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Stringent Response in Bacterial Growth and Survival

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Stringent Response in Bacterial Growth and Survival

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF PUBLICATIONS

The thesis is based on the following publications that will be referred to in the text by their Roman numerals:

Ref. I Putrinš M, Kogermann K, Lukk E, Lippus M, **Varik V** & Tenson T (2015). Phenotypic heterogeneity enables uropathogenic *Escherichia coli* to evade killing by antibiotics and serum complement. Infection and Immunity 83, 1056–67.

Ref. II **Varik V**, Oliveira SRA, Hauryliuk V & Tenson T (2016). Composition of the outgrowth medium modulates wake-up kinetics and ampicillin sensitivity of stringent and relaxed *Escherichia coli*. Scientific Reports 6, 22308.

Ref. III Andresen L*, **Varik V***, Tozawa Y, Jimmy S, Lindberg S, Tenson T & Hauryliuk V (2016). Auxotrophy-based High Throughput Screening assay for the identification of Bacillus subtilis stringent response inhibitors. Scientific Reports 6, 35824.

* Equal contribution

My contribution to the papers:

Ref. I Performed the experiments to set up the methodology for serum killing of the bacteria and flow cytometry assays and participated in writing the manuscript.

Ref. II Performed all the experiments, except the ampicillin killing assay, analyzed the data, participated in writing the manuscript, and as a co-corresponding author prepared the submission to the journal.

Ref. III Performed the experiments adjusting the assay to high throughput layout, wrote the automated data analysis script, analyzed the data and participated in writing the manuscript.

LIST OF ABBREVIATIONS

ACP	Acyl Carrier Protein
CFU	Colony Forming Unit
CRP	cAMP Response Protein
csr	Carbon storage regulatory (network)
GASP	Growth Advantage in Stationary Phase (phenotype)
HD	Histidine-Aspartate (conserved residues in active center of RSH enzymes)
HPF	Hibernation Promoting Factor
HTS	High Throughput Screening
MIC	Minimal Inhibitory Concentration
ppGpp	Guanosine tetraphosphate i.e. guanosine-5',3'-bispyrophosphate
pppGpp	Guanosine pentaphosphate i.e. guanosine-5'-triphosphate-3'-diphosphate
(p)ppGpp	ppGpp and/or pppGpp
RMF	Ribosome Modulation Factor
RSH	RelA/SpoT Homologues
SAH	Small Alarmone Hydrolase
SAS	Small Alarmone Synthetase
SCDI	Stationary phase Contact-Dependent Inhibition
UPEC	Uropathogenic *Escherichia coli*
VBNC	Viable But Nonculturable

INTRODUCTION

In the struggle for survival, the fittest win out at the expense of their rivals because they succeed in adapting themselves best to their environment.
Charles Darwin

Bacteria, free living single cellular organisms, are tightly exposed to their environment. Both biotic and abiotic forces shape their fate. During the time of plentiful, many bacterial species are able to grow astonishingly fast. This itself, however, leads to a rapid and inevitable change – the bacteria exhaust the growth supporting potential of the environment.

In changing environments, in order to adjust and to survive, several mechanisms are in place. One of the most widespread bacterial strategies against perturbations and survival during harsh times is a mechanism called the stringent response. In case of a bacterial infection, not surprisingly, the host is hostile. As expected, the stringent response is important for pathogenic bacteria both to establish an infection and to endure. Therefore, the stringent response is not only of great intellectual interest of a few, instead, it affects the general public, in health and in sickness. That is not to say, in life and death.

In the following pages, I will describe the life-style of bacteria in growth and survival with an emphasis on the role of the stringent response. No attempt was made to be exhaustive – way more is known about the subjects that could contain in the thesis – I will portray just inasmuch as is necessary to support the inquiries taken and follow the results obtained. As it is often the case in studies of bacterial physiology, although contemporary science is advancing into new bacterial species at ever increasing pace, the treatise will be heavily biased towards *Escherichia coli*. It becomes also obvious very quickly – out of all the major biological processes, the author is most familiar with the translation.

1. OVERVIEW OF LITERATURE

1.1. Growth

This treatise is much about bacterial cell physiology in batch culture. It is nonsense to talk about cell physiology without specifying the strain and the growth conditions – the nutritional, chemical, and physical environment. The resulting growth of bacterial culture can be described by extensive or intensive properties (**Fig. 1**). Extensive properties include the amounts of different components of the culture – protein, DNA, RNA etc. Intensive properties describe the distribution of the extensive properties i.e. distribution of cells in terms of total protein, DNA, RNA content. Other most common intensive properties are cell size and age. During unrestricted growth, *extensive* parameters increase by the same factor over the time, this condition was aptly termed 'balanced growth' by Campbell (Campbell, 1957). It should be perhaps emphasized that studies of balanced growth cultures describe an average cell, yet individual cells can be very different. During steady state, however, the *intensive* parameters are invariant. Thus, steady state is the most strict term and implies both exponential and balanced growth. The sloppiest one of the three terms – in addition to balanced and steady state growth – is exponential growth, it implies, if anything, that cells were growing experimentally unperturbed. All that said, it is hard to find the homogeneous, unaltered environment necessary for steady, balanced or exponential growth of bacterial population in their natural environment.

Cell growth comprises of increase in cell mass followed by cell division. One of the most remarkable and well known features of bacteria is the speed at which they – though, of course, not all of the species – are able to grow and divide, in favorable environments. Laboratory *E. coli* can form two new daughter cells astonishingly fast, in 20 minutes. As pointed out by Postgate (Postgate, 1994), if it were sustainable for long periods of time, *E. coli* culture would equal the mass of earth in mere three days. Instead, cells eventually run out of nutrients – more generally speaking, change their growth environment – and slow down the growth. Now we encounter the next key feature of bacterial cells, their endurance to withstand stasis, long periods in harsh environments of little or no growth. Not only do they survive the specific starvation which they encounter, but develop resistance to other stresses, as if adhering to the Nietzche's "That which does not kill us makes us stronger". An important aim as well as a direct measure of survival is the ability to resume growth when conditions become favorable.

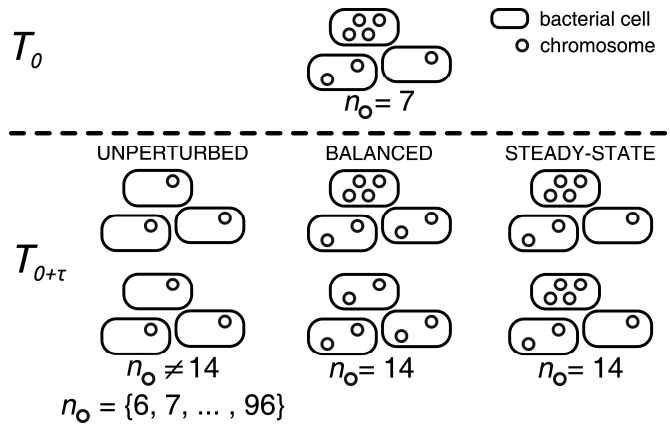

Figure 1. The unrestricted growth of bacterial cultures can be described by extensive or intensive properties. Let us suppose that we are interested in the amount of DNA in the culture, which is our extensive property of interest. Let it also hold true that cells have been growing some time in the medium and when we finally start our experiment (T_0), one third of the cells have either one, two, or four chromosomes. For the sake of the brevity, therefore, the cells shown on figure describe the exact distribution of the chromosomes among cell population. The very distribution of chromosomes is our intensive property of interest. Then, one culture doubling time later ($T_{0+\tau}$), we observe that the number of cells has faithfully increased twice. If the amount of chromosomes, however, has increased by anything else than factor of two, the growth is unperturbed at most. If the number of chromosomes has, indeed, increased by factor of two, but the distribution of chromosomes has changed, the cells are in balanced growth. Only if the number of chromosomes has increased twice and the distribution of the chromosomes between the cells stays unaltered, the culture is growing in steady state.

Next couple of chapters will briefly visit the key features of bacterial cell growth and stasis survival of non-sporulating bacteria.

1.1.1. Balanced growth and its rate

When bacteria have adjusted to the growth environment, after some cell divisions, they achieve a balanced growth i.e. all cell constituents begin to increase by the same proportion over the same interval of time. This situation can be approximated for some time in laboratory.

In batch culture, unchanged environment is often an assumption which can be checked empirically by following the increase in cell mass – exponential increase of cell mass by at least factor of 10 is a good starting point. Obviously, not all changes in cell physiology can be detected this way. Because of the often imperceptible changes, it is safe to assume that all the properties of the cells remain the same only if the density of the culture is sufficiently low, a long before the growth is slowing down. Usually less than 10^8 cells per milliliter is safe.

Although balanced growth does not occur for long periods in natural environments, it is desirable to achieve it in laboratory conditions for one reason: it is highly reproducible. First, one can sample the culture at any time point during the balanced growth and the sample content is identical. Second, if you know the growth rate, a single measurement of DNA, mRNA, ribosome content and so on, will tell you the absolute rate of synthesis (e.g. femtograms/ bacterium/minute). Third, given its highly reproducible nature, it is the only way to *directly* compare the results from different laboratories (Neidhardt, 2006).

In balanced growth culture, bacteria divide asynchronously. Even if starting from a single cell and sustaining the environment unchanged, small deviations in division time (coefficient of variation around 20% (Schaechter *et al.*, 1962)) will inevitably result in an asynchronous culture. Measurements at the level of cell culture, therefore, represent an average over all cells. This average does not necessarily describe even the majority of the cells, in fact, if the property in question has a binomial distribution, average does not describe almost any cell in the population. Fortunately, given the cumulative action of components of complex systems – to which biological ones belong, even if dissected to the level of single molecules – the rising distribution of a feature can often be approximated by a normal distribution. Note that chemical processes, to which biological ones rely on, are multiplicative and therefore a log transformation is often necessary to this approximation of a normal distribution (Galton, 1879; McAlister, 1879).

Bacterial cells divide by a binary fission, an auto-catalytic first-order reaction for which the rate constant can be derived from a simple exponential equation. Therefore, we can calculate the number of cells (or, in fact, any extensive property of cell culture by):

$$N_t = N_{t_0} 2^n \tag{1}$$

Where N_t is the number of cells at a timepoint of interest (at time t), N_t is the number of cells at some previous timepoint and n is the number of doublings the culture has gone through, during the time interval ($\Delta t = t - t_0$). Next, n can be substituted by a time it takes to double, a doubling time, τ ($n=\Delta t/\tau$), or better yet, with a reciprocal of doubling time, growth rate μ ($n=\mu\Delta t$). After taking $\log_2$ and rearranging the equation, we arrive at:

$$\mu = \frac{\log_2 N_t - \log_2 N_{t_0}}{\Delta t} \tag{2}$$

It is simple and straightforward, however, some care should be taken to distinguish μ and τ from μ_e and τ_e, the latter two can be derived by starting from:

$$N_t = N_{t_0} e^n \tag{3}$$

or from:

$$\frac{dN}{dt} = \mu_e N \tag{4}$$

after rearranging and integrating (from t_0 to t), in μ_e.

