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Role of the stringent response in antibiotic tolerance of *Escherichia coli*

Master's Thesis

30 ECTS

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Contents

Abbreviations

A-site - Aminoacyl site of the ribosome

ACT - Aspartokinase, Chorismate mutase and TyrA domain

AMP – ampicillin

ATP – Adenosine triphosphate

aa-tRNA – Aminoacylated tRNA

CAM – Chloramphenicol

CC - Conserved cysteines domain

CFU – Cloning-forming unit

CTD - Carboxy-terminal domain

DNA - Deoxyribonucleic acid

EF - Elongation factor

EF-G - Elongation Factor G

EF-Tu - Elongation Factor Tu

GDP - Guanosine diphosphate

GPP - 5'phosphorylase

GTP - Guanosine triphosphate

HD – Hydrolyze domain

HPLC - High pressure liquid chromatography

IF - Initiation factor

MOPS - (3-N-morpholino) propanesulfonic acid

MUP – Mupirocin

NTD - N-terminal domain

NTP - Nucleoside triphosphate

ppGpp - guanosine tetraphosphate

pppGpp - guanosine pentaphosphate

PTC - Peptidyl transferase centre

RNA - Ribonucleic acid

RNAP – RNA polymerase

rRNA – ribosomal RNA

RSHs - RelA/SpoT homologue proteins

SAH - Small Alarmone Hydrolases

SAS - Small Alarmone Synthases

SYNTH – Synthetase

TGS - ThrRS, GTPase and SpoT domain

tRNA – Transfer RNA

TA - Toxin – antitoxin

TET- Tetracycline

THIO – Thiostrepton

TRIM – Trimethoprim

Introduction

1. The Stringent Response

Bacterial cells face rapidly changing environmental conditions. In order to survive, they have to sense and adapt to the harsh conditions, and to that end they have evolved multiple survival mechanisms that modulate bacterial physiology in response to stress. The so-called the stringent response is one of such mechanisms (1). In *Escherichia coli* the stringent response is mediated by two enzymes – RelA and SpoT – through the regulation of the intracellular levels of the alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively referred to as (p)ppGpp (2). The synthesis of the alormones by RelA activated by the binding of uncharged tRNA to the ribosomal acceptor site (A-site) (3, 4) (**Figure 1A**); GDP/GTP and ATP are used as substrates (**Figure 1B**). Increased levels of (p)ppGpp rewire bacterial physiology, affecting translation, transcription, replication, persistence, and virulence (5, 6).

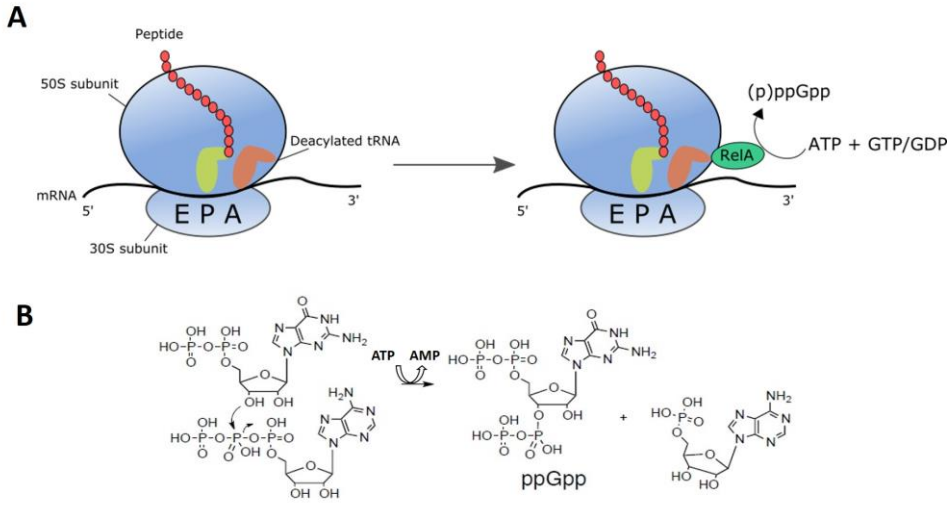


Figure 1 | Molecular mechanism of (p)ppGpp synthesis by *E. coli* RelA. (**A**) Amino acid starvation induces the synthesis of (p)ppGpp. As a consequence of amino acid starvation deacylated tRNA accumulates in the ribosomal A-site. This ribosomal state is recognized by RelA, leading to the activation (p)ppGpp synthesis by the enzyme using ATP and GDP/GTP as substrates. (**B**) The RSH-catalyzed reaction of (p)ppGpp synthesis. The figure is adapted from (1).

1.1 RelA/SpoT Homologue (RSH) protein family

In *E. coli* RelA/SpoT homologue proteins (RSHs) regulate the concentration of (p)ppGpp in response to several stress conditions (7, 8). The RSHs enzymes are classified in two types: 'short' enzymes that has a single domain and 'long' multi-domain RSH enzymes (2). The 'long' RHS proteins are represented in bacteria by either a combination of RelA and SpoT or by one protein, Rel. RelA and SpoT are found in γ- and β-proteobacteria and is has evolved via gene duplication and consequent diversification of Rel, an ancestral protein found in the vast majority of bacterial lineages (9) (**Figure 2**). Like SpoT, Rel is bifunctional with active SYNTH ((p)ppGpp synthesis) and HD ((p)ppGpp hydrolysis) domains. RelA also has present the HD but is not in an active state (2). The 'short' single-domain RSHs are specialized proteins that contain either SYNTH or the HD domain, Small Alarmone Synthases (SAS) and Small Alarmone Hydrolases (SAHs) (2).

Under amino acid starvation, and consequent uncharged tRNA, RelA uses ATP and GDP (or GTP) to synthesize ppGpp (or pppGpp) in the SYNTH domain (2, 10) (**Figures 1** & **2**). SpoT enzymes are required to respond to a various stress conditions such as phosphate, carbon, iron or fatty acid starvation (11). pppGpp is rapidly hydrolyzed to ppGpp by guanosine pentaphosphate phosphohydrolase (gpp) (12).

The HD and SYNTH domains are a part of the N-terminal domain (NTD) (**Figure 2**). The carboxy-terminal (CTD) region of 'long' RSHs contains four domains: ThrRS, GTPase and SpoT (TGS); helical; aspartokinase, chorismate mutase and TyrA (ACT), and conserved cysteines (CC) (2). CTD region of RSHs regulates the catalytic action of the NTD region (13).

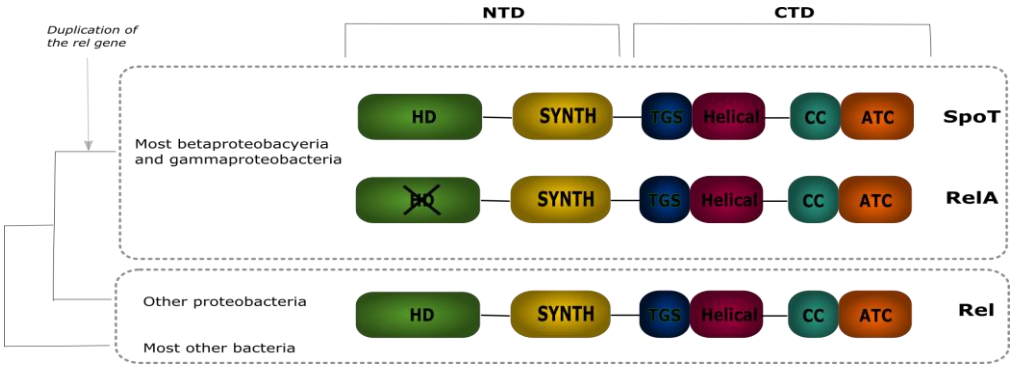


Figure 2 | Domain structure of the long RSHs and their distribution in bacteria. The coloured boxes representing each domain. The HD and SYNTH domains comprise NTD, while the TGS, helical, CC and ACT domains together involve the CTD. SYNTH domain in SpoT has a weak synthetic activity, whereas HD domain has no hydrolytic activity in case of RelA. Rel has hydrolytic and synthesis domains. The phylogenetic tree that illustrates the evolutionary relationships among bacteria that contain RSHs. The arrow indicates the duplication event that led to the emergence of RelA and SpoT from an ancestral Rel protein in the lineage of the γ- and β-proteobacteria. The figure is adapted from (2).

1.2 Regulation of transcription, translation and replication by (p)ppGpp

Acute accumulation of nucleotide (p)ppGpp coordinates bacterial physiology through an array of mechanisms: it causes an abrupt cessation of stable RNA production (14) while inducing transcription of amino acid biosynthesis genes (15, 16) and affects transcription of a number of other genes (14).

Regulation of transcription is achieved either directly or indirectly. In *E. coli* (p)ppGpp binds to to RNAP (RNA polymerase) (17, 18) and works together with a small protein DskA (DnaK suppressor A) that binds to the second channel of the RNAP (19) and amplifies the effect of the alormone (11). In *Bacilus subtilis* (p)ppGpp does bind to RNAP; instead it affects the balance of initiator nucleotides, iNTPs. The GTP pool is depleted via consumption of GTP for formation of pppGpp and via inhibition of IMP dehydrogenase, an enzyme that is crucial for GTP synthesis (20). The rRNA start with G nucleotide and are, therefore, downregulated by decrease of GTP levels. Similar strategy is utilized in *Thermus thermophiles* (21) as well as in Firmicute bacteria such as *Staphylococcus aureus* (22), *Streptococcus pyogenes* (23), *Streptococcus mutans* (24) and *Listeria monocytogenes* (25).

The alormone (p)ppGpp also regulates the translation directly by inhibition GTPase translational factors IF2 (6, 26), EF-Tu and EF-G (6). It also inhibits ribosomal assembly by inhibiting GTPases involved in that process (27).

The stringent response affects DNA replication as well in *E. coli* (6, 28-30) and *Bacillus subtilis* (28). Slow growth rates and nutritional downshifts inhibit replication initiation (28-30).

2. Antibiotics

The discovery of antimicrobial agents was one of the greatest medical triumphs of the 20th century that revolutionized the treatment of bacterial infections. The antibiotics most used are natural products, they come from species of microbe: bacteria or fungi. Antibiotics affect important cellular functions by inhibiting drug-target interaction (31) **(Appendix 1)**. Antimicrobial agents can be classified based on the cellular component or system they affect, in addition to whether bactericidal drugs, inducing cell death, or bacteriostatic that inhibit cell growth (31). However, the successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. A wide range of biochemical and physiological mechanisms may be responsible for persistence and resistance. Resistance allows bacteria continue reproduce under stressful conditions. Persistence allows a small fraction of the population escape from stress in a non-replicative conditions, which can be classified as dose-dependent and time-dependent persistence. When the bacterial population is exposed to high concentrations of antibiotic and the capacity of the bigger part of the cells in the population is reached, but not for the subpopulation – dose-dependent persistence. Whereas a bacterial subpopulation has longer lag time or slower growth rate than the majority of the population are classified as time-dependent peristence. The difference between tolerance and persistence that in the latter it is only a subpopulation that is tolerant to antibiotic challenge, but not the whole microbial culture (32).

