 and *fusA*1-15) which were previously found to be unfit *in vivo* were also unfit in competitions against the wild-type in the macrophage assay. However, *fusA*1-7 effectively competed with the wild-type in macrophages although it is unfit *in vivo*. This finding suggests that, in the more complex *in vivo* environment, *fusA*1-7 might encounter stresses that are not present in the macrophage assay which lower its fitness.

2. 1. 8. Fus^R mutants are sensitive to the presence of hydrogen peroxide *in vitro* and *in vivo* (Paper II)

One of the main determinants for killing of *Salmonella* by macrophages is H_2O_2 produced during respiratory burst (section 1. 1. 2.). We tested the survival of 10^6cells/ml of Fus^R mutants in glucose minimal medium supplemented with 70μM H_2O_2. This concentration was used because it approximates the concentration generated during respiratory burst (Gonzales-Flecha and Demple, 2000; Kaul and Forman, 1996; Vasquez-Torres et al., 2000a). Bacterial growth was initially inhibited for several hours after which a decrease in the viable count (c.f.u. /ml) was observed. For the wild-type and the fittest Fus^R strain (*fusA*1-1), number of viable cells decreased around two logs. For mutant carrying *fusA*1-15, decrease in number of viable cells was around 5 logs. After 18 hours of incubation, no viable cells of mutant with the least fit allele, *fusA*1, were observed. With exception of *fusA*1, each of the strain resumed growth and by 36 hours had reached a density of ~ 10^9 cells/ml. The relative sensitivity of different Fus^R mutants to H_2O_2 correlated with their relative *in vivo* fitness in the BALB/c mouse infection model.

To test whether Fus^R mutants are sensitive to the presence of H_2O_2 *in vivo*, we compared their competitive ability against the wild-type in two different mouse strains: a wild type C57BL/6 mice and an isogenic *Cybb* strain which is unable to undergo respiratory burst because of a targeted mutation in NADPH cytochrome oxidase. We find that the fitness of three unfit Fus^R (*fusA*1, *fusA*1-14 and *fusA*1-15) mutants is improved in the *Cybb* mice by about 40-fold. This result suggests that sensitivity to H_2O_2 is and important determinant of *in vivo* fitness of Fus^R mutants.

2. 1. 9. Fus^R mutants have reduced expression of *rpoS* (Paper II)

Perturbed levels of ppGpp, together with decreased fitness *in vivo* lead us to investigate *rpoS* expression in Fus^R mutants. Synthesis of RpoS, which is virulence factor of *Salmonella* and essential for infection, is positively regulated by ppGpp (section 1. 3. 1. C). Translational and transcriptional expression of *rpoS* during growth in LB medium was assessed using *rpoS-lacZ* fusions in genetic background of three Fus^R mutants and wild-type. Relative to the wild-type, each of the Fus^R mutants tested induced *rpoS-lacZ* expression to a lesser extent upon entry into stationary phase. In stationary phase, translational expression of unfit *fusA*1 was 23% of the wild-type level. Unfit GRC *fusA*1-15 mutant showed 54% and fit *fusA*1-1 76% of the wild type level of translational expression in stationary phase. Assays with transcriptional *rpoS-lacZ* fusions demonstrated that, of the three Fus^R mutants, only *fusA*1 mutation had significantly lower expression of around 50% of the wild-type level in stationary phase. Taken together, the measurements of *rpoS-lacZ* expression suggest that Fus^R mutants are defective in inducing *rpoS* upon entry into stationary phase and that this defect is more pronounced at translational level.

2. 1. 10. Fus^R mutants have reduced catalase activity (Papers II and III)

The sensitivity of Fus^R mutants to H_2O_2 suggested that they might have reduced catalase activity (section 1. 4.). Measurements of catalase activity revealed that Fus^R mutants generally have lower catalase activity than the wild-type. The most reduced catalase activity was associated with unfit strains carrying *fusA*1 and *fusA*1-15 mutations (35% and 45% of the wild-type catalase activity, respectively). To elucidate whether the reduced catalase activity in Fus^R mutants is associated with reduced activity of particular catalase species (HPI or HPII; reviewed in the section 1. 4.), strains carrying functional knockouts affecting either HPI or HPII were constructed in the genetic background of the wild-type and one representative Fus^R strain (*fusA*1-15). Catalase activity measurements showed that presence of *fusA*1-15 mutation was not associated with reduction of activity of one type of catalase but rather, it reduced both HPI and HPII activities to the less than half the value measured in the equivalent *fus*$^+$ strain.