Since μ_e is solved on base of e, it tells us *instantaneous* growth rate, i.e. how many times per hour does the culture increase e-fold (2.718-fold, unit: h^{-1}). Conventional μ – which can be denoted as μ_2 to distinguish it from μ_e and can be derived from the latter by simple division ($\mu_2=\mu_e/\ln 2$) – is more intuitive to biologists, because it tells how many doublings are the cells going through per hour (unit: doublings$\times h^{-1}$). Both growth rate notations are in use and almost never specified. Hereafter, μ refers to conventional growth rate i.e. expresses doublings per unit of time.

1.1.2. Exponential phase

As we saw in last chapter, growth rate constant (μ), the only parameter to solve for quantitative description of balanced bacterial growth, is located in exponent of equation for binary fission ($N_t = N_{t_0} 2^{\mu \Delta t}$). Bacteria of unrestricted balanced growth, i.e. cells grow at maximum growth rate achievable in particular medium, are therefore told to be in exponential growth phase, sometimes the word logarithmic is used instead (**Fig. 2**). Note that for practical purposes, however, it is mostly impossible to tell a difference between exponential and linear growth during one division cycle – the difference is just too subtle. Only after a couple of divisions, the pattern emerges.

Given the condition of balanced growth, the macromolecular composition of exponentially growing bacterial cells, on average, has a universal correlation with growth rate. Faster growing cells have more RNA, protein, DNA, and the cells are larger (Schaechter *et al.*, 1958). In this relationship, with a growth rate as independent variable, the most rapid change is for RNA, followed by cell mass and protein abundance and the slowest increase is for DNA content. It follows that faster growing cells have a higher RNA-per-protein ratio, an important hint for the reasons of some of the observed phenomena. Indeed, faster growing cells are enriched in RNA because the intracellular content is shifted in favor of more ribosomes per cell. As a result, the RNA-to-protein ratio increases because ribosomes are composed of more RNA than protein (~65% RNA and ~35% protein; in contrast, average *E. coli* cell at 40 minute doubling time, 21% of RNA and 55% of protein (Neidhardt & Umbarger, 1996)). The most common and long withstanding explanation for the positive correlation between the ribosome content and growth rate is that the speed of protein chain elongation by ribosome is kept maximal whenever possible (close to 22 amino acids/per second (Dennis & Bremer, 1974; Dennis & Nomura, 1974; Young & Bremer,

1976)) and thus, instead of making the ribosomes work considerably faster or slower, the cells adjust the amount of ribosomes. We will return to this question in chapter 1.2.2.

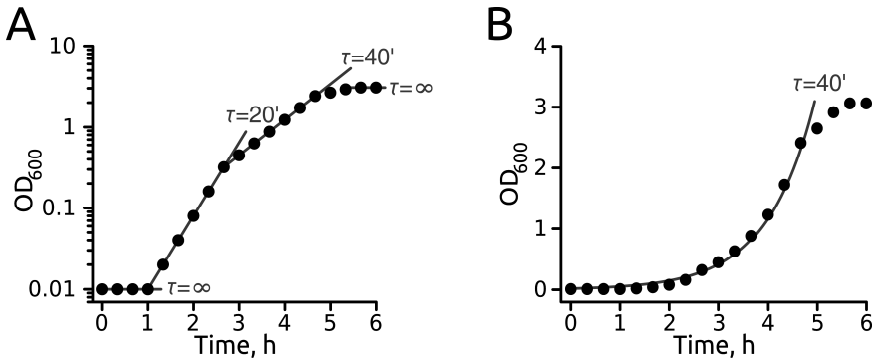

Figure 2. Bacteria of unrestricted balanced growth are growing exponentially. (A) When inoculated into a new medium, as described in chapter 1.1.3.3., cells often do not start to grow right away (first hour, doubling time, τ, is infinitely long), but when they finally do, the growth – followed here by measuring the culture turbidity at 600 nm – is exponential as is evident from the straight line in logarithmic scale (τ=20 min). Rapid exponential growth, however, changes the composition of medium, upon which cells can assume some different growth rate (τ=40 min) before they stop growing altogether (τ=∞). **(B)** Exactly the same growth curve as in (A), except plotted in linear scale. Importantly, most of the information contained in (A) is lost and, instead, experimentalist might be led to believe there is about two hour growth lag followed by exponential growth with a doubling time of 40 minutes.

In rich nutritious environment with less of an anabolic burden, *E. coli* cells can divide very rapidly – two new daughter cells are formed in 20 minutes. Yet the chromosome replication (called C period) itself takes about 40 minutes and cell division process (called D period) takes about 20 minutes in rapidly growing *E. coli* cells (Bipatnath *et al.*, 1998; Cooper & Helmstetter, 1968). In order to have cell division in every 20 minutes, therefore, cells start a new round of DNA replication before the previous round has been finished (Helmstetter & Cooper, 1968). This explains why there is more DNA in faster growing cells. It also implies that when *E. coli* is grown in rich medium (i.e. LB), for the chromosome region around the origin of replication the gene copy number, in some of the cells, is as high as 16 (Akerlund *et al.*, 1995; Hill *et al.*, 2012; Nielsen *et al.*, 2007). Finally, because the DNA replication is generally initiated when cells have acquired certain mass (Bipatnath *et al.*, 1998; Cooper, 1997; Donachie, 1968; Hill *et al.*, 2012; Wold *et al.*, 1994), cells with multiple rounds of replication must be larger. So, *E. coli* cells at 2.5 doublings/h are six times larger than when growing at 0.6 doublings/h (Schaechter *et al.*, 1958).

1.1.3. Stationary phase

1.1.3.1. Transition

During transition to stationary phase, the growth of bacteria becomes unbalanced. Some process – say, protein synthesis – becomes suboptimal, limiting, and slows down well before the others. This results in three things worth mentioning. First, the composition of the culture is changing and, therefore, sampling timepoints are not equal any more. Second, the culture composition depends on the particular circumstances of growth stop – i.e. starvation for carbon, or that for a nitrogen, accumulation of waste products and so on – each of which results in different culture composition and bacterial physiology (Peterson *et al.*, 2005). Variable combinations of circumstances can act simultaneously, further complicating the state of affairs. Finally, the cell-to-cell variability increases considerably, initially it is phenotypic, but eventually genetic heterogeneity will emerge, too (Finkel & Kolter, 1999).

If cells grow in a defined minimal medium, the run-out of a single essential component results in abrupt cessation of growth. In complex media, often of undefined composition, growth stop is usually more gradual as cells exhaust several components one after another (Sezonov *et al.*, 2007), also the pH might become unfavorable (Wilson *et al.*, 2003), upon which cells adjust and continue growing at slower rate, possibly in several subsequent steps, before final stop. In modern microbiologist's favorite medium, Lysogeny broth (LB), balanced growth can be disturbed already at OD_{600} 0.3 (Fig. 2A), most probably when cells run out of residual glucose (LB is not supplemented with glucose, it originates, in variable amounts, from the yeast cell extract). Thus some experimentalist prolong the first, hopefully balanced growth phase by supplementing glucose to the LB.

Regardless the reasons for stasis, there are still some overarching principles common to cells entering the stationary phase: (i) the number of chromosomes approaches an integer; (ii) cells get smaller in size; (iii) active ribosomes are converted into inactive ribosome dimers. If the exponential phase environment supports fast enough growth to have several copies of chromosome (see chapter 1.1.2), then, during transition to stationary, new rounds of DNA synthesis are not initiated yet the elongation continues to termination. During that time, cells do not grow much – it is, after-all, during conditions that do not allow growth of cell mass – but do still divide, therefore, cells get smaller in size, this process is called reductive cell division (Lange & Hengge-Aronis, 1991a; Nyström *et al.*, 1996). Besides reducing the number of chromosomes and increasing the number of cells, it improves surface-to-volume ratio – all of which might improve survival. Resulting stationary phase cells can still contain several copies of chromosome, in case of rich complex medium up to 8 chromosomes, with 2 and 4 being the most common (Akerlund *et al.*, 1995; Boye & Løbner-Olesen, 1991).

Excess protein synthesis is curtailed by converting ribosomes into translationally inactive 100S dimers, owing to the concerted action of ribosome

modulation factor RMF (Wada *et al.*, 1990) and hibernation promoting factor HPF (Ueta *et al.*, 2005). 100S dimers seem to function as a inactive storage form of ribosomes (Wada, 1998; Wada *et al.*, 1990, 1995; Yamagishi *et al.*, 1993), energetically expensive molecular machinery, to be rapidly utilized whenever the conditions permit a rapid growth again (Aiso *et al.*, 2005; Yamagishi *et al.*, 1993). The importance of 100S ribosomes is further emphasized by the fact that during prolonged starvation, 100S ribosome dissociation is correlated with the lost viability of the cells (Wada, 1998). In addition to ribosome storage in 100S, rRNA becomes less stable as rRNA degradation is activated (Gausing, 1977; Hsu *et al.*, 1994; Maiväli *et al.*, 2013).

Much of the transition of cells into and survival throughout the stationary phase depends on the *rpoS* which encodes a master regulator, stationary phase and starvation specific sigma factor σ^S (Loewen & Hengge-Aronis, 1994). Sigma factors bind the core RNA polymerase to program the pattern of promoter recognition and thus direct the transcription at genome-wide scale (Österberg *et al.*, 2011). σ^S is close relative to the exponential growth phase sigma factor σ^{70} and, accordingly, the two factors recognize similar, but not identical promoters (Typas *et al.*, 2007). Directly and indirectly, RpoS activates about 10% of *E. coli* genes (Weber *et al.*, 2005). For example, the cells produced during reductive cell division (see above) are often coccoid in shape, which depends on BolA morphogene upregulated by *RpoS,* during entry into stationary phase (Lange & Hengge-Aronis, 1991a).

1.1.3.2. Duration

Besides numerous morphological changes – to name some: cells are smaller in size, the cell wall is more highly cross-linked, cytoplasm is condensed, and periplasmic space is increased (Huisman *et al.*, 1996) – stationary phase cells are remarkably resistant against different stress factors, such as high salt (Jenkins *et al.*, 1990), heat shock and hydrogen peroxide (Jenkins *et al.*, 1988; Lange & Hengge-Aronis, 1991b; McCann *et al.*, 1991). The feature is called cross protection. Development of the resistance depends on protein synthesis during first couple of hours of starvation (Jenkins *et al.*, 1988) and requires RpoS (Hengge-Aronis, 1993). Accordingly, *rpoS* mutants have decreased viability in stationary phase (Boaretti *et al.*, 2003; Lange & Hengge-Aronis, 1991b). The σ^S transcription factor is therefore the master regulator of an important stasis survival regulon which comprises of diverse set of proteins involved in central metabolism, stress response, cell morphology, mutation rates and virulence (Schellhorn, 2014). In addition, several global regulators, sigma factors (σ^{70}, σ^{FliE}, σ^E and σ^{54}), flagellar master regulator FlhDC and small alarmones cAMP, (p)ppGpp, and c-di-GMP work in concert (Hengge, 2011).