2.1 Antibiotic tolerance and persister cells

In 1944, Joseph Bigger, discovered bacterial tolerance when he was explored how bacteria were killed by penicillin (33). When genetically identical bacteria were exposed to bactericidal antibiotic, the bulk of the population was killed. However, after a few hours of treatment, the killing rate decreased, revealing the existence of cells which were less sensitive to the antibiotic (34). These cells drug-tolerant were dubbed ‘persisters’.

In the recent years, virulence of pathogenic bacteria (35, 36), antibiotic resistance (18, 37) and persistence (38-42) were suggested to be under control of (p)ppGpp. At the same time (p)ppGpp

is also the key regulator of bacterial growth rate (43, 44), and the growth per se is a major factor affecting antibiotic tolerance **(Appendix 2)**. Slow growth explains bacterial drug tolerance since the cellular targets affected by antibiotics are much less susceptible in slow-growing than in fast-growing cells (34, 45). In the case of the β-lactam, ampicillin, the killing efficiency is directly proportional to the rate of growth (45). (p)ppGpp and DskA are global regulators of metabolism (15, 16) that are mediators of persister cells (38, 39, 41, 46-48). Initially (p)ppGpp was associated to the persistence through *hipA7*, a toxin mutant that required (p)ppGpp for high levels of persisters appears (48). Other works show that the native HipA has also shown that its impact on persistence requires (p)ppGpp (38-40). The alormone increase persistence levels through its inhibition of exopolyphosphatase (ppx), a modulator of the antitoxin degrading Lon (41).

2.2 RSH enzymes as targets for developing new antibacterials

The stringent response has a role in bacterial virulence and tolerance, inhibitors of RSH enzymes such as RelA are potentially very promising tools for disarming pathogenic bacteria (49, 50).
The frist approach to use inhibitors of translation. In the ribosome a deacylated tRNA at the A-site triggers the synthesis of (p)ppGpp (3, 4). Tetracycline (51-53) and thiostrepton both are A-site specific inhibiting the protein synthesis and inhibiting (p)ppGpp accumulation in bacteria. Tetracycline does so indirectly by preventing the stable binding of tRNA to the A-site of the ribosome (54). As the deacylated tRNA in the A-site of the ribosome is necessary for the stringent response and tetracycline prevents the binding of tRNA, leads to inhibition of (p)ppGpp production. Thiostrepton in other hand binds to the ribosome within the GTPase-associated center, in a cleft formed between the NTD of L11 and 23S rRNA – an important region for RelA binding and activation on the ribosome – and is suggested to inhibit RelA directly (55). Finally, any inhibitor of translation would inhibit consumption of aa-tRNA, resulting in increased acylation levels leading to an indirect inhibition of RelA. A prime example of this mechanism is chloramphenicol (56, 57).

The second approach for targeting RSHs is development of mimics of (p)ppGpp that would bind to the enzyme direct and inhibit it (58, 59). The prime example is Relacin that compounds that inhibits RSHs in the test tube and decreases (p)ppGpp production in bacterial cultures (50). That

compound affects the production of multicellular biofilm communities, impedes bacterial long term survival pathways, being a promising compound to inhibit the effects of stringent response (50, 58).

The third approach is to target (p)ppGpp itself rather than the enzymes that make it. A small cationic peptide called 1018 was proposed to do exactly that by degrading (p)ppGpp inside the cells of Gram-positive and Gram-negative bacteria, acting as a broad-spectrum biofilm inhibitor (49).

2.3 Cell wall synthesis as target for β-lactam antibiotics

Cell wall peptidoglycan metabolism is inhibited during the stringent response (60, 61). The peptidoglycan succulus are made of peptidoglycan chains crosslinked by short peptides and it is essential to maintain the cell shape and integrity from the osmotic phenomena and degradation of the cell wall (62, 63). Peptidoglycan is located outside of the cytoplasmic membrane of almost all bacteria (63). It is involved in the processes of cell growth and cell division (62). One key site of inhibition in peptidoglycan synthesis was identified as the terminal step in peptidoglycan polymerization corresponding to the activities of the penicillin binding proteins (PBP) (62, 64). PBP It is well known that amino acid-deprived bacteria are penicillin tolerant (61, 65). DD-transpeptidases generate crosslinks between D amino acids in peptidoglycan sacculus (62). The DD-transpeptidases belong to the PBP family and are inhibited by of β-lactam antibiotics (60, 62, 64). A small proportion of the cross-links are unlikely to be generated by PBPs since they involve two meso-DAP residues (66). *E. coli* produces five LD-transpeptidases with two distinct functions: ErfK, YcfS, and YbiS anchor the Braun lipoprotein to the peptidoglycan, whereas YcbB and YnhG form the meso-DAP meso-DAP peptidoglycan cross-links (66) and are β-lactams insensitive (62). These crosslinks are responsible for natural resistance to β-lactams in *E. faecium* (67).

Aim of the project

1) To characterize the connection between (p)ppGpp accumulation and abolishment and ampicillin tolerance in wild-type and *ΔrelA E. coli*.

Materials and Methods

1. Growth measurements

E. coli wild type BW25113 and Δ*relA* strains were grown at 37 °C in MOPS (68) supplemented with 0.4% glucose and 25 µg/ml 20 amino acids. The process started with pre-warmed medium in a 500 ml flask. Cells were diluted 100-fold from an overnight culture. Then placed in a shaker at 200 rpm. The cells grown until desired optical density at 600 nm (OD_{600}) of 0.5. The volume of the flask was divided into 10 ml in 125 ml flasks. The consequent growth was measured every hour for 3 to 5 hours (69). The following antibiotic concentrations were added: 70 µM muporicin (MUP) (Applichem), 20 µM and 8 µM chloramphenicol (CAM) (Sigma), 2 µM and 0.75 µM tetracycline (TET) (Sigma) and 16 µM and 19 µM trimethoprim (TRIM) (Sigma) and negative control where was no added.

2. Persister measurements

E. coli wild type BW25113 and Δ*relA* strains were also grown at 37 °C in MOPS (68) supplemented with 0.4% glucose and 25 µg/ml 20 amino acids. The process started with pre-warmed medium in a 500 ml flask, diluted 100-fold from an overnight culture. Then placed in a shaker at 200 rpm. The cells grown until desired optical density at OD_{600} of 0.5. The volume of the flask was divided into 10 ml in 125 ml flasks (69, 70). The following antibiotic concentrations were added: 70 µM MUP, 8 µM and 20 µM CAM, 0.75 µM and 2 µM TET, 16 µM and 19 µM TRIM.

After 30 min was taken the time point where is no ampicillin (AMP) (Sandoz) and the Colony Forming Unit (CFU) were measured. Then 200 µg/ml of ampicillin was added to all flasks, including the negative control. The cells were incubated for 5 hours and the CFUs were measured every hour. The CFU measurements were done adding 10 µl of cells from each flask in a 96-well plate. Dilutions were made until 10^6. From every dilution 5 µl were tranfered to a Luria-Bertani

(LB) agar medium plate. The plates were incubated at 37°C overnight. The CFU/mL were calculated.

To see if the effect of ampicillin was specific was used the same conditions and *E. coli* strains as described above we substituted ampicillin for norfloxacin (NOR) (Sigma) with a concentration of 5 µg/ml. However were only few antibiotic concentrations used.

In order to test the effect of imipenem (IMP) (USP Rockville), that is a Ldt inhibitor, the cells were killed with 200 µg/ml AMP + 4 µg/ml IMP. The pretreatment antibiotics and respective concentration were: 16 µM TRIM + 70 µM of MUP and 16 µM TRIM + 8 µM CAM.

Ampicillin killing was also performed with *E. coli* strain lacking functional Ldc genes *ynhG* and *ycbB* as described above. Except the pretreatment antibiotics were only: 16 µM trim + 70 µM of mup and 16 µM trim + 8 µM cam.

E. coli (p)ppGpp0 cells were grown in MOPS (68) 0.4% glucose 400 µg/ml Serine 40 µg/ml 19 amino acids (71) and LB medium, then diluted to OD_{600} of 0.1 in 50 ml of medium. Also the volume was divided into 10 ml to 125 ml flasks and incubated at 37 °C. The ppGpp0 cells were challenged with 70 µM of MUP; 19 µM TRIM; 70 µM of MUP + 16 µM TRIM. After 30 min, 200 µg/ml of ampicillin was added to all flasks, including the negative control. The CFUs measurements were taken and done as described above. This same set up also was performed for wild type BW25113 and Δ*relA* strains in defined medium to compare all the strains in the same conditions.

3. Analysis of muropeptide composition

From 1 ml of overnight culture the cells were grown in pre-warmed MOPS 0.4% glucose 25 µg/ml 20 amino acids. In the morning, the cells were diluted 100-fold to a final volume of 600 ml. The cells were placed in a shaker at 200 rpm and at 37°C and allowed to grow until OD_{600} of 0.5. For *E. coli* wild type BW25113 the antibiotic concentrations were 70 µM of MUP and the negative control (no antibiotics). Δ*relA* strain was treated with 70 µM of MUP; 70 µM of MUP + 2 µM TET; 70 µM of MUP + 16 µM TRIM; and the negative control. Followed by 30 minutes of

incubation, the cells were harvested by 10 min at 5000 g at room temperature, washed with 10 ml of phosphate buffered saline (PBS). Second wash was done and the pellet was frozen in liquid nitrogen. The UPLC analysis was done by Dr. Teresa Del Peso Santos in Felipe Cava lab at Umeå University following the protocol described by Cava and colleagues (2011) (72).

Results

1. Effects of RelA functionality on ampicillin tolerance in *E. coli*

(p)ppGpp was proposed to be the main dreiver of bacterial persistence (38-41). We set out to characterize the survival of wild-type BW25113 and an isogenic knock-out delta Δ*relA* strain upon treatment with β-lactam ampicillin, either alone or combined with antibiotics targeting translation, cloramphenicol and tetracycline – and thus inhibiting (p)ppGpp production. As one control we used mupirocin (73-77) – an inhibitor of isoleucyl-tRNA synthetase (78) that is inducing stringent response. DNA replication inhibitor, trimethoprim, was used as a negative contriol. All of the antibiotics were used in concentrations that cause 50% growth inhibition.