2. 1. 11. Fus^R mutants have reduced levels of heme (paper III)

In order to be active, HPI and HPII catalase require heme as co-factor (see section 1. 4.). Reduced activity of both catalases suggested that Fus^R mutants might be defective in production of heme. The expression of *hemA* which mediates the first commited step in the heme biosynthesis (section 1. 4.) was measured in the wild-type and two Fus^R mutants using *hemA-lacZ* operon fusion. We found that both Fus^R mutants tested have decreased level of transcription of the fusion relative to the wild-type (64% of the wild-type level of *hemA-lacZ* expression is measured in the strain with *fusA*1 and 85% with *fusA*1-15). In addition, growth of Fus^R mutants in the presence of precursor of heme biosynthesis, ALA, rendered cells tolerant to H_2O_2-mediated killing. Also, addition of glutamine or glutamate to growth medium has a large affect in ameliorating the sensitivity of *fusA*1 strain to H_2O_2. Since biosynthesis of heme begins with charging of tRNA^{Glu} with glutamate, the increased sensitivity of *fusA*1 to H_2O_2 may be due to combination of decreased *hemA* expression and reduced intracellular levels of glutamate available for tRNA^{Glu} charging. Taken together, these results suggest that increased

sensitivity of Fus^R mutants to hydrogen peroxide is associated with reduced flow through heme biosynthesis pathway.

2. 1. 12. Fus^R mutants are defective in aerobic respiration (Paper III)

One consequence of decreased heme production might be respiratory defect since cytochromes of respiratory chain require heme as cofactor (reviewed in the section 1. 4.). Measurements of rate of oxygen consumption during exponential growth revealed that Fus^R mutants respire with reduced rate relative to the wild-type. The aerobic respiration rate associated with *fusA*1 is 44% of the rate measured in the wild-type and 77% for *fusA*1-15.

2. 1. 13. Iron mediated DNA damage in Fus^R mutants (Paper III)

Defect in respiration are associated with increased rate of iron-mediated DNA damage. This is because respiratory deficiency results in accumulation of cytosolic reductants, such as NADH, which can reduce free iron. In the presence of H_2O_2, the ferrous iron thus formed will donate an electron and generate a hydroxyl radical which can damage DNA (reviewed in the section 1. 4.). Sequestration of ferrous iron in respiratory-deficient strains results in their improved survival in the presence of H_2O_2. We incubated cultures of wild-type and Fus^R mutant with 2, 2'-dipyridil (an iron chelator that penetrates cells and chelates intracellular ferrous iron) prior to exposure of bacteria to H_2O_2. Results suggested that decreased survival of Fus^R mutants in the presence of H_2O_2 is associated with iron mediated DNA damage since reduction of intracellular ferrous iron resulted in improved survival in the presence of H_2O_2.

2. 1. 14. Fus^R mutants are sensitive to several classes of antibiotics (Paper IV)

We examined survival of Fus^R mutants upon exposure to UV light and observed that *fusA*1 strain is more sensitive in this assay than the wild-type. We than tested whether Fus^R mutants are sensitive to agents that generate strand breaks in DNA, such as the antibiotic norfloxacin. The assay was made as an eighteen-hour competition in LB medium with a starting inoculum of 10^6 cells/ml of wild-type and either Fus^R mutant (permitting about 10 generations of growth). The influence of norfloxacin concentrations in the range of 0.1 - ~1 x MIC value (MIC determined by E-test) on strain competitiveness was monitored. For each of the three strains norfloxacin MIC was identical. We defined the ratio of Fus^R/wild-type after eighteen hours of growth in the absence of antibiotic as a normalised competitive index (NCI) of 1. Fot the *fusA*1 mutant we observed that in the range 0.1 - ~0.5 x wild-type MIC (MIC_{wt}) the NCI decreased from 1 down to 10^{-4}. Thereafter, the NCI improved because the wild-type was also killed at higher norfloxacin concentrations. A similar pattern of decreasing NCI was also observed with *fusA*1-15 although the decrease was not as great as with *fusA*1. We concluded that the two Fus^R mutants are hypersensitive to the fluoroquinolone antibiotic norfloxacin in this assay, even though a standard MIC test showed no significant differences in susceptibility between the wild-type and mutant strains.

Altered ppGpp levels can result in global alterations of transcriptional patterns in Fus^R mutants. We reasoned that these alterations could affect different cellular processes, including transcription, translation and cell wall synthesis. We therefore measured the susceptibility of Fus^R mutants to

antibiotics that inhibit these cellular processes. Competition assays were made, as described above for norfloxacin, between Fus^R mutants and the antibiotic-sensitive wild-type in the presence of various concentrations of rifampicin, streptomycin, chloramphenicol, penicillin G and ampicillin up to MIC_{wt}. Rifampicin is an antibiotic that inhibits RNA transcription. In the presence of rifampicin, the NCI of the *fusA*1 strain decreased to $\sim 10^{-4}$ at 0.5 x MIC_{wt} showing that the strain is hypersensitive to sub-MIC levels of this antibiotic.