A common theme in stationary phase survival is that cells fight with the accumulation of oxidative damage. Accordingly, omitting oxygen protects *E. coli* cells from losing viability during starvation (Dukan & Nystrom, 1998) and

rpoS mutants display elevated levels of oxidative damage (Dukan & Nystrom, 1998; Dukan & Nyström, 1999). Interestingly, some proteins are more susceptible than others (Dukan & Nystrom, 1998) and translational accuracy of the ribosome has been proposed to be responsible for some of the effects (Ballesteros *et al.*, 2001). Oxidative damage appears to be the bacterial counterpart of the free radical hypothesis of aging in case of higher organisms, accumulation of oxidative damage by reactive oxygen species produced by normal metabolism (Finkel & Holbrook, 2000).

Among oxidative damage, carbonylation is of special interest. Carbonylation happens to arginine, lysine, proline and threonine and appears to be irreversible, so that the only way to get rid of potentially damaged proteins is via degradation (Nyström, 2005). Furthermore, protein turnover, providing material for *de novo* protein synthesis in growth arrested cells, is suggested to be necessary for long term survival and development of general resistance to multiple stresses (Matin, 1991; Reeve *et al.*, 1984; Weichart *et al.*, 2003). Regardless of removal by degradation, accumulation of carbonylated proteins has been described in stationary phase *E. coli* (Desnues *et al.*, 2003).

After some time in stationary phase (about 1–2 days in LB, but longer in minimal media), during which the number of CFUs stays unaltered, the CFUs start to decline few orders of magnitude. This phase is called the death phase. Some of the phenomenon can be accounted for conversion of cells into viable but nonculturable state (VBNC, see below) – a fraction of the population loses the capability to form colonies on agar medium plates yet stays viable, as assessed by membrane potential, membrane integrity, and measures of intracellular enzymatic activity. Accordingly, VBNC cells have higher levels of irreversible oxidative damage, proteins are more carbonylated (Desnues *et al.*, 2003). Nevertheless, in some cells, eventually also in VBNC cells, respirations stops and cells lose membrane potential. Again, RpoS is important to enhance the growth resumption and prolong the VBNC duration before cell death (Boaretti *et al.*, 2003).

Long-term starvation conditions come with their own set of interesting phenomena, too numerous to cover here in detail and will therefore be just mentioned. First, the GASP phenotype – as stationary phase cell cultures are highly dynamic (Zambrano & Kolter, 1996), populations evolve, some that are so adapted to stationary phase that they take over the culture. Furthermore, genetic instability is induced by RpoS driven expression of error-prone DNA polymerases to increase the chances for useful mutations (Saint-Ruf & Matic, 2006). Second, there is VBNC mentioned above – cells lose the ability to form colonies, but remain viable and potentially able to restart growth (Oliver, 2005). Third, Lemonnier and colleagues described stationary phase contact-dependent inhibition (SCDI, (Lemonnier *et al.*, 2008), which manifests itself in emerging variants that appear to kill or inhibit the growth of parent strain. Although there are some similarities with GASP, the authors claim the processes to be functionally and genetically different.

1.1.3.3. Exit

After transfer to a new medium, composition of which is different from previous, cells do not necessarily start to grow and divide right away at the maximum speed supported by the new conditions. Instead, there is a certain period of delay during which the cells adjust to the new environment (Buchanan, 1918; Monod, 1949). This period is also called a lag phase. In comparison with exponential and stationary, way less is known about the lag phase despite its perceived importance to infection development and food safety.

Deceivingly trivial, however, there are several definitions of the lag phase (Madar *et al.*, 2013) and even more ways to quantify it (Swinnen *et al.*, 2004). There are two main reasons for the multitude of definitions of what exactly comprises a lag phase. First, after transition to a new medium, cells initially grow bigger and only then, at some point, start to divide. This results in a period when turbidity of the culture increases, but the number of cells stays the same. Second, cells do not start to grow right away at maximal growth rate, there is an acceleration period. Note that before any indication of growth, regardless if defined by increase in mass or cell number, once the new substrate becomes available, starving cells respond with increased respiration and proteins synthesis almost instantaneously (Albertson *et al.*, 1990; Flardh & Kjelleberg, 1994). This fact makes use of some biochemical or molecular marker to define lag phase equally ambiguous.

The duration of the lag phase depends on the extent of the adjustments necessary to start the growth. Length of a lag phase is therefore in positive correlation with the length of a stationary phase (Albertson *et al.*, 1990; Amy *et al.*, 1983). This substantiates the suggestions that besides induction and/or activation of new enzymes at levels appropriate to new condition, some of the lag phase might account for repairing of the damage that has accumulated during stationary phase (Dukan & Nystrom, 1998; Dukan & Nyström, 1999).

Two specific and characteristic regulators of growth resumption are certainly worthy of note. First, RMF protein, responsible for ribosome dimerization (see chapter 1.1.3.1), facilitates faster growth resumption of *B. subtilis* (Akanuma *et al.*, 2016). Second, a small basic DNA-binding protein Fis regulates several processes important during growth resumption, *viz.* initiation of DNA replication (Filutowicz *et al.*, 1992), and transcription of rRNA (Nilsson *et al.*, 1990; Ross *et al.*, 1990). Further, *fis* expression – both mRNA and protein – is upregulated during growth resumption right before the number of cells starts to increase both in *Salmonella* and in *E. coli* (Ball *et al.*, 1992; Ninnemann *et al.*, 1992; Osuna *et al.*, 1995). Yet the knock-out mutant strains of *Salmonella* show relatively small (about +20 min per otherwise 120 min lag phase) delay in LB, absent in glucose minimal medium (Osuna *et al.*, 1995), and in *E. coli*, too, Fis protein appears dispensable for growth resumption (Rolfe *et al.*, 2012).

Regulation of lag phase correlates with the invasiveness of bacterial cells (Bättig *et al.*, 2006; Hathaway *et al.*, 2012) and affects antibiotic treatment of bacterial infections (Fridman *et al.*, 2014; Frimodt-Møller *et al.*, 1983). Most

bactericidal antimicrobials need some active target and thus are way less effective on non-growing bacteria, say, those of a lag phase. Indeed, wake-up kinetics determine the abundance of persister cells (Balaban *et al.*, 2004; Jõers *et al.*, 2010; Luidalepp *et al.*, 2011) – phenotypically different subset of parental population that is highly tolerant to antibiotic in question – which can result in failure of antibiotic treatment (Harms *et al.*, 2016; Lewis, 2007, 2010). Here, a cautionary note is appropriate – lag phase is just one facet of the persister phenomena, which probably is comprised of multiple heterogeneous states of bacterial physiology (Allison *et al.*, 2011b; Kaldalu *et al.*, 2016). We will briefly return to the issue of persisters in chapter 1.2.5.

1.2. The stringent response

In rapidly growing bacterial cells, proteins and stable RNA account for three fourths of the dry weight of the cell (Neidhardt & Umbarger, 1996) and, accordingly, about 70% of the cellular energy is devoted to translation (Russell & Cook, 1995). Thus, growth rate can be approximated by the concentration of ribosomes multiplied by the rate of peptide chain elongation (Dennis *et al.*, 2004). One would expect, therefore, that perturbations in translation machinery trigger a response that adjusts cell physiology to new conditions. Further, since production of ribosomes is controlled by the synthesis of rRNA (Paul *et al.*, 2004), one would expect the regulatory mechanism to affect most strongly the very process of rRNA transcription. That global response, indeed, is in place. It is termed the stringent response, adjustment program that, upon perturbations in environment, adjusts the physiology of the cell and results in a new, appropriate growth rate – including zero growth rate i.e. growth stop, if appropriate.

The stringent response is orchestrated by an accumulation of a nucleotide alarmones ppGpp and pppGpp, collectively (p)ppGpp. Acute accumulation of the alarmones re-allocates cellular resources away from rapid proliferation towards stress resistance and survival. The hallmark of the stringent response is, upon amino acid starvation, rapid curtailment of stable RNA synthesis (Paul *et al.*, 2004) and upregulation of amino acid biosynthesis (Traxler *et al.*, 2008). The wild-type strains are termed stringent and mutant strains that fail to do so, are called relaxed – the mutant cells continue to synthesize high levels of stable RNA when environmental conditions have become unfavorable for rapid growth, therefore, for rapid translation (see above). However, (p)ppGpp is a global regulator affecting all major synthetic activities of the cell: transcription, translation and replication. Moreover, (p)ppGpp exerts its regulatory role during unperturbed conditions, too.

As a result, stringent response has been implicated in virulence (Dalebroux *et al.*, 2010) and antibiotic survival (Doherty *et al.*, 2010; Harms *et al.*, 2016).

1.2.1. RelA

In many bacteria, upon sensing of uncharged tRNA in the ribosomal A-site (Haseltine & Block, 1973; Pedersen *et al.*, 1973), an adjustment program, termed stringent response, is activated (Stent & Brenner, 1961) (**Fig. 3**). Although uncharged tRNA binds the A-site with smaller affinity than charged tRNA (Rheinberger *et al.*, 1981; Schilling-Bartetzko *et al.*, 1992), the binding is facilitated by a drop in tRNA charging from 80–100% (Dittmar *et al.*, 2005; Ezekiel, 1964; Yegian *et al.*, 1966) to 5–40% during amino acid starvation (Böck *et al.*, 1966; Dittmar *et al.*, 2005; Ezekiel, 1964; Morris & DeMoss, 1965; Sørensen, 2001; Yegian *et al.*, 1966). Further, the transient interaction with the ribosome, in contrast to more permanent, is optimal for stringent response (Wendrich *et al.*, 2002).

In *E. coli*, the enzyme responsible for sensing uncharged tRNA in ribosomal A-site is RelA (Haseltine & Block, 1973), which, upon activation, catalyzes a pyrophosphoryl group transfer from ATP to 3' position of either GTP or GDP (Cochran & Byrne, 1974; Haseltine *et al.*, 1972; Haseltine & Block, 1973; Sy & Lipmann, 1973) resulting in pppGpp or ppGpp, respectively (**Fig. 3**). k_{cat} and K_m of RelA are similar for both GDP and GTP (Cochran & Byrne, 1974; Justesen *et al.*, 1986; Pedersen & Kjeldgaard, 1977). Given that the K_m is about 0.3–0.5 mM, i.e. higher than concentration of intracellular GDP and lower than GTP (Bochner & Ames, 1982; Buckstein *et al.*, 2008), it follows that pppGpp should be the main product. *In vivo*, during amino acid starvation, it is therefore mostly pppGpp that is synthesized (Fiil *et al.*, 1977; Weyer *et al.*, 1976). In most laboratory conditions studied, however, the pppGpp is rapidly hydrolyzed to ppGpp by an enzyme GppA (Somerville & Ahmed, 1979)(**Fig. 4A**). The only physiological difference know between the guanosine tetra- and pentaphosphate is quantitative, not qualitative, *viz.* ppGpp appears more potent in *E. coli* (Maciag *et al.*, 2010; Mechold *et al.*, 2013; Rymer *et al.*, 2012) (however, see also (Steinchen *et al.*, 2015)), therefore the two nucleotides are often considered together and collectively called (p)ppGpp.