In the **Figure 3A** (wt) is observed that for almost all the cases that we added pre-treatment antbiotics the cells are killied. However, when the cells are pre-treated with mupirocin is shown that this antibiotic protects the cells when we try to kill them with ampicillin. In the **Figure 3D** (Δ*relA*) the same antibiotics and concentrations were apllied. In that graph we can see that all antibiotics killed the cells efficiently. This observation tells us that the persisters formation in wild type strain is dependent on strigent response, dependent on RelA. Then we tested the cells while are challeged to produce (p)ppGpp, with mupirocin, and at the same time inhibiting that production **Figures 3B** (wt) **and 3E** (Δ*relA*). In the wild-type strain **(Figure 3B)** we got a strong antibiotic tolerance in all the experiments. Nonetheless, in Δ*relA* strain we obtained cell protection with combination of mupirocin with trimethoprim **(Figure 3E),** telling us that ampicillin tolerance is also related to RelA. Since mupirocin is a translation inhibitor and trimethoprim a DNA replication inhibitor, we tested trimethoprim in combination with the translational inhibitors, i.e., chloramphenicol and tetracycline **(Figures 3C** and **3D).** In wild type strain these combinations give ampicillin tolerance to the cells. However, in the relaxed strain the combination of trimethoprim with tetracycline confer high levels of persistence, but for trimethoprim with chloramphenicol the ampicillin tolerance is not present.

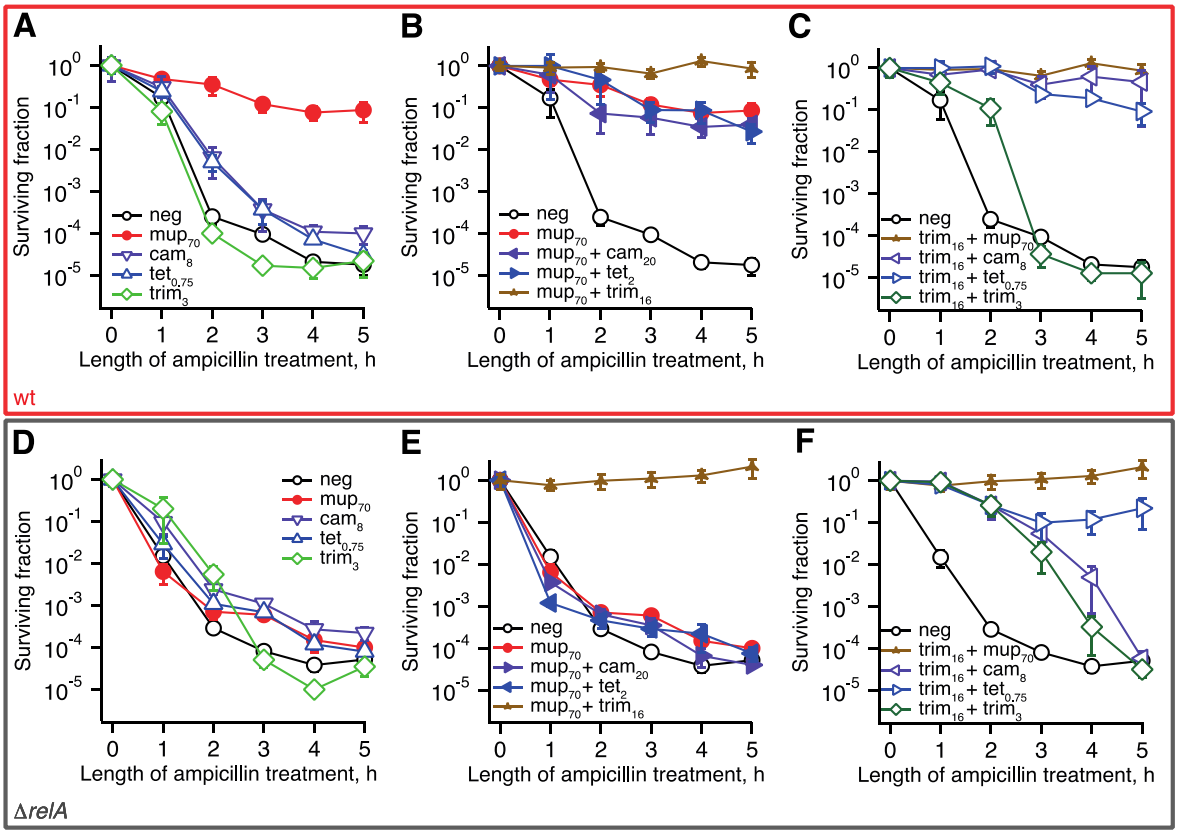


Figure 3 | Effects of RelA functionality and antibiotic treatment on ampicillin tolerance in *E. coli*. The experiments were performed in BW25113 *E. coli* wild-type strain (wt, **A**-**C**) and in BW25113 *E. coli relA* knock-out (Δ*relA*, **D**-**F**). Antibiotic concentrations are in μM, e.g. mup_{70} designates pre-treatment with 70 μM mupirocin. The bacteria grown in MOPS 0.4% glucose supplemented with 25 μg/ml at 37 °C. Error bars indicate standard error. (**A**) Effects of pre-treatment with individual antibiotics on ampicillin tolerance of wild-type BW25113 *E. coli*. The antibiotics were used at certain concentrations causing 50% growth inhibition. (**B**) The antibiotics were added in concentrations that inhibit stringent response. Moderate effects of chloramphenicol, tetracycline and trimethoprim on ampicillin tolerance induced by mupirocin in wild type *E. coli*. (**C**) Effects of chloramphenicol, tetracycline and mupirocin combined with trimethoprim ampicillin tolerance of wild-type. (**D**) Absence of effects of pre-treatment with mupirocin, chloramphenicol, tetracycline and trimethoprim on ampicillin tolerance of Δ*relA E. coli*. (**E**) Induction of *relA*-independent ampicillin tolerance of Δ*relA E. coli* by the combination of mupirocin and trimethoprim. (**F**) Effects of chloramphenicol, tetracycline and mupirocin combined with trimethoprim on ampicillin in wild type Δ*relA E. coli*.

1.1 Effects if combinations of antibiotics on *E. coli* growth

Since bacterial slow growth is an important basis for antibiotic tolerance to β-lactam, ampicillin (34, 45), we tested the effects of same antibiotic combination on growth in stringent and relaxed *E. coli* **(Figure 4)**. So, was expected that in wild type strain we see growth on the antibiotic combinations, in the cells that are treated only with mupirocin, and in the relaxed strain observe growth in mupirocin with trimethoprim and trimethoprim with tetracycline.

The results of the growth measurements for all the combinations and single antibiotic do not show bacterial growth, neither in wild-type **(Figures 4A** and **4B)** or in Δ*relA* **(Figures 4C** and **4D)** strains. It is no significant difference between the combinations that we see high persiters levels from the combinations that we do not see any tolerance to ampicillin. We can say that the growth measurements are not associated to the tolerance to ampicillin that we go, not associated to the slow bacterial growth.

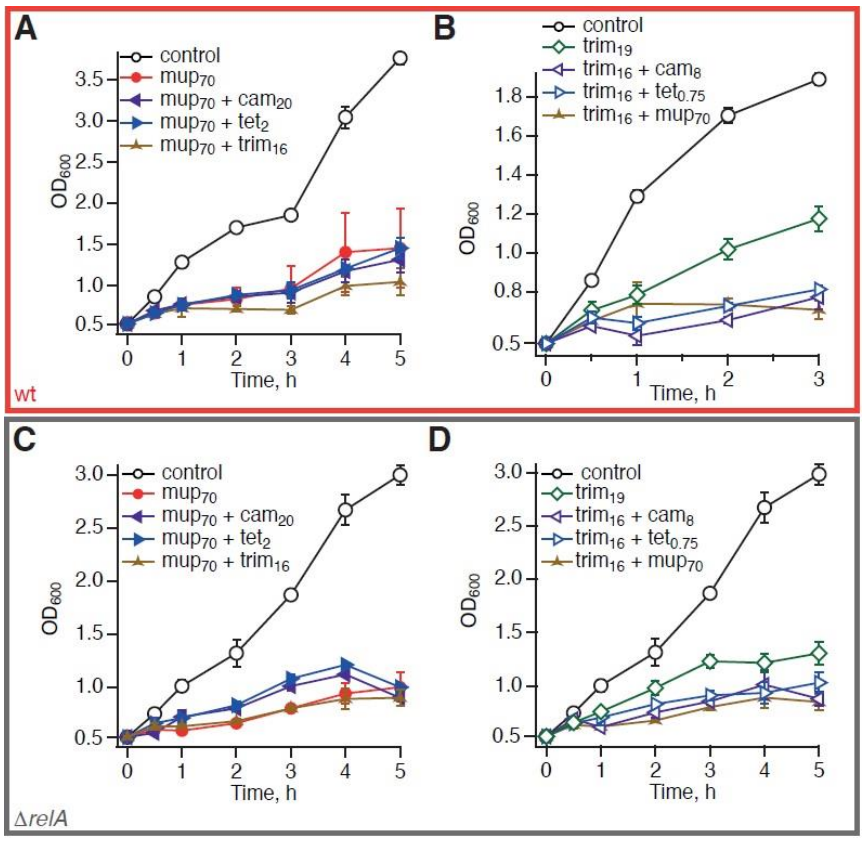


Figure 4 | Effects of antibiotic combinations on growth in *E. coli.* The experiments were done in BW25113 *E. coli* wild-type strain (wt, **A** and **B**) and in BW25113 *E. coli relA* knock-out (Δ*relA*, **C** and **D**). Antibiotic concentrations are in μM. Error bars indicate standard error. (**A** and **C**) Antibiotic concentrations that inhibits stringent response. No significant bacterial growth in mupirocin induction combinations. (**B** and **D**) Antibiotic concentrations that inhibits the growth 2 times combined with 16 μM of trimethoprim, concentration that inhibits stringent response. Here also no significant growth in any of the combinations that we can associate to the ampicillin tolerance observed in **Figure 3C** and **3F.**

2. Effects of RelA functionality on norfloxacin tolerance

Since we obtained a strong protection of the cells when challenged with ampicillin, and to comprehend if the sensitivity of the cells is there ampicillin we decided to test a fluoroquinolone, norfloxacin, bactericidal that affects DNA gyrase (79). The experiments were done with the exactly the same setup for stringent and relaxed *E. coli* of the killing experiments with ampicillin, the only difference was substituting ampicillin for norfloxacin.