Streptomycin binds to the ribosome in the vicinity of the decoding A-site. E-test showed that *fusA*1 (MIC 16) is more sensitive than wild-type (MIC 32) to streptomycin. In the competition assay the NCI was 4 x 10^{-4} at 0.25 x MIC_{mutant} and decreased further to $\sim 10^{-5}$ at MIC_{mutant}. At MIC_{wt} (where the starting inoculum of 10^6 wild-type cells was viable), no Fus^R cells remained viable. Thus the *fusA*1 mutant is extremely sensitive to sub-MIC concentration of streptomycin. In contrast, the *fusA*1-15 mutant was relatively unaffected, with NCI values close to 1 at all concentrations.

Chloramphenicol is another antibiotic that inhibits protein synthesis, in this case by binding to the 50S ribosomal subunit and inhibiting peptide bond formation. E-tests showed that *fusA*1 is marginally more sensitive to chloramphenicol than the wild-type. In the growth competition assay the NCI of *fusA*1 was decreased approximately one hundred fold in the range 0.25 – 0.5 x MIC_{wt}.

β-lactam antibiotics inhibit peptidoglycan synthesis and thus prevent cells from building the cell walls they require for growth. Sensitivity to β-lactams results in lysis of growing cells. E-tests showed that *fusA*1 is slightly more sensitive to the β-lactam antibiotics ampicillin and penicillin G than is the wild-type. Liquid growth competition assays in the presence of ampicillin or penicillin G showed that for *fusA*1 the NCI values decreased to between 10^{-2} and 10^{-3} in the range of 0.1 – 1 x MIC_{wt}. Taken together, these results with various antibiotics suggest that Fus^R mutants are more sensitive than an isogenic antibiotic-sensitive wild-type to antibiotics that inhibit replication, translation and cell wall synthesis.

2. 2. The picture

I put together the pieces of my jigsaw with great care…..my only problem is choice.
Salvador Dalí, "Dali" by Robert Descharnes and Gilles Néret

The results presented in this study are based on fusidic acid resistance (Fus^R). This is used as a model system of antibiotic resistance mediated by a chromosomally-encoded target alteration, in genetically well defined and extensively studied organism *Salmonela typhimurium*. The generality of findings presented in this study, in terms of their relevance to other target alterations and antibiotics, remains to be determined. Accordingly, the ideas and suggestions discussed in this concluding remark should be considered as concepts which are inviting further examination in other bacterial species and/or with other types of antibiotic resistance.

A general conclusion emerging from the results presented in this study is that Fus^R phenotypes are pleiotropic and are associated with broad physiological changes. In the case of this model system of Fus^R *Salmonella typhimurium*, physiological alterations so far identified occur at three levels:

-First, changes are associated with the mechanism of resistance itself. In the case of Fus^R mutations, it is alteration of the target of antibiotic binding, i.e. EF-G. Alterations in EF-G are associated with a defect in GTP binding which results in reduced rate of regeneration of active EF-G·GTP complex off the ribosome. The consequence of this defect is reduced translation elongation rate which results in slower growth of Fus^R bacteria *in vitro* and *in vivo*.

-Second, changes occur in the synthesis of global regulatory molecules. Fus^R mutants have perturbed levels of global transcriptional regulator ppGpp, which can result in altered expression patterns of many genes. One identified consequence of reduced ppGpp levels in Fus^R mutants is reduced production of RpoS, a master regulator of stress response which is mandatory for survival of *Salmonella in vivo*. Reduced production of RpoS observed in Fus^R mutants can explain their reduced *in vivo* fitness and virulence.

-Third, in Fus^R mutants, the synthesis of enzyme co-factor heme is reduced. Heme is necessary for the activity of catalases and cytochromes of respiratory chain. Reduced synthesis of heme results in reduced ability of Fus^R mutants to withstand oxidative stress, a reduced aerobic respiration rate, and cell death caused by DNA damage.

The different levels at which these physiological changes occur may be connected and, in some cases, it is possible that alterations at one level are consequences of defects observed at other levels. For example, reduced levels of ppGpp in the most extreme Fus^R phenotype encoded by *fusA*1, could be explained by its translational defect which results in slow elongation rate. The model is that if the cells translate slowly, than the proportion of time that A-site of the ribosome is empty, and can accept uncharged aa-tRNA, is smaller. The presence of uncharged tRNA at A-site is signal for ribosome-

associated RelA to synthesize ppGpp. The production of ppGpp in this mutant is perturbed via the *relA*-pathway. This model predicts that one consequence of slow translation elongation would be reduced production of ppGpp. However, this model can not explain altered ppGpp levels observed in GRC Fus^R mutants with restored translation rates. An alternative proposal that could account for perturbed ppGpp levels in GRC Fus^R mutants is that EF-G may interact with RelA on the ribosome and thus directly influence the amount of ppGpp produced. There are indications that both EF-G and RelA might be present in close proximity on the ribosome. EF-G is positioned close to ribosomal protein L11 which was shown to be required for ppGpp synthesis *in vivo*.