Besides cognate uncharged tRNA in the ribosomal A-site, the synthesis activity of RelA needs, for yet to be determined reasons, ribosomal protein L11 (Friesen *et al.*, 1974; Parker *et al.*, 1976; Wendrich *et al.*, 2002). Finally, RelA synthetase activity is induced by alcohols, both *in vivo* (Mitchell & Lucas-Lenard, 1980) and *in vitro* (Sy *et al.*, 1973) Methanol stimulated activity of purified RelA without any ribosomes (Sy *et al.*, 1973) was the early indication that it is RelA and not the ribosome itself – for example, upon stimulation by RelA – that catalyzes the reaction of (p)ppGpp formation.

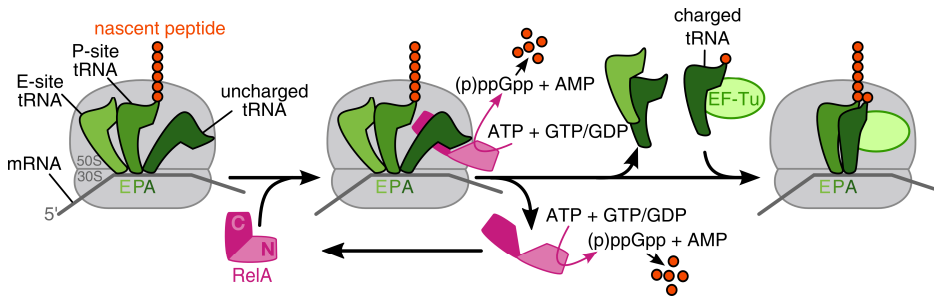

Figure 3. The defeat of the tRNA aminoacylation to keep up with the demand of protein synthesis triggers a regulatory adjustment process in bacteria called the stringent response. Amino acid starvation results in accumulation of uncharged tRNA which binds to the ribosomal A-site. Such ribosomes, with nonenzymatically bound uncharged cognate tRNA in A-site, are recognized by RelA protein. The C-terminal regulatory part of RelA binds to the ribosome and wraps around the tRNA establishing the tRNA in distorted shape as compared to enzymatically accommodated A-site tRNA, whereas the N-terminal part of RelA synthesizes (p)ppGpp from ATP and GTP or GDP. It is still open to dispute whether most of the (p)ppGpp synthesis takes part while RelA is still bound on the ribosome or off the ribosome. Nonetheless, (p)ppGpp acts as a global and pleiotropic regulator which ultimately results in the the restoration of amino-acylation of tRNAs. Once again charged, cognate tRNAs are delivered to the ribosome the usual enzymatic way by EF-Tu and replace the weakly bound uncharged tRNA in A-site. Note that it results also concomitant release of the E-site bound tRNA.

The structure of RelA bound to the ribosome reveals a highly distorted tRNA (Agirrezabala *et al.*, 2013; Arenz *et al.*, 2016; Brown *et al.*, 2016) with the C-terminal part of RelA buried deep into the ribosome and wrapped around tRNA (**Fig 3**). Since the N-terminal part of RelA has catalytic functions (Schreiber *et al.*, 1991; Svitil *et al.*, 1993) and the C-terminal part has regulatory functions (Gropp *et al.*, 2001; Schreiber *et al.*, 1991; Svitil *et al.*, 1993; Yang & Ishiguro, 2001a), it has been proposed that embedding of C-terminus facilitates the active conformation of RelA (Arenz *et al.*, 2016). Regardless of embedding, interference with regulation by tagging the C-terminus might still account for some of the controversies in reports. Earlier biochemical studies, with native RelA, suggested that RelA stays on the ribosome (Richter, 1976). Then, working *in vitro* with C-terminal histidine tagged RelA, it was suggested that RelA comes off from the ribosome upon activation (Wendrich *et al.*, 2002). In addition, it was speculated that RelA binds back quickly, "hops" from ribosome to ribosome – inferred from the fact that (p)ppGpp synthesis of RelA was six times faster when ribosomes were in vast excess (10-fold). The following *in vivo* single-molecule tracking study, using C-terminal fluorescent label, supported the view of RelA being active off the ribosome, however, it argued against hopping (English *et al.*, 2011). However, next *in vivo* single molecule study, this time comparing couple of fluorescent labels in C-terminus and having longer linkers between the label and RelA, suggested that RelA is off the ribosome in

unstressed conditions, amino acid starvation induces binding to the ribosome and (p)ppGpp is synthesized while RelA is bound to the ribosome (Li *et al.*, 2016).

The molecular model of binding and activity, when finally solved, must take into account a couple of things. First, there are way more ribosomes than RelA molecules in the *E. coli* cell (one molecule per 200 ribosomes (Pedersen & Kjeldgaard, 1977)). Second, it should account for at least some of the (p)ppGpp-independent effects of starvation. For example, protein synthesis is inhibited already by low level accumulation of uncharged tRNA long before RelA is stimulated (Rojiani *et al.*, 1989, 1990). Third, RelA is somehow regulated by oligomerization (Gropp *et al.*, 2001; Yang & Ishiguro, 2001a), possibly by forming dimers (Yang & Ishiguro, 2001b), and feedback-stimulated by its product (Shyp *et al.*, 2012), although, inhibited at yet higher (p)ppGpp levels (Beljantseva *et al.*, 2017a). Finally, it might be crucial to include the role of toxin-antitoxin systems in the model (Christensen & Gerdes, 2004; Diderichsen *et al.*, 1977; Maisonneuve *et al.*, 2013; Tian *et al.*, 2016).

When does a bacterial cell have to deal with a lot of uncharged tRNA? In laboratory conditions, stringent response is elicited by either (i) reducing the availability of amino acids or (ii) reducing the aminoacylation of tRNA. In addition to uncharged tRNA, cell wall driven regulation of RelA activity has also been proposed (Kusser & Ishiguro, 1987). Finally, (p)ppGpp accumulation is induced by heat shock (Braedt & Gallant, 1977; Lemaux *et al.*, 1978; Lund & Kjeldgaard, 1972), though how exactly and to what extent is RelA activated, is yet unclear. In unperturbed conditions and during slow transitions, in fact, (p)ppGpp has a role too, probably it is fine tuning the bacterial physiology in accordance with growth potential of the environment. Accordingly, in exponentially growing *E. coli* cells with disruption of the RelA function, (p)ppGpp is still present (although at about 3-fold lower levels) (Gallant *et al.*, 1970) – most of the (p)ppGpp in unperturbed conditions originates from yet another enzyme, SpoT which will be portrayed in the next chapter.

1.2.2. SpoT

Early studies with mostly non functional (p)ppGpp synthetase version of RelA (encoded by a *relA1*, an allele that has an amino terminal IS*2* insertion (Metzger *et al.*, 1989) and is wide-spread among laboratory strains of *E. coli*) suggested that there must be an alternative, perhaps a weaker, source of (p)ppGpp synthesis activity (Atherly, 1979; Friesen *et al.*, 1978). Indeed, in *E. coli* and in other gamma-proteobacteria, (p)ppGpp can also be synthesized by a homologous protein, SpoT (Laffler & Gallant, 1974; Stamminger & Lazzarini, 1974). *E. coli* strains devoid of both RelA and SpoT lack ppGpp altogether, a phenotype designated (p)ppGpp^0, which renders cells to severe multiple amino acid auxotrophy (Xiao *et al.*, 1991).

SpoT synthetase activity is triggered in response to starvations of various nature: carbon (Lazzarini *et al.*, 1971), iron (Vinella *et al.*, 2005), fatty-acid (Seyfzadeh *et al.*, 1993), and phosphate (Lazzarini *et al.*, 1971; Spira *et al.*, 1995) (**Fig. 4A**). In addition, osmotic (Harshman & Yamazaki, 1972) and heat shock (Gallant *et al.*, 1977) induce the (p)ppGpp synthesis activity of SpoT. In good accordance with the more moderate nature, SpoT is responsible for maintaining the ppGpp levels during steady-state growth conditions (Murray & Bremer, 1996; Ryals *et al.*, 1982).

Besides weak synthesis activity, SpoT also hydrolyses (p)ppGpp and is responsible for the main (p)ppGpp degradation activity in the cell (An *et al.*, 1979; Heinemeyer *et al.*, 1978; Heinemeyer & Richter, 1978). In wild-type *relA* background, therefore, *spoT* appears essential (Xiao *et al.*, 1991) – since high (p)ppGpp levels stop the cell growth (see chapter 1.2.4), most simple explanation is that one just does not reap $relA^{+}$ $spoT^{-}$ cells, they do not grow. Experimental validation to that claim, i.e. by *spoT* knock-down, has not been reported. The hydrolysis domain of SpoT consists of conserved His-Asp (HD) residues (Aravind & Koonin, 1998) and requires Mn^{2+} for the activity (Johnson *et al.*, 1979; Sy, 1977).

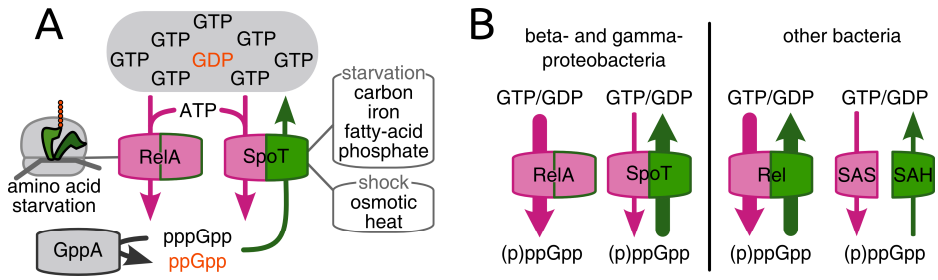

Figure 4. (p)ppGpp has a role beyond the amino acid starvation and family of proteins responsible for its metabolism. (A) In *E. coli*, in addition to the RelA, there is a homologous protein SpoT that is responsible for both (p)ppGpp production (pink) and hydrolysis (green). At least for RelA, specificity constants for GTP and GDP are similar, yet there is about seven GTP molecules per one GDP in exponentially growing bacterial cells, therefore, mostly likely pppGpp is the major product. pppGpp, nonetheless, is rapidly turned into ppGpp by GppA. While synthesis activity of RelA is triggered by amino acid starvation, SpoT is regulated by various environmental cues, net outcome of which is determined by the balance between its hydrolysis and synthesis activities. Note that RelA and SpoT are very similar, so that former has also (p)ppGpp hydrolysis domain albeit inactive. **(B)** In contrast to RelA (strong synthetase, thick pink arrow) and SpoT (weak synthetase, thin pink arrow; strong hydrolase, thick green arrow) which are common only in beta- and gamma-proteobacteria, most of the bacterial species contain but one homologous bifunctional protein (with fairly strong activity of (p)ppGpp synthesis and hydrolysis). Furthermore, many of the bacteria have accessory small proteins, with only synthetase or hydrolase domain.