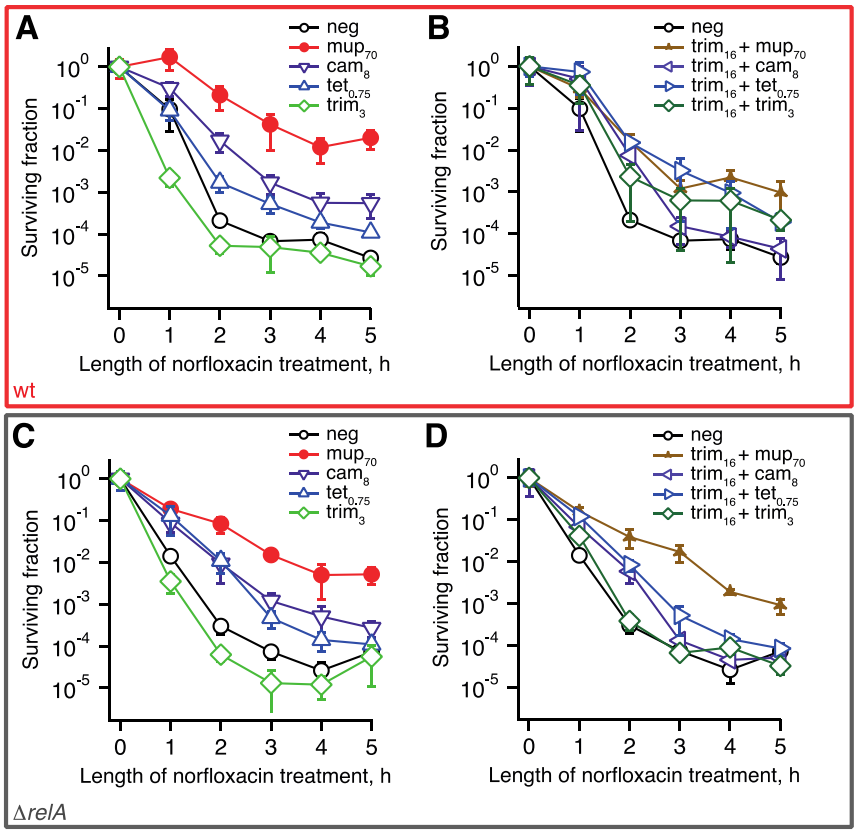


Figure 5 | Effects of RelA functionality on tolerance to norfloxacin. The cells grown in MOPS 0.4% glucose 25 µg/ml 20 amino acids at 37 °C for 5 hours upon adding norfloxacin. (**A** and **C**) Single antibiotics concentrations represent 50 growth inhibition of the bacteria. Mupirocin doesn't protect dramatically the cells either in wild-type or *rel*A knock-out. (**B** and **D**) Antibiotic combinations with trimethoprim. 16 µM of trimethoprim goes to the concentration that inhibits stringent response. In both of the strains we do not have strong protection to norfloxacin killing as we saw for ampicillin tolerance experiments.

When *E. coli* wild type is killed with norfloxacin with single antibiotics we do not see such a dramatic protection for mupirocin as we have when the cells are killed by ampicillin **(Figure 5A)**. The antibiotic combinations the strong protection is not there either **(Figure 5B)**. In relaxed strain **(Figure 5C** and **5D)** the fluoroquinolone has a modest effect that lead us to assume that RelA is not crucial for norfloxacin tolerance either. This suggests a specific mode of action for ampicillin tolerance.

3. Cell wall remodeling upon ampicillin treatment

The high levels of tolerance in case of the cell wall inhibitor **(Figure. 5)**, ampicillin, is not present when the cells are killed with inhibitor of DNA gyrase, norfloxacin **(Figure 5)**. We can say that the effect of stringent response and its inhibition on antibiotic tolerance is antibiotic specific. The ampicillin tolerance can be originated from several mechanisms (80) and also was correlated to the growth (45). However, our growth rates measurements **(Figure 4)** are not correlated to the ampicillin tolerance observed **(Figure 3)**. This suggests a specific mode of action for ampicillin tolerance. Ampicillin's molecular target is PBPs (81), DD-transpeptidases generating crosslinks between D amino acids in peptidoglycan (62). So, was tested the possibility when the antibiotic challenging alters the peptidoglycan composition. The samples were sent to specialists in cell wall analysis – Dr. Teresa Del Peso Santos at Felipe Cava's lab at, Umeå University – to test that possibility. An UPLC analysis of peptidoglycan composition was done, where the cells were exposed to either to mupirocin only or mupirocin together with trimethoprim. The results revealed that the percentage of crosslinks between DAP moieties in the third position - DAP-DAP, or 3→3 crosslinks - are significantly high in both wild type and relaxed strains upon antibiotic treatment, especially in the case of mupirocin and trimethoprim combination **(Figures 6A** and **6B)**. Also was performed a kinetic analysis of DAP-DAP accumulation in relaxed strain **(Figure 6C)**. In case of

mupirocin with trimethoprim combination the fraction of DAP-DAP crosslink increasing more than six times from 1.76 % prior to antibiotic challenge to 11 %. However, an expected result came out, since a significant increase in the DAP-DAP crosslink is observed upon mupirocin challenge only (non-tolerance condition), indicating that the relationship between peptidoglycan remodeling and ampicillin tolerance is not direct.

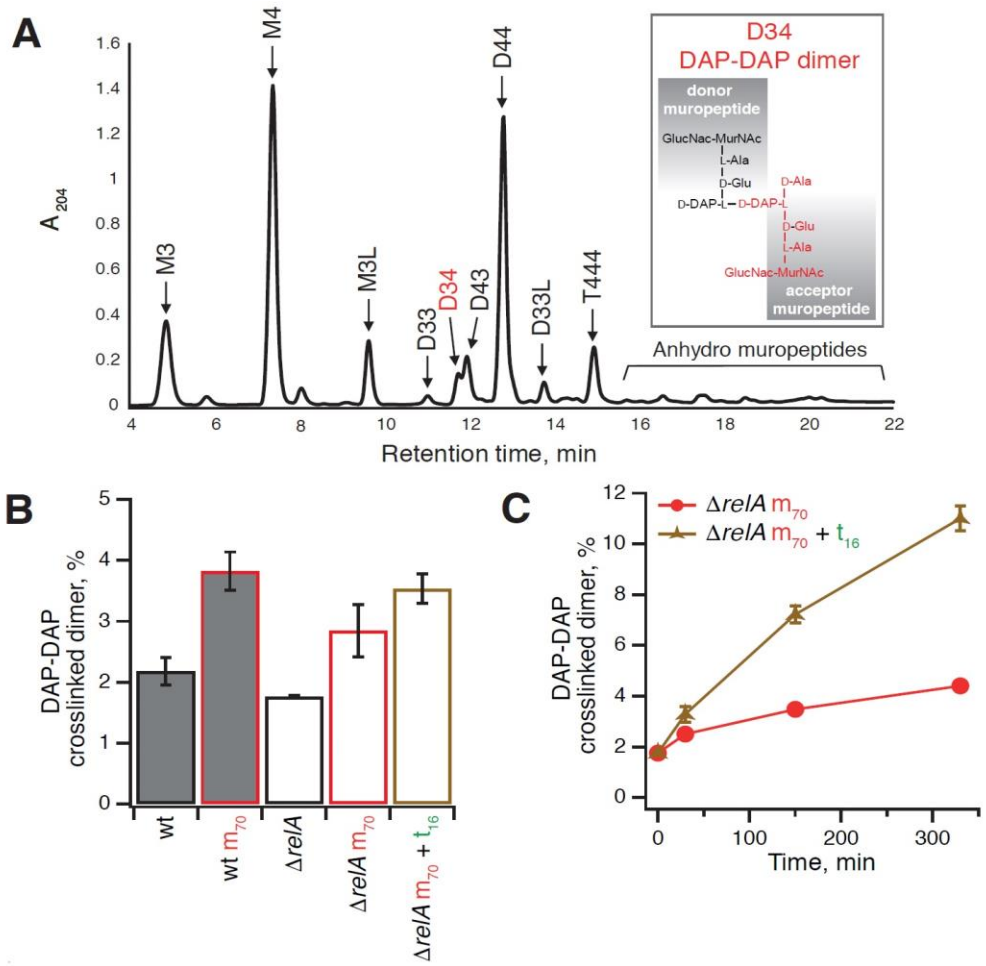


Figure 6 | Cell wall remodeling and ampicillin tolerance. The experiments were performed using *E. coli* BW25113 wild-type and Δ*relA* strains. Both strains were submitted to 70 µM mupirocin, single antibiotic, and Δ*relA* was also challenged with the combination of 70 µM mupirocin plus 16 µM of trimethoprim. (**A**) Chromatogram where several dimers 70 µM mupirocin of the muropeptides are separated. D34 DAP-DAP dimer (red) was the one that was observed accumulation. The YcbB and YnhG LD-transpeptidases generate the meso-DAP3-meso-DAP3 peptidoglycan cross-links. These enzymes cleave he meso-DAP3-D-Ala4 peptide bond of a donor muropeptide and link the carbonyl of meso-DAP3 with meso-DAP3 of the side chain amine in an acceptor muropeptide. (**B**) The percentage of DAP-DAP crosslinked dimer is variable for between strains and between antibiotic pretreatments. The cells challenged with mupirocin, single antibiotic and through high levels of persiters dependent on RelA, in both strains is visible an

increase of the cross linked dimer. However, is not observed high persister levels when the cells are pretreated with mupirocin in Δ*relA* strain. In case of antibiotic combination mupirocin with trimethoprim (high ampicillin tolerance independent on RelA) is there a significant increase of the DAP-DAP dimer crosslinked. (**C**) The kinetics analysis of DAP-DAP accumulation in Δ*relA* strain indicates a difference of 9.24% DAP-DAP crosslinked dimer between the single antibiotic treatment and the combination of mupirocin and trimethoprim.

3.1 RelA-dependent and RelA-independent tolerance to ampicillin and imipenem

The DD-transpeptidases are inhibited by of β-lactam antibiotics and LD-transpeptidases are inhibited by imipenem (64). A combination of ampicillin and imipenem could be efficient in *E. coli* with the different modes of transpeptidation and lysate the cells. In accordance to that statement and the increase of DAP-DAP crosslinks **(see section 3)** come out the question if the LD crosslinks would be responsible for the high levels of ampicillin tolerance observed **(Figure 3)**. To test that we used the usual *E. coli* strains, the combination of imipenem and ampicillin with our pretreatment combinations. In our case the effect of the killing in both strains show us that the LD crosslinks do not have a role in ampicillin tolerance.

The experiments were performed in *E. coli* wild type **(Figure 7A)** and relaxed strains **(Figure 7B)** with the combination of ampicillin and imipenem. Previously in ampicillin tolerance tests **(Figure 3)** the pre-treatment combination of mupirocin and trimethoprim gave an independent protection of RelA. However, when the cells are killed with ampicillin plus imipenem in both strains the protection remains. Regarding the combination of chloramphenicol and trimethoprim protection to ampicillin tolerance the protection was not deal with the stringent response. When the cells are challenging the in this case with this pretreatment combination the cells are not harshly killed either in wild type or Δ*relA* **(Figure 7)**. The results suggest that the increase of LD crosslinks levels are not the source of the effect.