Reduced ppGpp levels result in reduced levels of RpoS. Interestingly, effects of *fusA* mutations on expression of *rpoS* are more pronounced on translational then transcriptional level. One possible explanation is that the effect of ppGpp level on translational expression of *rpoS* is indirect. Thus, ppGpp may be required for synthesis of some additional factor, such as Hfq, HU or DsrA, which can bind to *rpoS* mRNA and facilitate translational initiation.

Reduced production of co-factor heme can not be fully explained by perturbations in ppGpp levels, since *relA*21::*Tn*10 and *fusA*1 strains have similar basal ppGpp levels but different levels of *hemA-lacZ* expression (89% and 64% of the wild type level, respectively). However, the slow rate of protein synthesis associated with *fusA*1 might independently affect the level of heme.

We also studied how these physiological changes affect fitness of Fus^R mutants. In the cases where fitness cost *in vitro* was observed, it was accompanied with reduced fitness *in vivo*. Growth rates of Fus^R mutants *in vitro* strongly correlate with elongation rates and translational defect which result in reduced elongation rates of Fus^R mutants can, in principle, explain their reduced fitness *in vitro* and *in vivo*. However, a more complex situation is observed in which mutants with no apparent fitness cost *in vitro* have reduced fitness *in vivo*. GRC Fus^R mutants with restored growth rates in laboratory medium are still unfit in the mouse infection model. This difference in fitness reflects difference in nutrient availability between laboratory medium and more complex *in vivo* environment. Also, it suggests that demands for expression of genes which are important for survival and growth of *Salmonella in vivo* are not fulfilled in Fus^R mutants. Inefficient induction of *rpoS* which is important for survival of *Salmonella* in nutrient-limited environment of host can explain why there is a fitness cost associated with *in vivo* growth of Fus^R mutants. There is also a possibility that Fus^R mutants are defective in induction of other important virulence factors, which might require ppGpp for their expression. Observed phenotypes of reduced fitness of Fus^R mutants in macrophage infection model and in the presence of H_2O_2 *in vivo,* as well as improved fitness in the absence of H_2O_2 *in vivo* suggest that these mutants might be defective in the expression of SPI-2 type III secretion system (TTSS) which mediate exclusion of NADPH oxidase from *Salmonella* containing vacule within macrophages (reviewed in the section 1. 1. 2.).

The results presented in this thesis lead to formulation of the term “physiological cost of resistance”, which refers to the nature of fitness cost of an antibiotic resistant bacterium in a particular environment. This term emphasizes the importance of identification of the actual physiological change

that causes reduced fitness under given conditions of growth. The knowledge of physiological cost could be important for two reasons:

-It could suggest a spectrum of targets for design of novel antimicrobial agents

Altered production of global regulatory molecules clearly affects bacterial fitness. One can imagine development of the novel class of antibiotics which as a target have global regulators. For example, molecules which bind to RpoS and reduce its free concentration in the cells could be used as antimicrobial agents against *Salmonella* and other enteric bacteria in which RpoS plays role of virulence factor. Also, ppGpp (in form which allows uptake of intact molecule by bacterial cells) could be used as an antimicrobial agent, since at present it seems that no analogues of this molecule are found in human cells and maintenance of proper ppGpp levels is mandatory for bacterial growth. Alternatively, molecules that alter ppGpp production through interaction with ppGpp-synthesizing enzymes could have negative effect on bacterial fitness. Another type of antibiotic would be those that affect the status of enzyme co-factors. For example, molecules that block heme synthesis, reduce its free concentration or prevent iron binding by heme could be used to increase sensitivity of intracellular bacteria to oxidative stress during respiratory burst.

-It could help in establishing the strategy for treatment of resistant bacteria

Identifying whether there are any physiological changes associated with resistance to a particular antibiotic and determining actual alterations could be used in assessing the possibility to successfully treat particular resistant mutants with other antibiotics. If there is a physiological cost associated with resistance mutation, sensitivity to antibiotic that target altered process can be tested. Alternatively, competition assays between resistant mutants and wild-type in sub-MIC range of different antibiotics could be done without any prior knowledge of physiological cost. The prediction is that, if particular resistant strain is unfit in sub-MIC range of an antibiotic, it is more likely that it can be effectively treated with clinically relevant antibiotic concentrations which usually exceed MIC several folds.

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