Regulation of the SpoT activities is complex and only some of the interaction partners of SpoT have been elucidated. A GTPase Obg (also called ObgE, YhbZ or CgtA) seems to inhibit the (p)ppGpp synthesis activity of SpoT in rapid growth conditions (Jiang *et al.*, 2007; Raskin *et al.*, 2007; Wout *et al.*, 2004). In addition, acyl carrier protein (ACP) stimulates SpoT-dependent (p)ppGpp accumulation during fatty acid starvation (Battesti & Bouveret, 2006).

Regardless of the interaction partners, the balance between (p)ppGpp hydrolysis and synthesis activities is the key mechanism for adjustment of (p)ppGpp levels by SpoT (Gallant *et al.*, 1972; Murray & Bremer, 1996). For example, during carbon source shift-down, both ppGpp synthesis and degradation are inhibited, but the degradation is reduced to greater extent giving the net expansion of (p)ppGpp pool (Friesen *et al.*, 1975; Murray & Bremer, 1996). In case of single amino acid starvation, both activities are again inhibited, but the synthesis is inhibited more (accumulation of uncharged tRNA inhibits degradation (An *et al.*, 1979; Richter, 1980), however, since overall (p)ppGpp levels drop in amino acid starved *relA* mutants (Ryals *et al.*, 1982), it can be deduced that synthesis is inhibited to greater extent). In contrast, during multiple amino acid starvation, the synthesis is stimulated and degradation inhibited (Murray & Bremer, 1996).

The wide variety of environmental inputs that result in the regulation of SpoT has been perplexing. Complicating the studies, extracts of *E. coli* have no SpoT dependent synthetase activity (Heinemeyer & Richter, 1977). In an attempt to explain most of the observed phenomena, the following model has been proposed (Bremer & Dennis, 2008; Dennis *et al.*, 2004; Ehrenberg & Kurland, 1984). SpoT monitors the functioning of the ribosome so that its synthetase activity is stimulated whenever the peptide chain elongation rate is suboptimal – due to molecular crowding or decreased charging of several tRNAs (see chapter 1.2.4). There is, however, an additional constraint – SpoT synthetase activity is unstable (Murray & Bremer, 1996), it requires protein synthesis and thus disappears when protein synthesis stops altogether. The model definitely has its appeal as it is capable to adjust other sensory inputs and interaction partners (*viz.* Obg, ACP, see above), i.e. is open to refinement in details, yet explains a wide variety of observations.

1.2.3. The rest of the RelA/SpoT family

Sequence analysis reveals that combination of RelA and SpoT has very limited phylogenetic distribution – they are present only in among beta- and gamma-proteobacteria (Atkinson *et al.*, 2011). In the majority of bacterial species, there is but one full-length protein, with both synthetase and hydrolase activity, termed Rel, *viz*. Rel_{Bsu} or Rel_{Mtb} for the one of *B. subtilis* and *Mycobacterium tuberculosis*, respectively (**Fig. 4B**). In addition, in many species, the (p)ppGpp levels are regulated by small alarmone synthetase (SAS), which contain only of (p)ppGpp synthetase domain, and small alarmone hydrolases (SAH), which

contain only hydrolase domain (**Fig. 4B**). Not much is known about bacterial SAHs, however, SASs have been studied in *B. subtilis* (Nanamiya *et al.*, 2008; Steinchen *et al.*, 2015)*, Enterococcus faecalis* (Abranches et al., 2009; Beljantseva et al., 2017b; Gaca et al., 2015), *Streptococcus mutans* (Lemos *et al.*, 2007), and *Vibrio cholerae* (Das *et al.*, 2009).

Similarly to RelA, (p)ppGpp synthesis activity of Rel is activated in response to amino acid starvation in some bacterial species including *E. faecalis* (Gaca *et al.*, 2012), *Streptomyces coelicolor* (Martínez-Costa *et al.*, 1998; Strauch *et al.*, 1991), and *Myxococcus xanthus* (Harris *et al.*, 1998). In *M. tuberculosi*s, Rel_{Mtb} synthetase is regulated by its product (Syal *et al.*, 2015), activated by uncharged tRNA in ribosomal A-site (Avarbock *et al.*, 2000), however, *in vivo* experiments with amino acid starvation failed to induce (p)ppGpp accumulation, instead, removal of all nutrients was necessary (Primm *et al.*, 2000). Regardless of the reasons for the discrepancy, examples of species that do not produce (p)ppGpp upon amino acid starvation are well documented and include *Rhodobacter sphaeroides* (Acosta & Lueking, 1987; Eccleston & Gray, 1973)*, Rhizobium meliloti* strain 41 (Belitsky & Kari, 1982)*, Rhizobium tropici, Azobacter vinelandii, Azomonas agilis* (Howorth & England, 1999). Some of the species are still stringent, *R. meliloti* 41 and *R. sphaeroides* restrict stable RNA synthesis upon amino acid starvation, yet do it without the involvement of (p)ppGpp (Acosta & Lueking, 1987; Belitsky & Kari, 1982; Eccleston & Gray, 1973). Furthermore, they do produce (p)ppGpp, *R. meliloti* 41 in carbon and ammonium deprivation (Belitsky & Kari, 1982), and *R. sphaerodies*, photosynthetic bacterium, upon decrease in light intensity (Eccleston & Gray, 1973).

In *Caulobacter crescentus* (Chiaverotti *et al.*, 1981) and *Helicobacter pylori* (Scoarughi *et al.*, 1999) the amino acid starvation does not trigger (p)ppGpp synthesis nor reduction in stable RNA transcription. Again, however, those species are able to synthesize (p)ppGpp. In *C. crescentus,* (p)ppGpp is accumulating in response to starvation for carbon or nitrogen but not for phosphate (Boutte & Crosson, 2011; Chiaverotti *et al.*, 1981; Lesley & Shapiro, 2008; Ronneau *et al.*, 2016). Interestingly, although the synthetase activity was indifferent to amino acid starvation, the functioning of ribosomes was still important for the control of the activity of Rel_{Cc}, aligning well with the proposed role of SpoT in governance of translational machinery (see chapter 1.2.2). In *H. pylori*, (p)ppGpp accumulates in response to carbon and serum starvation and acid stress (Wells & Gaynor, 2006; Zhou *et al.*, 2008). Both bacteria are able to restrict stable RNA synthesis when (p)ppGpp is synthesized (Amemiya, 1991; Wells & Gaynor, 2006).

Given the differences in stresses that trigger stringent response in various bacteria, be it assessed by stable RNA curtailment on (p)ppGpp accumulation, it has been proposed that these follow the adaptations to particular lifestyles of each species (Boutte & Crosson, 2013).

1.2.4. (p)ppGpp in growth and survival

Growth rate control is a term used to describe the systematic variation of bacterial cell composition at different growth rates, most remarkably described by seminal work of Schaechter *et al.* (Schaechter *et al.*, 1958): given the balanced growth, there is exponentially more RNA, DNA and cell mass per cell at higher growth rates (see chapter 1.1.2). This entails that the synthesis rates, per cell, are higher (see chapter 1.1.1). The largest difference is for RNA, followed by cell mass and DNA. When cells are shifted from low to high growth rate, or vice versa, per cell, the RNA and protein production are regulated first, while DNA synthesis and the speed of cell division follow later (Brunschede *et al.*, 1977; Kjeldgaard *et al.*, 1958).

Growth rate and (p)ppGpp levels are inversely correlated, e.g. slowly growing cells have higher (p)ppGpp levels (Ryals *et al.*, 1982). Higher (p)ppGpp levels result in lower amounts of stable RNA synthesis and this reduction in translation apparatus results in slower growth (amount of ribosomes × amino acid synthesis rate ~ growth rate; see introduction to chapter 1.2). However as trivial as it might seem, there are couple of complications to establish such a simple causal relationship. First, transitions, necessary to alter (p)ppGpp levels, will have profound secondary effects on cellular metabolism in itself. To that end, studies with inducible expression of (p)ppGpp synthesis without any starvation (Rodionov & Ishiguro, 1995; Schreiber *et al.*, 1991; Svitil *et al.*, 1993) and experiments with ppGpp^0 strain are instrumental. Note that the ppGpp^0 strains are genetically instable, however, expert advice for working with them can be found from the literature (Potrykus *et al.*, 2010). Second, regulation at transcription level, the hallmark of stringent response, can not act quickly. This implies that other processes – toxin-antitoxin systems (Harms *et al.*, 2016), trans-translation (Christensen & Gerdes, 2003, 2004; Li *et al.*, 2008), to name a few – are integral part of the regulation and will introduce other inputs. Third, direct and indirect effects of (p)ppGpp are very likely to act simultaneously.

Much of the growth rate control by (p)ppGpp can be explained by assuming that the translational speed of ribosome, although it can vary two-fold (Bremer & Dennis, 1996), is kept close to maximal possible. Therefore, the major mode to increase the production of protein at higher growth rates is to increase the amount of ribosomes. When the speed of ribosome becomes suboptimal, probably detected by SpoT (see chapter 1.2.2), (p)ppGpp starts to curtail the amount of stable RNA therefore reducing the abundance of translational machinery (Bremer & Dennis, 2008; Dennis *et al.*, 2004; Ehrenberg & Kurland, 1984). Some recent analysis has suggested that molecular crowding might be a major factor that limits the maximum speed of translation making it to deviate from constant speed at all times (Klumpp *et al.*, 2013).