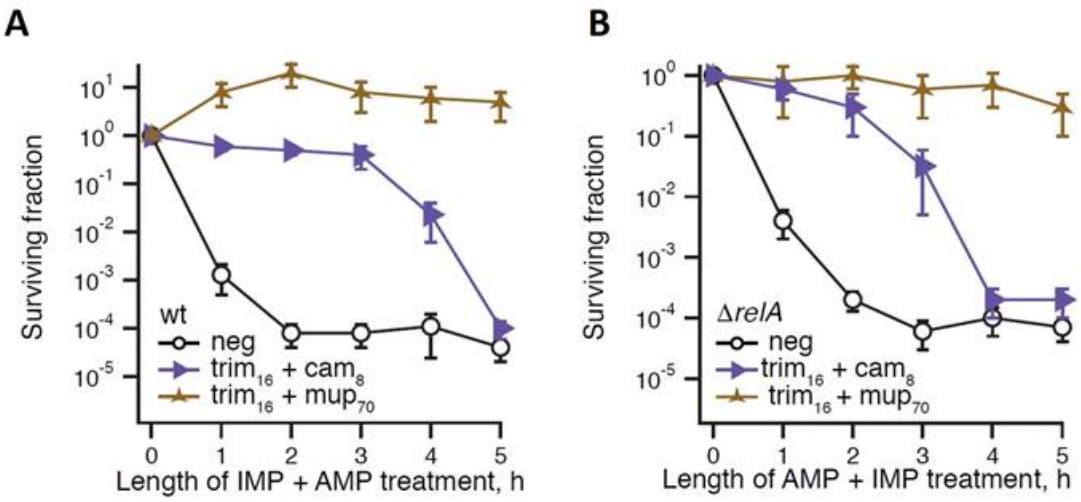
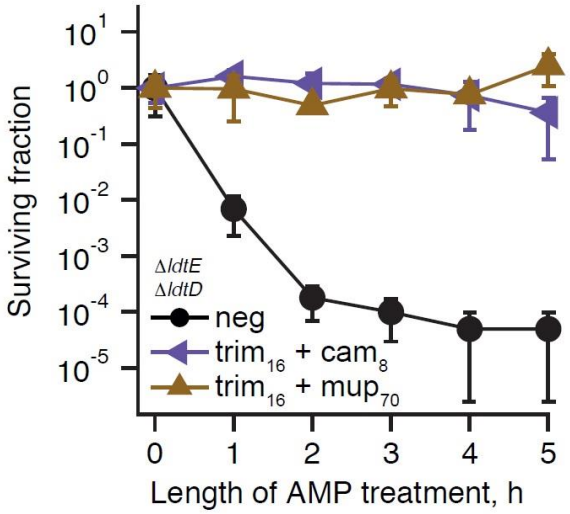


Figure 7 | Effects of RelA functionality on *E. coli* killing kinetics by IMP and AMP combination. The cells were challenged with imipenem plus ampicillin, 4 µg/ml, 3 times MIC, and 200 µg/ml respectively. Same experimental conditions as the experiments of ampicillin tolerance. The bars represent the standard error. (**A**) The experiment was performed in BW25113 *E. coli* wild type. Even with the plus of imipenem the cells in case of the combination of trimethoprim and mupirocin is not noticeable cellular lysis. (**B**) *E. coli rel*A knock-out was also used in that test. Also here with the combination of the ampicillin and imipenem we still observe a strong protection with trimethoprim and mupirocin when are combined.

To further support this statement, we used an *E. coli* strain lacking functional Ldt genes c (*ldtD*) and *ycbB* (*ldtE*) and consequently unable to form 3-3 crosslinks (82). In that case with the same previous pretreatment antibiotics setup we obtained strong protection to ampicillin **(Figure 8)**.

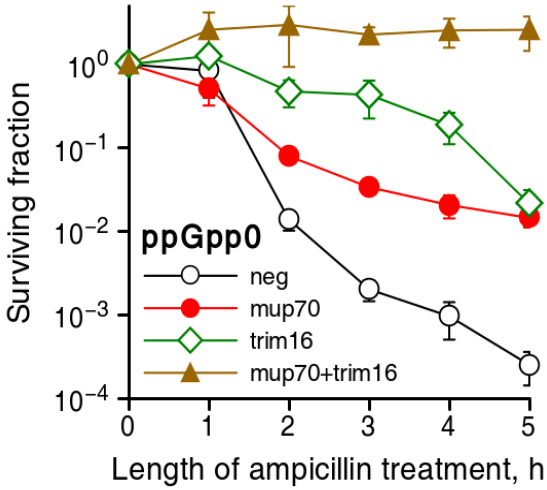


Therefore, we can settle that the accumulation of LD crosslinks are not linked to our ampicillin tolerance.

Figure 8 | Ampicillin tolerance on *E. coli* Δ*ldt*E Δ*ldt*D. The experiment was performed in *E. coli* Δ*ldt*E Δ*ldt*D – Δ*ldt*E goes to *ycbB* and Δ*ldt*D goes to *ycbB*, nomenclature for LD-transpeptidases in *E. coli* that is in accordance with other bacteria – the cells grew in MOPS 0.4% glucose 25 µg/ml amino acids at 37 °C. The cells were pre-treated with the 16 µM trimethoprim combinations with 8 µM chloramphenicol and 70 µM mupirocin and challenged by 200 µg/ml ampicillin. In both combinations is remarked high levels of ampicillin tolerance. The lacking of this two Ldt genes do not interfere with the ampicillin tolerance observed in *E. coli* wild-type. In our case infers that also LD crosslinks have no role in ampicillin tolerance.

4. The role of SpoT on ampicillin tolerance

The RSH family of proteins are the essential players on (p)ppGpp synthesis **(see Introduction section 1.1)**. Since we discarded the potentials of the ampicillin tolerance be related to the functionality of RelA and either to LD crosslinks accumulation, one possibility left was to test if the bifunctional enzyme SpoT is the key of the ampicillin tolerance. We used a strain named ppGpp0 that is lacked of the genes *relA* and *spoT*, thus the strain do not produce (p)ppGpp. Came out that (p)ppGpp has no role in ampicillin tolerance observed previously **(Figure 3)**. The single antibiotics added, mupirocin and trimethoprim, are not strongly killed. However, the combination mupirocin with trimethoprim has high levels of ampicillin tolerance **(Figure 9)**. We can



accomplish that also SpoT is not responsible for the high levels of persistence that we observe when *E. coli* is challenge by ampicillin.

Figure 9 | Ampicillin killing of *E. coli* ppGpp0 strain. This ampicillin tolerance test was performed in *E. coli* ppGpp0 lacking both genes *relA* and *spoT*. MOPS 0.4% glucose 400 μg/ml Serine 40 μg/ml amino acids at 37 °C were the conditions used. The antibiotics applied in a single approach were 70 μM mupirocin and 16 μM trimethoprim, and also the combination of these two antibiotics. The cells were challenged with the usual 200 μg/ml ampicillin. The bars represent the standard error. Meant for the single pre-treatment antibiotics the killing was not as hard as for *E.coli* Δ*relA*. Concerning the combination of mupirocin with trimethoprim the high levels of persister cells still being observed as for wild-type or knock-out *relA* strains. SpoT is not the key for ampicillin tolerance.

The original discovery of mechanism connecting (p)ppGpp and antibiotic tolerance in *E. coli* reported increased ampicillin sensitivity for ppGpp0 cells (41, 83). We tried perform our experiments with that strain in defined medium, MOPS supplemented with 0.4% glucose and 25 μg/ml amino acids, which was used to perform all the prior experiments. We failed using that medium for ppGpp0 strain, we observed restricted growth and for consequence high level of persistence (data not shown). Increase the concentration of the amino acids especially the amino acid serine was an option. Since, serine might contain an inhibitor that prolongs the lag phase before growth resumes and a low concentration of the rest of amino acids also helps the growth yield be low (71). The minimal medium to perform the experiments with ppGpp0 strain was supplemented with 40 μg/ml 19 amino acids and 400 μg/ml of serine. However, persister experiments by Maisonneuve and colleagues were done originally in LB medium. It is a complex medium where bacteria can alter their physiology to several changes of limiting nutrients and affect the growth (84, 85). As was mention previously in case of persistence the growth conditions are crucial (45). Therefore, we have performed the following ampicillin killing experiments with ppGpp0 strain both in LB and MOPS media comparing the three *E. coli* used in the entire study.

In the graph that shows the experiments that were perform in MOPS medium **(Figure 10A)** is observed that the surviving fraction of the three strains do not differ much after the usual 5 hours of killing with ampicillin. Thus, in the MOPS supplemented with high concentration of serine doesn't affect differently the physiology of the *E. coli* strains. Regarding the test in LB medium also the cells of the all different strains are pretty much killed at the same order of magnitude **(Figure 10B)**. Comparing the values of surviving fraction between MOPS and LB filtrated media

the difference of orders of magnitude after of the 5 hours killing by ampicillin is not significant. In our case the different medium affect similarly the physiology of the strains that we were working with.

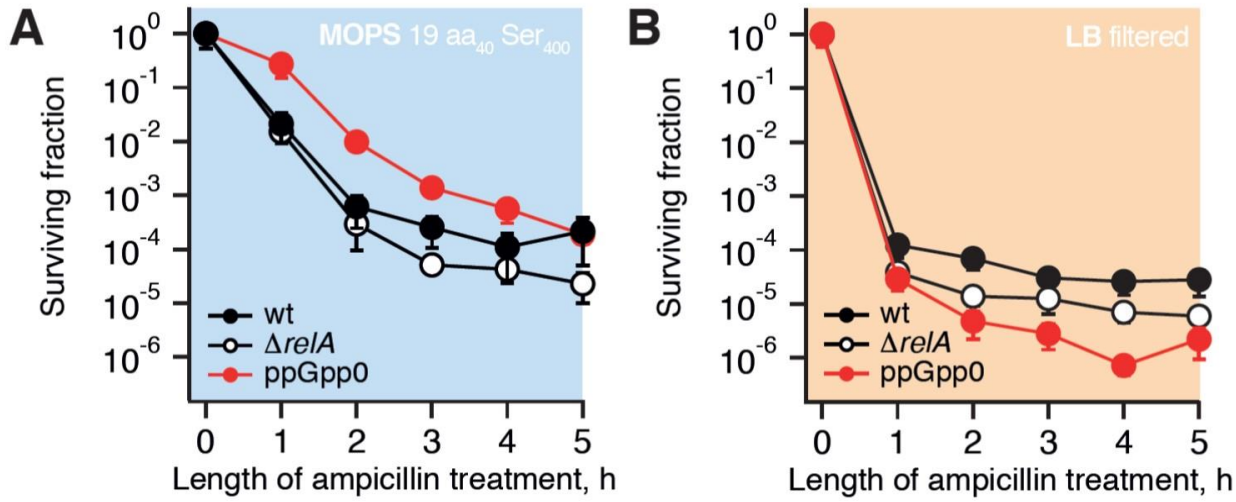


Figure 10 | Influence of growth medium on ampicillin killing of *E. coli*. Three *E. coli* strains – BW25113 wild-type, Δ*relA* and ppGpp0 – were used in both experiments with MOPS 0.4% glucose 40 µg/ml 19 amino acids 400 µg/ml Serine (**A**) and LB filtrated (**B**). No pre-treatment was applied, the cells were only treated with 200 µg/ml ampicillin for 5 hours. (**A**) The strains grown in MOPS 19 aa_{40} Ser_{400} are killed at the same level. (**B**) In LB filtrated the cells are killed better than in MOPS 19 aa_{40} Ser_{400} medium (**A**). However, that difference is not significant. Comparing between the surviving fraction in LB filtrated medium the three strains have the same level of killing by ampicillin.

Discussion

Persisters cells represent a small subpopulation of cells that enter a nondividing state and are tolerant to antibiotics. When a population is treated with a bactericidal antibiotic, regular cells die, whereas persisters survive. The cells reach this state without undergoing genetic change. The antibiotic tolerance to several types of bactericidal and their common incidence may contribute to the intractability of chronic and periodic infections. (p)ppGpp is a global regulator and mediator of antibiotic tolerance and acute accumulation of (p)ppGpp – the stringent response – was suggested to be the driver of persister cell formation. A well-known molecular model (41), where (p)ppGpp and TA system play an important role for antibiotic tolerance.

When we induce the stringent response with mupirocin in *E. coli* wild-type and in the *relA* knockout strains protection against ampicillin is strictly dependent on RelA **(Figures 3A** and **3D)**. This suggests that the stringent response can, indeed, cause antibiotic tolerance. However, when we inhibit the stringent response by treating bacteria with antibiotics inhibiting translation – cloramphenicol and tetracycline – we do not eradicate the ampicillin tolerance **(Figure 3B)**. The most surprisingly result when we use antibiotic trimethoprim, inhibitor of DNA synthesis, together with the stringent response inducer mupirocin we see potent and RelA-independent protection from ampicillin **(Figure 3E)**. The effects described above are specific to *E. coli* tolerance to β-lactam antibiotic ampicillin and were not observed in control experiments with another bactericidal antibiotic – fluoroquinolone norfloxacin **(Figure 5)**.

Next we attempted to figure out the molecular mechanism behind the RelA-independent ampicillin tolerance induced by antibiotic pretreatment. The main molecular target of ampicillin is PBPs enzymes forming crosslinks in cell wall peptidoglycan. We hypothesized that peptidoglycan may have undergone a remodeling when the cells are exposed to combinatorial pretreatments with antibiotics rendering cell-wall formation PBP-independent. While we did observe the remodeling of the cell wall i.e. accumulation of so-called DAP-DAP crosslinks **(Figure 6)**, we showed that the said remodeling is not the causative agent of ampicillin tolerance since both genetic (via disruption of the Ldt enzymes forming DAP-DAP bridges) or chemicals (using antibiotic imipenem that inhibits the Ldt enzymes) did not eradicate the ampicillin tolerance **(Figures 7** and **8)**. Next we hypothesized that maybe (p)ppGpp is, after all, the key to observed tolerance – but it

is not RelA, but another RSH enzyme, SpoT, which is responsible for its production under our experimental conditions. We have tested a so-called ppGpp0 strain lacking both RelA and SpoT **(Figure 9)** and it still displayed ampicillin tolerance upon combined treatment of mupirocin and trimethoprim.

Taken together, my data suggest that while (p)ppGpp is important for ampicillin tolerance, it is, first, not the only mechanism at play and, second, the connection between (p)ppGpp and antibiotic tolerance is complex. Several studies connect tolerance is motivated by the slow growth (34, 45). That is connected with bacterial transition from dormancy in stationary phase to new growth (86) that process is affected by the production of (p)ppGpp in a media specific way (69). A better covered studies regarding the role of RelA SpoT Homologue enzymes connecting the antibiotic tolerance and the transition from dormancy to new round of growth are necessary, then link to the establishment of the bacterial infection. This is the direction I am keen on following up during my PhD studies.

Conclusions

1. High levels of (p)ppGpp induce ampicillin tolerance in RelA-dependent manner.
2. However, pre-treatment of antibiotics can cause RelA-independent ampicillin tolerance.
3. The exact mechanism of this tolerance is unclear.

Summary

The stringent response is a near-universal bacterial adaptation system control mediated by accumulation of two guanine nucleotides ppGpp and pppGpp, collectively known as (p)ppGpp. The response monitors several environmental stress inputs, such as nutrient limitation and heat shock and remodels bacterial physiology in order to overcome the challenges. In *Echerichia coli* (p)ppGpp levels controlled by two enzymes – RelA and SpoT, the namesakes of RelA SpoT Homologue (RSH) protein family. The stringent response is associated to induction of virulence, antibiotic resistance and was recently suggested to be the driving force behind the formation of so-called persister cells – antibiotic-tolerant phenotypic variants in antibiotic-sensitive population. Since drug resistance and tolerance constitute a significant public health threat, understanding the connection amongst (p)ppGpp, antibiotic treatment and persistence is of great importance.

For this thesis I studied the role of presence and absence of accumulation of (p)ppGpp in antibiotic tolerance, especially in case of the β-lactam ampicillin that kills cell via inhibition of cell wall biosynthesis. The bacterial growth rate is a key factor affecting bacterial tolerance antibiotics, and in the case of ampicillin the killing efficiency is directly proportional to the rate of growth. (p)ppGpp is expected to contribute to ampicillin tolerance in several ways: first, is a key regulator of growth rate, second, third it regulates cell wall synthesis via direct effects on several key enzymes involved in the process, third, (p)ppGpp was proposed to induce persistence via activation of so-called toxin-antitoxin modules. By following ampicillin killing in *E. coli* strains either having active (wild-type) or inactive RelA (Δ*relA*) under various conditions I have dissected RelA-dependent and RelA-independent aspects of ampicillin tolerance. I conclude that, first, while elevated (p)ppGpp does drive bacterial tolerance against ampicillin, the effect is specific to protection to this antibiotic as opposed to fluoroquinolone norfloxacin and, second, that challenging bacteria by antibiotics can cause RelA-independent ampicillin tolerance.

Key words: stringent response, antibiotic tolerance, bacteria, RelA SpoT Homologue enzymes

Resümee

Poomisvastuse roll *Escherichia coli* antibiootikumide taluvuses

Poomisvastus on bakterite seas laialt levinud kohanemismehhanism, mille puhul tõuseb rakus kahe guaniinnukleotiidi, ppGpp ja pppGpp ehk (p)ppGpp tase. Selle mehhanismi käivitavad muutused kasvukeskkonnas, nagu näiteks toitainete puudus ja kuumašokk; protsessi tulemusel seadistub rakufüsioloogia muutunud tingimustele vastupidavaks. *Escherichia coli*-s kontrollivad (p)ppGpp taset kaks ensüümi – RelA ja SpoT, mille järgi on nimetatud ka terve nende valguperekond: RelA ja SpoT-ga Homoloogsed (RSH) ensüümid. Poomisvastust seostatakse nii virulentsuse kui antibiootikumide resistentsusega. Lisaks on hiljuti pakutud välja, et poomisvastus võiks olla peamiseks käivitavaks jõuks nn persister-rakkude – antibiootikumi taluva (ehk tolerantse) fenotüübiga üksikrakud muidu antibiootikumi-tundlikkus rakupopulatsioonis – moodustumisel. Kuna nii antibiootikumide resistentsus kui tolerantsus kujutavad endast tõsist ohtu inimkonna tervishoiule, on äärmiselt oluline uurida (p)ppGpp ja antibiootikumi toime vahelisi seoseid.

Käesoleva töö käigus uurisin (p)ppGpp akumuleerumise mõju antibiootikumi taluvusele peamiselt beetalaktaamse ampitsilliini näitel. Ampitsilliin tapab rakke, inhibeerides rakukesta biosünteesi. Bakteri kasvukiirus on tegur, mis määrab sageli antibiootikumide toime tõhususe; ampitsilliini põhjustatud tapmise efektiivsus on võrdelises seoses kasvukiirusega. Võib oletada, et (p)ppGpp mõjutab bakterirakkude ampitsilliini taluvust mitmel moel: (1) reguleerib rakkude kasvukiirust; (2) mõjutab rakukesta sünteesis osalevaid ensüüme; (3) käivitab persister-rakkude moodustumise, aktiveerides bakteris nn toksiin-antitoksiin süsteeme. Uurides *E. coli* metsik-tüüpi ja RelA-deletsiooniga rakke (Δ*relA*) erinevates kasvutingimustes, olen toonud selgust, millised ampitsilliini taluvuse ilmingud sõltuvad RelA-st ja millised mitte. Kokkuvõttes järeldan: (1) kuigi (p)ppGpp rakusisese taseme tõus tagab suurema ampitsilliini taluvuse, on tegemist spetsiifilise efektiga, mis ei taga kaitset näiteks fluorokinoloonse norfloksatsiini vastu; (2) teatud antibiootikumide eel-töötlus võib viia suurema ampitsilliini taluvuseni, mis ei sõltu RelA-st.