During severe starvation, when the zero growth rate is appropriate, besides the curtailment of activities required for rapid proliferation, (p)ppGpp induces the increase in σ^S levels (Gentry *et al.*, 1993), transcription of the ribosome dimerization factors *rmf* (Izutsu *et al.*, 2001), downregulation of the *fis*

promoter (Ninnemann *et al.*, 1992). Also osmoprotectants and/or storage compounds, such as glycogen and polyphosphate, accumulate (Rao & Kornberg, 1996; Wei *et al.*, 2000), both being upregulated by (p)ppGpp (Kuroda *et al.*, 1997; Rao *et al.*, 1998). Polyphosphate, however, has an additional role in activation of toxin-antitoxin systems (Maisonneuve *et al.*, 2013), which are connected to stringent response very intimately (Harms *et al.*, 2016). All these and numerous other factors are important for stasis survival and the outgrowth following (see chapter 1). Accordingly, (p)ppGpp has been reported important for stasis survival in *H. pylori* (Mouery *et al.*, 2006), *C. crescentus* (Lesley & Shapiro, 2008), *M. tuberculosi*s (Dahl *et al.*, 2003; Primm *et al.*, 2000), *Borrelia burgdorferi* (Drecktrah *et al.*, 2015), *Legionella pneumophila* (Trigui *et al.*, 2014), and *Campylobacter jejuni* (Gaynor *et al.*, 2005).

1.2.5. (p)ppGpp in virulence

Given the general themes of stringent response – dormancy and endurance – and the global nature of the (p)ppGpp elicited changes in bacterial physiology, it does not perhaps come as a surprise that the alarmone has implications for virulence. The importance of (p)ppGpp for invasion and survival during infection has been indicated for numerous bacterial species. Attenuated infection in mice, for stringent response defective strains, has been reported for *M. tuberculosi*s (Dahl *et al.*, 2003; Stallings *et al.*, 2009), *V. cholerae* (Silva & Benitez, 2006), *S. typhimurium* (Webb *et al.*, 1999), *Yersinia pestis* (Sun *et al.*, 2009), *Streptococcus pneumoniae* (Hava & Camilli, 2002; Kazmierczak *et al.*, 2009), and *Brucella* sp. (Dozot *et al.*, 2006).

One of the reoccurring principles – in studies where stringent response is implicated in virulence – is that of a dormant, stationary phase like phenotype (Boes *et al.*, 2008; Song *et al.*, 2004; Webb *et al.*, 1999). This leads us to one of the most controversial and peculiar connection between stringent response and virulence – the persister cell. An interested reader should consult numerous excellent recent reviews on the topic (Amato *et al.*, 2014; Balaban, 2011; Brauner *et al.*, 2016; Cohen *et al.*, 2013; Harms *et al.*, 2016; Kaldalu *et al.*, 2016; Lewis, 2010; Maisonneuve & Gerdes, 2014), which is way too broad and complex to be discussed in its entirety here. Instead, only few carefully selected issues pertinent to current study will be discussed next.

Already at the earliest days of antibiotic usage, Bigger noticed that bactericidal antibiotics cannot be used for sterilization purposes and called the survivors peristers (Bigger, 1944). Turns out that in growing bacterial population, one which is susceptible to certain bactericidal antibiotic, there are always some cells that are not killed and resume growth once antibiotic treatment is discontinued (Balaban *et al.*, 2004; Jõers *et al.*, 2010; Roostalu *et al.*, 2008). Given the fact that persisters form exactly similar, mostly susceptible culture once the antibiotic has been removed (Bigger, 1944; Keren *et al.*, 2004a), verifies that the cells are not mutants but phenotypic variants, a form of phenotypic hetero-

geneity. In contrast to resistant bacteria, in the presence of antibiotic, persisters are not growing but survive. There is subtle yet important difference between non-growing and persister cells. In growth-supporting environment, the persister cells belong to the subpopulation that is non-replicating and can thus survive, for example, ampicillin treatment. Importantly, however, not every non-growing cell is persister, in fact, only a tiny fraction of these non-growing and not killed cells might be able to resume growth and form colonies (less than 1% in some conditions (Roostalu *et al.*, 2008)) as required by the definition of persisters.

In principle, once the antibiotic treatment is discontinued, persisters can be the source of recurrent infection. This has put forth in a lot of studies, theoretical and experimental, to elucidate the mechanism, meaning and ways to kill persister cell. A large body of work on persister cells, however, has been performed in laboratory conditions, using batch culture. Yet in infection sites, non-growing persister cells could be imagined to be cleared off by the host's immune system. Furthermore, not all antibiotics are bactericidal – some are bacteriostatic, but still work, in concert with immune system. Importantly therefore, in recent years, there is a slowly accumulating body of evidence that persistence phenomenon does indeed have some role in real infection – persister level appears to increase during the course of antimicrobial therapy in case of *E. coli* (Schumacher *et al.*, 2015), *P. aeruginosa* (Mulcahy *et al.*, 2010), *M. tuberculosis* (Torrey *et al.*, 2016), or *Candida albicans* (Lafleur *et al.*, 2010), a yeast.

A diverse set of physiological states are probably responsible why some cells of certain bacterial species in specific conditions survive particular antibiotic treatment (Kaldalu *et al.*, 2016), accordingly, persister cell formation pathways are numerous (Hansen *et al.*, 2008; Torrey *et al.*, 2016). Nevertheless, toxin-antitoxins pairs – capable of interfering with essential cellular processes and thereby inhibiting bacterial growth – are likely involved, it is yet another question if stringent response happens before, after or throughout the induction of toxins (Aizenman *et al.*, 1996; Cheverton *et al.*, 2016; Christensen & Gerdes, 2004; Germain *et al.*, 2015; Kaspy *et al.*, 2013; Keren *et al.*, 2004b; Maisonneuve *et al.*, 2013). Finally, (p)ppGpp has been suggested to orchestrate cell wall modulation to protect from β-lactam antibiotics (Goodell & Tomasz, 1980; Kusser & Ishiguro, 1985; Pisabarro *et al.*, 1990; Rodionov & Ishiguro, 1995; Vanderwel & Ishiguro, 1984).

AIMS OF THE STUDY

We set out to study the relationship between the growth state, intracellular (p)ppGpp levels and the outcomes of antibiotic treatment. The experimental part thus consists of three linked studies with following objectives:

Ref. I To study the relationship between growing, non-growing bacterial cells, antibiotic susceptibility and innate immune system.

Ref. II Since the non-growing state in growth supporting environment was protective against both antibiotic treatment and action of the immune system (Ref. I), we set out to elucidate mechanisms controlling the growth resumption.

Ref. III As the stringent response emerged as a key player in growth resuscitation (Ref. II) and given its reported importance to bacterial virulence, we set up a high-throughput search for inhibitors of the stringent response.

2. RESULTS AND DISCUSSION

2.1. Relationship between bacterial growth, action of antibiotic and innate immunity (I)

It is known that after antibiotic treatment, urinary tract infections by uropathogenic *E. coli* (UPEC) can reoccur caused by the very same strain (Russo *et al.*, 1995). The involvement of phenotypic heterogeneity, i.e. persister cell formation has been suggested as a mechanism behind the survival of bactericidal antibiotic treatment and recurrent infection (Harms *et al.*, 2016). In addition to antibiotic treatment, however, surviving bacterial cells have to endure the insult of immune system. Yet the connection between bacterial physiology and immune system remains largely uncharted. We therefore set out to investigate if heterogeneity of bacterial culture affects survival of the killing by immune system and how does the simultaneous antibiotic treatment affect the eradication of bacteria. To that end, we used (i) UPEC strain CFT073 (O6:K2:H1) (Mobley *et al.*, 1990; Welch *et al.*, 2002), isolated from a patient with acute pyelonephritis i.e. a strain capable of causing bacteremia, and (ii) human serum as a model for innate immunity.

First, bacterial cells were treated with human serum (at 50% final concentration) and course of the treatment was followed by sampling, plating and counting CFUs. A subpopulation of bacterial cells turned out to be tolerant to the complement system of human serum (I, Fig. 1). In addition, as we used stationary phase cells to start the experiment, it was evident that killing by serum coincides with the time of growth resumption (I, Fig. 1) suggesting that the lag phase CFT073 cells are refractory to complement killing. However, when exponentially growing culture was stopped by addition of chloramphenicol, the cells were still efficiently killed by serum (I, Fig. 6B) warranting the caution to be executed when interpreting results merely based on dualistic growth no-growth axis – just as growing cells, non-growing cells do not necessarily have to be alike.

Next, we factored in the antibiotic treatment using bactericidal antibiotics from different classes – cell wall targeting ampicillin, the DNA replication inhibiting norfloxacin, and the translation inhibiting amikacin. These antibiotics are often used to study persisters and persistent infections (Allison *et al.*, 2011a; Balaban *et al.*, 2004; Bigger, 1944; Keren *et al.*, 2004b; Moyed & Bertrand, 1983). None of the antibiotics killed non-growing CFT073 cells even if added at several times the MIC (resuscitated in PBS) (I, Fig. 2; SFig 3A and 3B). Adding ampicillin to the serum around at its MIC (2 μg/ml) reduced the number of surviving cells about an order of magnitude suggesting that some cells, not killed by serum, could be killed by ampicillin (I, Fig. 2A). In case of norfloxacin, around its MIC (0.125 μg/ml), the number of cells killed by serum alone compared to simultaneous treatment with antibiotic were identical (I, Fig. 2B), suggesting – but not establishing – a possible overlap between cells killed by

norfloxacin and human complement system. Finally, adding amikacin to the serum around its MIC (8 μg/ml) and higher, decreased the number of survivors by 1–2 orders of magnitude (I, Fig. 2C), suggesting that some cells, not killed by serum, could be killed by amikacin. Interestingly, both active and heat-inactivated serum seemed to promote killing at lower amikacin concentrations (sub-MIC, < 8 μg/ml)(I, Fig. 2C). The reasons for that serum-promoted sub-MIC killing still await discovery. Finally, note that for all the antibiotics at concentrations of several times the MIC, it was the antibiotic that determined the outcome of the treatment, serum complement system did not add eradication efficiency to the antibiotic (I, Fig. 2A-C).

To interrogate the possible impact of population heterogeneity on serum mediated killing, we investigated the effect of growth in further detail. We used a previously established IPTG inducible GFP reporter system (Roostalu *et al.*, 2008) that allowed us to study cell division and growth resumption at the single cell level. We found that three classes of bacterial cells could be distinguished. Importantly, they all were equally well detected by complement system (based on the opsonization) (I Fig.5). Yet only the most rapidly proliferating and the dormant cells were protected from the action of serum whereas majority of the cells, belonging to the group of intermediate growth rate, were susceptible to serum (I, Fig. 3). In case of the simultaneous application of antibiotic treatment and serum incubation, only the dormant cells were surviving – the rapidly growing cells were efficiently eradicated by the action of antibiotics of different classes (I, Fig. 4). Note that norfloxacin and amikacin seemed to inhibit the growth resumption (I, Fig. 4A and 4C) and, as CFUs revealed, latter was actually pretty effective in killing the dormant cells in growth supporting environment (I, Fig. 4B and 4D). In contrast, amikacin was not killing bacterial cultures in environments not supporting growth (I, SFig 3A), reminding thus, again, that non-growing cells are not necessarily alike.

In summary, human serum complement mediated killing eradicates most of the growing population of UPEC strain CFT073, only the very rapidly growing and the dormant cells survive the insult. During simultaneous application of serum and various antibiotics from different classes, however, only dormant cells survive as antibiotics result in clearance of the rapidly growing cells. The reasons why bacterial cells are recognized uniformly by complement yet not killed, remains to be elucidated.

2.2. The role of stringent response in growth resumption (II)

Adjustments of the length and physiology of the lag phase are involved in antibiotic tolerance and persistence (Balaban *et al.*, 2004; Fridman *et al.*, 2014; Jõers *et al.*, 2010; Luidalepp *et al.*, 2011). As we had established that killing by the complement system correlates with the growth resumption of UPEC, i.e. the

lag phase cells were not killed by serum (I, Fig. 1), we next set out to investigate potential genes involved in growth resumption of *E. coli*. Of several target genes initially studied, we eventually focused on the stringent response factor RelA. Notably, the very field of stringent response research was once initiated by isolation of mutants unable to resume growth after amino acid starvation (Alföldi *et al.*, 1963; Diderichsen *et al.*, 1977; Fiil & Friesen, 1968; Raskó & Alföldi, 1971).

We constructed the *relA* deletion strain of *E. coli* K-12 lineage (BW25113), and tested the ability of the relaxed cells to resume growth after being some time in stationary phase. There was no difference between wild-type and Δ*relA* culture when resuscitated in undefined rich medium (LB) (II, Fig. 1A). The relaxed culture, however, showed about four hour growth resumption delay when diluted into fresh defined minimal medium (M9 with 0.4% glucose) (II, Fig. 1B and Table 1). Since turbidity of the culture (II, Fig. 1) and number of CFUs (II, SFig 2) were in good agreement, Δ*relA* cells are not losing viability in the stationary phase. It is still possible, however, that there is enhanced accumulation of damage in Δ*relA* cells, therefore it takes more time for the to recover. Indeed, increased level of mistranslation has been described in relaxed cells (O'Farrell, 1978; Wagner & Kurland, 1980) although the role of (p)ppGpp in it seems to be indirect – (p)ppGpp induces translational pausing, this results in lower abundance of mRNA due to enhanced decay. Limited protein synthesis, in turn, increases the charging of tRNAs, together with lower abundance of mRNA, hungry codons are encountered less frequently and, therefore, translational fidelity increases (Sørensen *et al.*, 1994; Wagner & Kurland, 1980).

The stringent response is induced during shiftdowns in growth environment including transition to stationary phase (Lazzarini *et al.*, 1971). Thus, it is somewhat surprising that the regrowth delay was determined by the regrowth medium instead of the medium where Δ*relA* cells were growing from exponential into stationary phase (II, Fig. 1C and 1D). Note that this does not rule out explanation that inappropriate entry into stationary and/or accumulated damage during the phase (see the above paragraph) are the reasons behind the observed phenotype. Nonetheless, to begin with, we next focused exclusively on growth resumption conditions.

Primarily, we considered two major differences between the undefined rich (LB) and glucose minimal medium: (i) LB contains amino acids and (ii) supports faster growth rate. The first aspect seemed relevant given the central importance of (p)ppGpp for regulation of amino acid anabolism (Paul *et al.*, 2005; Tedin & Norel, 2001; Traxler *et al.*, 2008). As for second aspect – given that (p)ppGpp levels and growth rate are inversely correlated (Potrykus *et al.*, 2010; Ryals *et al.*, 1982)and, in exponential phase, (p)ppGpp levels are lower in Δ*relA* compared to wild-type cells (Gallant *et al.*, 1970), (Potrykus *et al.*, 2010; Ryals *et al.*, 1982) relaxed cells might therefore be primed for growth in environments supporting rapid proliferation, but fail to do so in slower growth conditions. Following experiments revealed that a full set of natural amino acids, when supplemented to minimal glucose medium, could indeed abolish

the growth resumption delay of Δ*relA* culture (II, Fig. 2B). In order to examine the second aspect, we supplied the amino acids, but decreased the growth rate support by changing to poorer carbon source, switched from glucose to glycerol. Again, Δ*relA* cells resumed growth equally to wild-type (II, SFig. 3) suggesting that growth speed might be irrelevant and what matters is the lack of or presence of amino acids. Thus, we made an effort to find out if Δ*relA* cells were deprived of some certain single amino acid. To that end, we supplied (II, Fig. 3D-E) or omitted (II, Fig. 3A-C) just one out of 20 natural amino acids to the growth resumption medium However, both approaches were perturbing the growth resumption of just the wild-type (II, Fig. 3A and 3D). Notably, most of the effects were absent when same conditions were applied to exponential phase cells (II, Fig. 4). Overall, we could establish that, among other effects, probably the ones caused by aspartate-pyruvate family of amino acids (Aspartate family: aspartate, asparagine, methionine, threonine, and lysine; Pyruvate family: alanine, valine, leucine, and isoleucine) were most prevalent. Simplest speculation is, since these amino acids are among the most abundant ones in the cell (Bogatyreva *et al.*, 2006; Okayasu *et al.*, 1997) (furthermore, as synthesized very closely related pathways, collectively very abundant), the cells might suffer from the lack these amino acids the first/strongest during starvation. One could also argue that those amino acids stand out due to (p)ppGpp involvement in regulation of branched-chain amino acid biosynthesis (Tedin & Norel, 2001; Traxler *et al.*, 2008). The issue is definitely open to further studies.

There is an earlier work on *Vibrio* sp. strain 14 suggesting the role of amino acid starvation in growth resumption (Flardh & Kjelleberg, 1994). The authors found that during growth resumption, protein synthesis is initiated fast yet without much of an amino acid biosynthesis (Flardh & Kjelleberg, 1994). This leads into amino acid starvation which triggers stringent response necessary to initiate transcription of genes of amino acid biosynthesis. Importantly, control of rRNA synthesis is relaxed immediately after reversal of starvation i.e. if upshift is coupled to amino acid deprivation, rRNA synthesis is not curtailed (Jacobson & Gillespie, 1968; Nakada & Marquisee, 1965). The reason for this temporary relaxed phenotype awaits elucidation, but together with the body work done in Gourse's lab (i.e. (Murray *et al.*, 2003)) a following picture emerges: early in growth resumption, initiating nucleotide is the main regulator of rRNA transcription, (p)ppGpp becomes important later and reacts to shiftdowns.

While interrogating the role of amino acids and/or growth rate in growth resumption, we made a serendipitous discovery that glycerol itself allowed the Δ*relA* to resume growth as fast as wild-type (II, Fig. 5B). The reasons for that are far from clear, however, some connections between the nature of carbon source and stringent response are well known. Most notably, stringent response is involved in regulation of glycogen accumulation together with carbon storage regulator (*csr*) network (Edwards *et al.*, 2011; Romeo & Preiss, 1989). When growing on glucose, for example, *relA* gene is required for glycogen accumulation upon amino acid starvation, however, when glycerol is the carbon source, high cellular levels of cyclic AMP can replace the requirement for RelA (Leckie

et al., 1980; Taguchi *et al.*, 1980). Relatedly, (p)ppGpp regulates negatively cAMP response protein (CRP) (Johansson *et al.*, 2000). Moreover, cAMP, (p)ppGpp and CRP are all involved in regulation of the branched-chain amino acid biosynthesis (Freundlich, 1977). Finally, the activity of SpoT also governs the usage of carbon source, monitors the energetic status of the cell (Harshman & Yamazaki, 1971; Lazzarini *et al.*, 1971; Murray & Bremer, 1996) and can thus be involved in growth resumption phenomena we have observed.

Finally, we studied the role of the delayed growth resumption of Δ*relA* strain in susceptibility to bactericidal antibiotic treatment. Indeed, Δ*relA* cells were better protected from ampicillin treatment during regrowth on glucose (II, Fig. 5C). Surprisingly, though the Δ*relA* cells resumed growth on glycerol similarly to wild-type (II, Fig. 5B), they were still better at surviving ampicillin treatment (II, Fig. 5D). This warrants the notion that study of bacterial physiology starts from inspection of growth rate, but should not end there (see also chapter 2.1).

2.3. A quest for a stringent response inhibitor (III)

As we saw in chapter 2.1., non-growing cells were protected from both antibiotic action and immune system. Importantly, growth resumption correlated with the bactericidal action of complement system (I, Fig. 1). Next, we learned that, depending on the growth conditions, the growth resumption of bacteria was impaired by missing the function of RelA (II, Fig. 1). This could, in fact, mean that inhibition of RelA would result in better survival both innate immunity and antibiotic treatment. Indeed, Δ*relA* cells were better protected against ampicillin killing than wild-type (II, Fig. 5C and 5D). Yet there are several lines of evidence that stringent response is important to efficient infection (see chapter 1.2.5). Furthermore, decreasing the levels of (p)ppGpp – e.g. combinatorial treatment with subinhibitory levels of chloramphenicol – could render wild-type cells as susceptible to cell wall inhibitors as Δ*relA* strain (Kusser & Ishiguro, 1985). Quite some effort is therefore put into search for stringent response inhibitors. Regardless if the inhibitors would be of some immediate value to medicine, specific inhibitors of stringent response would be powerful tools for studies of bacterial physiology.

Although some nucleotide (Wexselblatt *et al.*, 2012) and peptide (de la Fuente-Núñez *et al.*, 2014) based inhibitors are reported, there is more work to be done as they either lack the potency (nucleotide Relacin is required at around 1–5 mM (Gaca *et al.*, 2015; Wexselblatt *et al.*, 2012)) or lack the specificity altogether (Andresen *et al.*, 2016). Therefore, we set up a High Throughput Screening (HTS) assay for stringent response inhibitors. We decided to overcome both purified target-based HTS and phenotype-based HTS limitations and opt for target-based whole-cell HTS (Farha & Brown, 2015) as described in the following.

We used *B. subtilis* because intracellular uptake, a major hurdle for drug discovery, is more efficient through Gram-positive than Gram-negative cell

envelope (Brown & Wright, 2016; Payne *et al.*, 2007; Tommasi *et al.*, 2015). *B. subtilis* has one long bifunctional Rel_{Bsu} and two small alarmone synthetases RelQ (synonyms: SAS1, YjbM) and RelP (synonyms: SAS2, YwaC) (Atkinson *et al.*, 2011; Nanamiya *et al.*, 2008). To reduce the obscuring redundancy in sources of (p)ppGpp, we used the strain that lacks both SASs (designated Δ*SAS*). For the strategy of screening, we took advantage of the amino acid auxotrophy of *B. subtilis* that lacks (p)ppGpp altogether – in accordance to published report (Kriel *et al.*, 2014), Δ*SAS* and $ppGpp^0$ strains (Δ*rel*Δ*SAS*) grow efficiently in defined medium (S7) containing all amino acids except lysine (S7-K) (III, Fig. 1A). In contrast, Δ*SAS* strain grows equally well yet $ppGpp^0$ strain fails to do so when medium lacks valine (S7-V) (III, Fig. 1B). This allowed us to opt for two-stage strategy using Δ*SAS* strain. Initially, we screened in S7-V medium, in which case we picked up not only potential Rel_{Bsu} inhibitors but also off-target and general inhibitors of cell growth. Hits from the first stage, were then subjected to screening in both S7-V and S7-K media, the chemical compounds inhibiting growth equally well in the two media were considered as off-target. In other words, only the ones inhibiting the growth in S7-V but not in S7-K should be considered as specific inhibitors of Rel_{Bsu} and subjected to further dose-response characterization.