Märksõnad: bakteriraku füsioloogia; poomisvastus; antibiootikumi taluvus; RelA ja SpoT-ga

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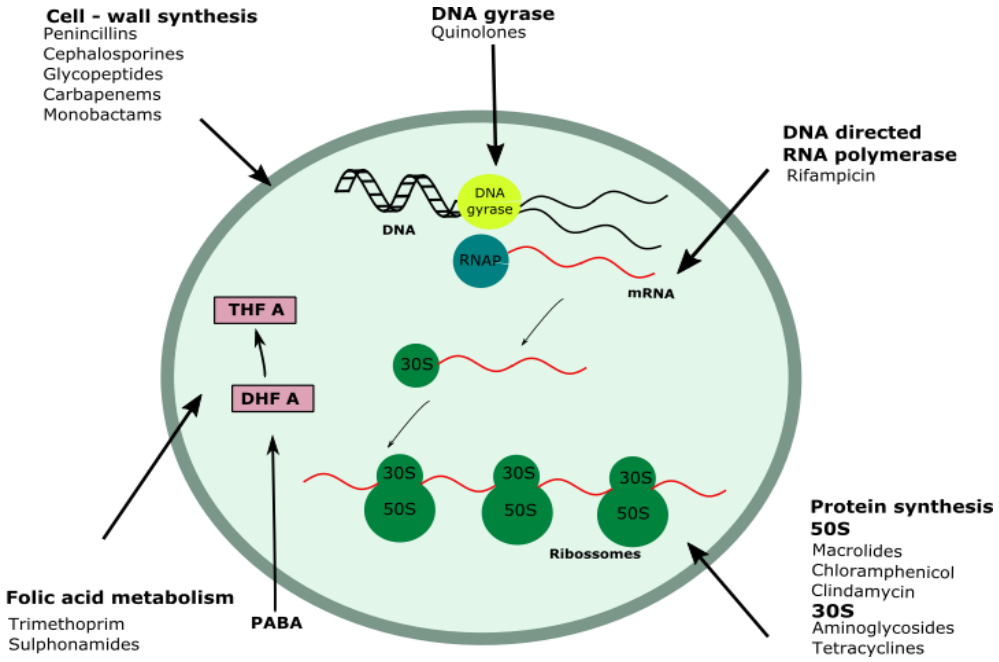
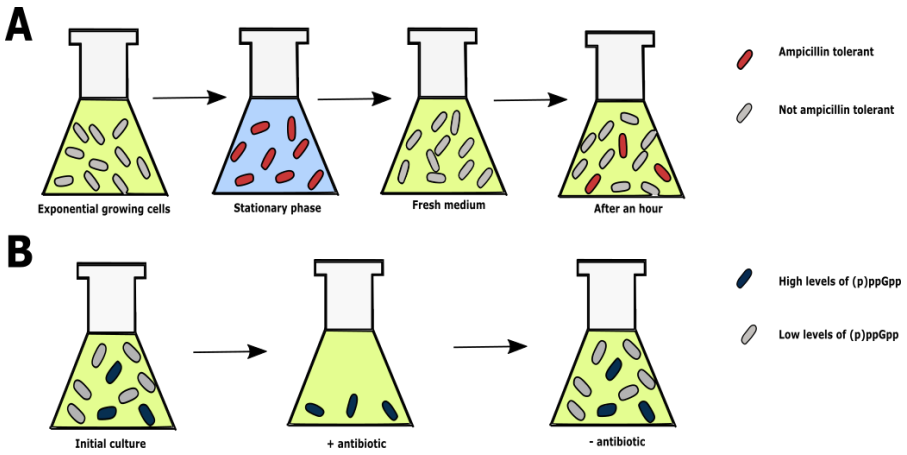
Appendices

Appendix 1



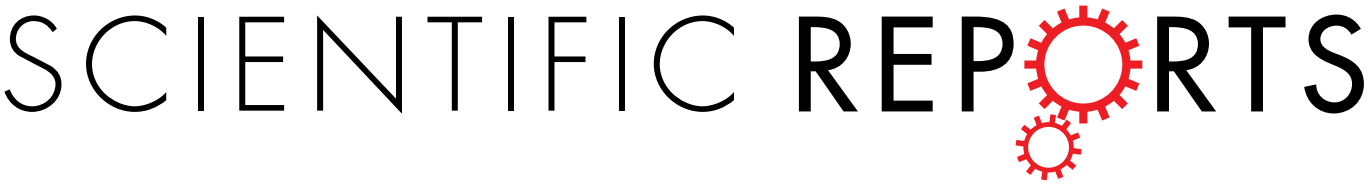
Appendix 1 | Antibiotic targets in bacterial cell. The β-lactams antibiotics target the penicillin binding proteins blockading crossing enzymes in peptidoglycan layer of cell walls, e.g. ampicillin and imipenem. Vancomycin also targets cell wall at D-Ala-D-ala termini of peptidoglycan and lipid II. The quinolones interfere DNA replication by trapping a complex of DNA bound to the enzyme DNA Gyrase, a type II topoisomerase, e.g norfloxacin. Protein biosynthesis at the ribosome is targeted by several classes of antibiotics, including macrolides, tetracyclines, aminoglycosides and oxazolidinones, which block one or more steps involving rRNA and the proteins of the ribosome at the peptidyl transferase centre. Rifamycin inhibits DNA-dependent transcription by stable binding, with high affinity, to the subunit of a DNA-bound and actively-transcribing RNAP. Trimethoprim and sulphonamides act through folic acid metabolism to damage DNA.

Appendix 2



Appendix 2 | Antibiotic tolerance induced by transition to stationary phase and by high (p)ppGpp levels. (**A**) At stationary phase cells arrest their growth when treated with ampicillin (red cells). Next the transfer to fresh medium cells turn to cells not ampicillin tolerant (grey cells). After an hour the few cells become tolerant to ampicillin, although non-tolerant still growing too. (**B**) The initial culture has levels of high (blue cells) and low (gray cells) levels of (p)ppGpp. When the cells are treated with an antibiotic the cells with high level of (p)ppGpp survives – persisters. Upon regrowth in a media with no antibiotic the culture gives a heterogenic population with the same antibiotic sensitivity as in the initial culture. This image is adapted from (5, 87).

Appendix 3



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Composition of the outgrowth medium modulates wake-up kinetics and ampicillin sensitivity of stringent and relaxed *Escherichia coli*

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The transition of *Escherichia coli* from the exponential into the stationary phase of growth induces the stringent response, which is mediated by the rapid accumulation of the alarmone nucleotide (p)ppGpp produced by the enzyme RelA. The significance of RelA's functionality during the transition in the opposite direction, i.e. from the stationary phase into new exponential growth, is less well understood. Here we show that the relaxed strain, i.e. lacking the *relA* gene, displays a relative delay in regrowth during the new exponential growth phase in comparison with the isogenic wild type strain. The severity of the effect is a function of both the carbon source and amino acid composition of the outgrowth media. As a result, the loss of RelA functionality increases *E. coli* tolerance to the bactericidal antibiotic ampicillin during growth resumption in fresh media in a medium-specific way. Taken together, our data underscore the crucial role of medium composition and growth conditions for studies of the role of individual genes and regulatory networks in bacterial phenotypic tolerance to antibiotics.

Bacteria face rapid changes in nutrient availability to which they have to adapt: in periods of famine they need to slow down their metabolism and growth, and when the food source is abundant again they need to resume their rapid production of biomass. The simplest laboratory model of feast-to-famine transition is bacterial stationary phase liquid culture diluted into fresh media. The renewed availability of nutrients allows the starved bacteria to transition to exponential growth after an initial lag phase. To exercise this metabolic maneuver efficiently, both adequate responses to nutrient limitation during the stationary phase and to nutrient abundance upon re-dilution are of importance.

One of the key players coordinating bacterial metabolism is the intracellular alarmone (p)ppGpp (see several excellent recent reviews on the subject)[1–3]. In *Escherichia coli* two enzymes RelA and SpoT, the namesakes of the widely distributed RelA/SpoT Homolog (RSH) protein family[4], control the intracellular concentration of this messenger nucleotide. RelA is a ribosome-associated factor that senses amino acid limitation by directly inspecting the aminoacylation status of the A-site tRNA[5]. Deacylated tRNA activates RelA's strong (p)ppGpp synthesis activity[6], and increased (p)ppGpp levels initiate a multilayered adaptation program. On the transcriptional level production of ribosomes is halted[7] while expression of amino acid biosynthesis genes is induced[8–10]. At the same time diverse molecular targets are directly engaged by (p)ppGpp[11], affecting protein synthesis, DNA replication and nucleotide biosynthesis[1]. While RelA is a one-trick pony, SpoT is a bifunctional enzyme capable of both (p)ppGpp synthesis[12] and degradation[13], which mediates (p)ppGpp accumulation during the response to various stimuli such as fatty acid[14], iron[15] and carbon source[12] starvation. In addition to responding to nutritional downshifts, SpoT maintains basal (p)ppGpp levels during steady state growth[16].

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Rapid RelA-dependent accumulation of ppGpp is dubbed the stringent response[17] and leads to cessation of stable RNA synthesis, inhibition of translation and growth arrest[18]. Loss of function *relA* mutants display a so-called relaxed phenotype characterized by a waste of cellular resources on continuous production of stable RNA during amino acid starvation[18], diminished antibiotic tolerance[19], and reduced production of glycogen[20].

The classical growth curve of bacteria in batch culture contains a lag phase, an exponential phase and a stationary phase[21]. RelA mediates rapid accumulation of (p)ppGpp during the exit from exponential phase to entry into the stationary phase[22], preparing the bacteria for starvation and cessation of growth. Interest in the physiology of relaxed (Δ*relA*) strains has been reignited in the last decade, since the functionality of ribosome-dependent RSH enzymes i.e. RelA in Beta- and Gammaproteobacteria and Rel in the rest of bacterial clades[4] has been linked to bacterial virulence[23] and antibiotic tolerance[19]. Given the multiple roles played by (p)ppGpp during bacterial stationary phase physiology (for review see Navarro Llorens and colleagues[24]) we set out to systematically characterize how RelA functionality affects re-growth of *E. coli* from an overnight stationary culture in fresh media, a step involved in virtually all microbiological experiments, specifically focusing on the role of amino acids and carbon source composition of the outgrowth media.

Results

The growth resumption delay of a Δ*relA* strain is dependent on the outgrowth medium and can be abolished by the addition of the complete set of 20 amino acids. We used two standard types of microbiology media: chemically defined minimal medium M9[25] and complex Lysogeny Broth (LB) medium[26]. LB is based on a mixture of nutrients originating from a pancreatic digest of casein from cow's milk and autodigest of *Saccharomyces cerevisiae*, and as different nutrients are sequentially consumed, *E. coli* cultures undergo a succession of diauxic shifts along the growth curve[27]. M9 in its simplest formulation consists of a buffering system, a mixture of essential inorganic salts and a carbon source – usually glucose, as is used here – and it satisfies minimal nutrient requirements for growth of *E. coli*, while supplements such as amino acids and vitamins can be added separately.

To test RelA's role in growth resumption, K-12 *E. coli* wild type strain BW25113[28] and isogenic relaxed Δ*relA* were grown through exponential phase into stationary phase, kept in stationary phase (defined as less than 10% increase in OD_{600} within 1 hour) for 15 hours and diluted into fresh medium. The OD_{600} of cultures was followed throughout the time course. During the initial growth to stationary phase there is no substantial difference in the growth of the two strains, both in LB (designated with light beige shading) and M9 (designated with light blue shading) (Supplementary Figure 1), just as there is no difference in growth resumption of the wild type and the relaxed strain upon LB-to-LB transition (Fig. 1A, quantification of lag and doubling times is summarized in Table 1). At the same time, the Δ*relA* strain showed a pronounced – around five hours – growth resumption delay during transition from LB to M9 medium supplemented with 0.4% glucose without additional supplements such as amino acids (Fig. 1D). As a simple numerical measure of the differences in growth resumption, we have plotted the ratio of OD_{600} for Δ*relA* to wild type strain (Fig. 1A–F, red trace).