Out of 17,500 synthetic drug-like low molecular weight compounds, we continued with 480 to the second stage. In second stage, twelve compounds were identified as general antibacterials since they inhibited *B. subtilis* Δ*SAS* equally well in both S7-V and S7-K media (III, STable 1). Five compounds, however, were slightly inhibitory in S7-V while there was no effect in S7-K, suggesting that these could be the specific inhibitors. Importantly, all the five shared the same core, 4-(6-(phenoxy)alkyl)-3,5-dimethyl-1H-pyrazole (III, Table 1). Unfortunately, both dose-response (III, Fig. 3 and SFig. 5) and *in vitro* reconstituted ppGpp synthesis assay (III, SFig. 6) indicated those five compounds were also general antibacterials.

In summary, we developed a robust and specific assay for stringent response inhibitors which resulted in discovery of some general antibacterials and awaits applications to other chemical libraries or natural products (Harvey *et al.*, 2015).

CONCLUSIONS

- A subpopulation of uropathogenic *E. coli* cells growing in human serum is refractory to killing by complement system
- Non-growing and rapidly growing UPEC cells, despite being recognized by the complement system, are protected from the action of human serum
- Only non-growing UPEC cells survive simultaneous treatment with serum and different classes of antibiotics (ampicillin, norfloxacin, and amikacin)
- A culture of stringent response deficient *E. coli*, i.e. relaxed strain, is defective in growth resumption rendering cells non-growing for longer periods of time in growth supporting environment
- The growth resumption defect of relaxed strain of *E. coli* is a function of both the amino acid and carbon source composition of the medium
- In comparison with wild-type, relaxed strain survives ampicillin treatment better even if the growth resumption of the two strains is equal
- A screening system was established for compounds specifically inhibiting stringent response, it failed to yield the desired compound but resulted in identification of novel class of antibacterials, derivatives of 4-(6-(phenoxy)alkyl)-3,5-dimethyl-1H-pyrazole

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SUMMARY IN ESTONIAN

Poomisvastus bakterite kasvus ja elus püsimises

Bakterid peavad ellu jäämiseks pidevalt kohanema oma väliskeskkonnaga. Sobivates tingimustes kasvavad paljud bakteriliigid väga kiiresti. Kiire kasv iseenesest viib aga kasvutingimuste muutumiseni. Nüüd kohtame järgmist bakteritele iseloomulikku omadust – jaksu pikka aega elus püsida kasvuks mittesobivates tingimustes. Seejuures säilitavad nad olulise võime kiiresti taas kasvama hakata, kui keskkonnatingimused paranevad. Muutuvate keskkonnatingimustega kohanemiseks on bakteritel evolutsiooni käigus välja kujunenud hulganisti mehhanisme. Üks selline, keskne ja pea kõigis bakterites esinev mehhanism on poomisvastus. Poomisvastust kutsuvad esile järsud muutused keskkonnas, mis nõuavad kasvu aeglustumist, sageli peatub kasv esialgu täielikult, rakk kohaneb ja kui võimalik, jätkab kasvamist muutunud tingimustes paraja tempoga. Vähemaks reguleeritakse näiteks valgusünteesi masinavärk ning rohkemaks elus püsimise ja autonoomsuse tarbeks oluline – hulganisti kahjustuste eest kaitsevaid süsteeme ja tarvilikud anaboolsed protsessid. Poomisvastust orkestreerivad signaalmolekulid, guanosiin nukleotiidid pppGpp ja ppGpp, koondnimega (p)ppGpp. Nende nukleotiidide sünteesi eest vastutavad *Escherichia coli*-s kaks valku, RelA ja SpoT. Viimane neist hoolitseb ka selle eest, et (p)ppGpp-d oleks rakus parasjagu, s.t SpoT on kahefunktsionaalne, omab ka (p)ppGpp-d lagundavat aktiivsust. Mitmetes teistes bakterites (näiteks *Bacillus subtilis*) on poomisvastuse tarbeks vaid üks peamine kahefunktsionaalne ensüüm (Rel_{Bsu}), aga ka hiljuti avastatud väikesed valgud, millest on veel vähe teada ja mis omavad kas sünteesi või hüdrolüüsi aktiivsust.

Arvestades poomisvastuse ulatuslikku mõju bakteriraku füsioloogiale, ei tule vast üllatusena, et see protsess mõjutab bakterite võimet põhjustada haigust ja antibiootikumide võimet infektsiooni ravida. Antibiootikumide kasutamise algusaegadest peale pandi tähele, et sugugi mitte kõik bakterirakud ei sure baktereid tapva antibiootikumi toimel, üksikud bakterid jäävad ikka elama. Erinevalt antibiootikumi resistentsusest ei kasva sellised rakud antibiootikumi juuresolekul, nad lihtsalt taluvad, elavad üle, ja neid nimetatakse persistoriteks. Ka persistorite moodustumises on nähtud poomisvastuse rolli – kui suurem osa bakteritest kasvab jõudsalt, lülitub üksikutes siiski millegipärast sisse poomisvastus. Oletatakse, et persistorid võivad antibiootikumi kuuri lõppedes põhjustada haiguse taastekkimist. Haigusest jagu saamisel on aga antibiootikumiga võrdväärne roll kanda immuunsüsteemil, mis võiks ju jagu saada sellistest mittejagunevatest persistoritest. Samas on vähe teada selliste persistor-rakkude ja immuunsüsteemi vahelistest seostest, mida asutigi käesolevas töös kõigepealt uurima.

Selgus, et nn kaasasündinud immuunsüsteem inimese vere seerumi komplemendi näol ei tapa sugugi kõiki uropatogeense *E. coli* rakke. Kui nüüd samaaegselt seerumile rakendati ka antibiootikumi töötlust, sõltus tulemus konkreetsest antibiootikumist. Ampitsilliini (rakukesta sünteesi inhibiitor) või amikat-

siini (translatsiooni inhibiitor) lisamisel vähenes seerumis ellujäävate bakterirakkude hulk ühe-kahe suurusjärgu võrra, mis lubab oletada, et mõned rakud, mida seerum ei hävita, tapeti antibiootikumi poolt. Lisaks võimendas seerum amikatsiini toimet subinhibitoorsete kontsentratsioonide puhul. Norfloksatsiini (DNA replikatsiooni inhibiitor) lisamine seerumile ei põhjustanud mingit muutust ellu jäänud bakterirakkude arvukuses, mistõttu võib spekuleerida, et komplement ja norfloksatsiin tapavad ühesuguseid rakke.

Uurimaks bakteripopulatsiooni võimaliku heterogeensuse mõju komplemendi süsteemi vahendatud tapmisele, analüüsiti järgmiseks bakterirakkude jagunemist üksikraku tasemel. Katsetulemused näitasid, et kuigi komplement tunneb ära kõik bakterirakud, ja suurem osa bakterirakkudest sureb, jäävad elama keskmisest oluliselt kiiremini kasvavad ja mittekasvavad rakud. Kui nüüd samal ajal rakendada antibiootikumi töötlust (ampitsilliini, amikatsiini või norfloksatsiiniga), jäävad alles vaid mittekasvavad rakud ning kiiremini kasvavad hävitatakse.

Kuivõrd katsed seerumiga näitasid muuhulgas, et bakterite suremisel on positiivne korrelatsioon rakkude seerumis kasvama hakkamisega ja mittejagunevad rakud on kaitstud nii antibiootikumi toime kui komplemendi eest, uuriti järgmisena *E. coli* rakkude kasvama hakkamise regulatsiooni ja poomisvastuse rolli selles. Selgus, et rakud, kus puudub peamine poomisvastuse valk RelA (edaspidi Δ*relA* tüvi), hakkavad soodsate kasvutingimuste saabudes kasvama neli tundi hiljem metsiktüüpi rakkudest, kui keskkonnas puuduvad aminohapped. Lisaks aminohapetele mõjutas kasvama hakkamist ka süsinikuallikas – Δ*relA* tüvi toibus metsiktüüpi tüvest hiljem süsinikuallikana glükoosi sisaldaval söötmel, ent võrdväärselt glütseroolil kasvades. Selgus, et selline RelA funktsiooni puudumine ja toibumisdefekt võib mõjutada antibiootikumi toimet – ampitsilliin tappis glükoosil toibuvaid metsik-tüüpi rakke efektiivsemalt kui Δ*relA* rakke. Mõnevõrra üllatuslikult elasid Δ*relA* rakud paremini üle ka ampitsilliinitöötluse glütseroolil toibudes. Igatahes, teatud tingimuste korral võib poomisvastus olla vajalik rakkude kiiresti kasvama hakkamiseks, mis omakorda võib mõjutada antibiootikumi toimet neile rakkudele.

Eelpool nägime, et poomisvastusel on roll bakterirakkude kasvama hakkamisel ja see mõjutab antibiootikumi toimet neile rakkudele. Tõsi küll, teatud üsnagi kitsastes tingimustes oli funktsionaalse poomisvastuse puudumine ampitsilliini toime üle elamiseks kasulik. Samas on küllaldaselt töid, mis näitavad, et poomisvastuse puudumise korral on vähenenud bakterite võime haigust põhjustada. Koos antibiootikumi resistentsuse hirmuäratava levikuga otsitakse seepärast ka spetsiifilisi poomisvastuse pärssijaid üsna palavikuliselt. Isegi kui neist ei ole peatset ja vahetut kasu meditsiinile, oleksid spetsiifilised inhibiitorid oluline töövahend bakteriraku füsioloogia uurimiseks. Sestap soovisime järgmiseks leida poomisvastuse inhibiitoreid.

Neid otsiti keemiliste ühendite raamatukogust (17500 ühendit), kasutades testsüsteemina bakterit *B. subtilis*, sest ainete sisenemine rakku on gram-positiivsetel bakteritel hõlpsam kui gram-negatiivsetel. Otsingu tulemusel leiti 17 uut antibakteriaalset ühendit, kahjuks polnud ükski neist piisavalt spetsiifiline

poomisvastuse suhtes. Jääb üle vaid loota, et välja töötatud kõrge läbilaskevõimega poomisvastuse inhibiitorite testsüsteem annab positiivse tulemuse mõne teise keemiliselt sünteesitud ja/või loodusest isoleeritud ühendite raamatukogu puhul.

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LIST OF PUBLICATIONS

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