The growth resumption delay of the Δ*relA* strain could, in principle, stem from lower effective inoculum size as measurements of colony forming units (CFU) do show slightly lower cell count of the Δ*relA* strain compared to the wild type during the stationary phase (Supplementary Figure 2A). However, cross-inoculation experiments LB-to-M9 and M9-to-LB show that the appearance of the growth resumption delay in the Δ*relA* strain is specific to the nature of the *outgrowth* medium, specifically it is present in M9 but not LB (Fig. 1C,D), suggesting that reduction of the inoculum size is not the cause of the phenomenon. Washing the cells with M9 during the LB-to-M9 transition in order to remove traces of LB has a dramatic effect on the relative growth delay of Δ*relA* strain: when this step is omitted the effect is considerably less pronounced (compare Fig. 1D,F). However, the wash *per se* is not responsible for the delay, since addition of the wash step during LB-to-LB transition, if anything, promotes an earlier regrowth of the Δ*relA* strain (compare Fig. 1E,A).

Eventual regrowth of the Δ*relA* strain in M9 medium could, in principle, be mediated by a sub-population harboring compensatory mutations – a well-documented phenomenon for *E. coli* strains unable to produce (p)ppGpp due to a simultaneous disruption in both *relA* and *spoT* genes[29]. However, passage of the wild type and Δ*relA* strain through a second regrowth phase faithfully replicated the growth delay effect (Fig. 2A), supporting the idea of composition of the outgrowth medium being responsible for the effect. Since the growth resumption lag was not apparent in LB medium, which has a high concentration of easily metabolizable amino acids[27], we have tested whether amino acid supplementation of M9 rescues delayed outgrowth of the Δ*relA* culture. Indeed, the growth resumption delay is rescued by addition of a full set of 20 amino acids (each at 100 μg/ml) to the outgrowth minimal medium (Fig. 2B), suggesting that amino acid limitation in M9 is, indeed, responsible for the effect. Measurements of CFUs are in good agreement with the OD_{600} trace (Supplementary Figure 2B).

Deprivation of methionine, valine and leucine in the outgrowth medium causes a relative delay in growth resumption of Δ*relA* strain. To test whether any specific amino acid is the limiting factor responsible for the delay in the resumption of the Δ*relA* strain we tested growth recovery in M9 minimal media supplemented with single amino acid drop out sets, M9 supplemented with 0.4% glucose and 19 individual amino acids added at final concentration of 100 μg/ml. Deprivation of methionine, lysine or any of the branched-chain amino acids (BCAA) – isoleucine, leucine and valine – resulted in a growth resumption delay in both strains, although to a somewhat different degree in each case (Fig. 3A,B). The effect, however, was substantially stronger in the case of the Δ*relA* strain (compare Fig. 3A,B,C).

In order to separate amino acid dropout effects on bacterial growth *per se* from specific effects on growth resumption we have performed the same set of experiments using inoculum of *E. coli* cells from exponential, rather then stationary, phase – an approach that was used in the past to study auxotrophy of *relA* mutants[30–32]. When switched from minimal M9 medium lacking amino acid supplements into a 19 amino acid medium neither

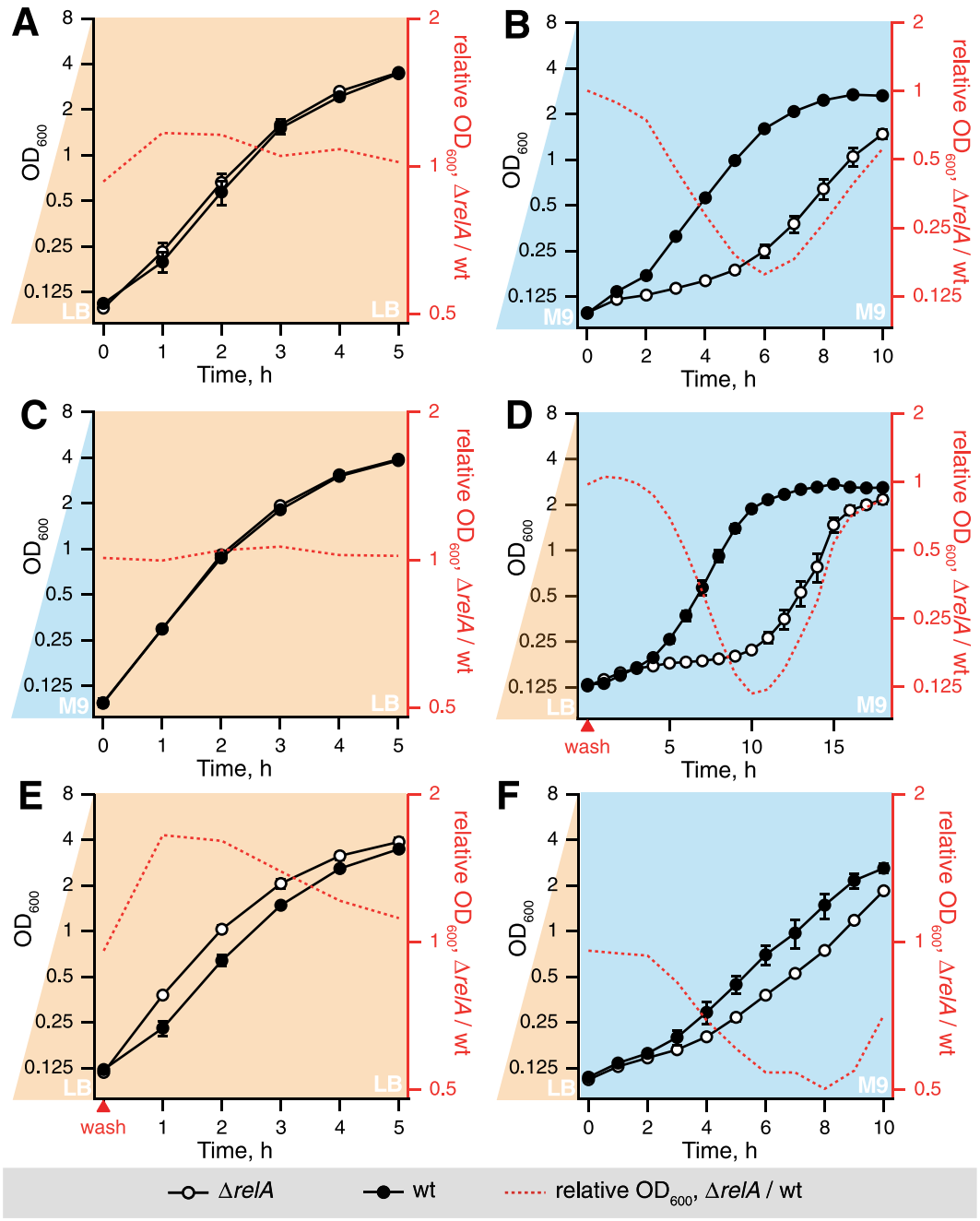

Figure 1. The outgrowth medium defines the growth resumption delay in Δ*relA E. coli* strain. The OD_{600} values of a wild type BW25113 strain (filled circles) and an isogenic Δ*relA* strain (empty circles) were followed in LB (**A, C** and **E**, light beige shading) or M9 medium supplemented with 0.4% glucose (hereafter M9, light blue shading) (**B**,**D**,**F**). The ratio of OD_{600} for Δ*relA* to OD_{600} of wild type strain (red dotted line) serves as a numerical measure of the difference in growth resumption kinetics between the two. Prior to inoculation, the seeder culture was kept for 15 hours in stationary phase in either LB (light beige shading) (**A**,**D**,**E**,**F**) or M9 (**B**,**C**) media. Cross-inoculation experiments M9-to-LB (**C**) and LB-to-M9 (**D**) demonstrate that the growth defect of Δ*relA* is specific to the outgrowth medium, i.e. present only in M9. During the LB-to-M9 transition (**D**), cells were washed with M9 (indicated by the red triangle on the x axis) to reduce carry-over of medium. The washing procedure itself had only mild effect on cells, and if anything, favored growth resumption of Δ*relA* cells (**E**). Results are shown as mean values of biological replicates ($n \geq 3$) and error bars (too small to be seen for some of the points) indicate standard error of the mean.

the wild type nor the Δ*relA* strain were able to resume growth in isoleucine dropout media for 24 hours of observation: a well-known phenotype of the K-12 strains[33–35] (Fig. 4A,B). This is in stark contrast with the stationary phase cultures, which did start regrowth after 3.1 ± 0.1 (wt), 3.4 ± 0.3 (Δ*relA*) hours (Fig. 3A,B, Table 1).

Figure	Initial medium	Wash step	Outgrowth medium	Doubling time, min		Lag phase, h	
				Δ*relA*	wt	Δ*relA*	wt
S1A	**LB**	*NA*	*NA*	26 ± 0.4	25 ± 0.3	*NA*	*NA*
1A	LB	–	**LB**	39 ± 0.1	40 ± 1	0.2 ± 0.1	0.4 ± 0.1
1E	LB	+	**LB**	36 ± 1	40 ± 1	−0.1 ± 0.01	0.2 ± 0.1
1C	M9	–	**LB**	37 ± 1	37 ± 2	0.03 ± 0.02	0.04 ± 0.05
S1B	**M9**	*NA*	*NA*	58 ± 1	67 ± 2	*NA*	*NA*
1B	M9	–	**M9**	82 ± 7	70 ± 3	4.5 ± 0.2	1.1 ± 0.03
5A	M9	+	**M9**	102 ± 0.5	68 ± 0.5	4.3 ± 0.3	1.0 ± 0.5
1F	LB	–	**M9**	91 ± 3	94 ± 7	3.6 ± 0.07	1.7 ± 0.3
1D	LB	+	**M9**	82 ± 2	87 ± 6	9.1 ± 0.3	3.4 ± 0.5
2B	M9	–	**M9 + AA**	43 ± 0.3	44 ± 1	0.4 ± 0.1	0.6 ± 0.06
5B	M9	+	**M9gly**	109 ± 5	123 ± 3	3.8 ± 0.3	2.9 ± 0.2
S3	M9	–	**M9gly + AA**	77 ± 3	73 ± 3	1.0 ± 0.2	1.2 ± 0.1

Table 1. Quantification of the growth kinetics data of wild type and relaxed BW25113 *E. coli*. Lag phase is estimated by fitting the data points used to estimate the doubling time to an exponential growth model as per Monod[21]. M9glc corresponds to M9 medium supplemented with 0.4% glucose; AA indicates to the addition of the 20 amino acids set; gly indicates substitution of glucose for glycerol. Detailed description of the media composition and growth conditions is provided in the corresponding Figure legends. Bold letters indicate the step for which the parameters are quantified. *NA* signifies that corresponding parameter is not applicable for the experiment. SEM is rounded up to one significant digit. Results are reported as mean values of biological replicates ($n \geq 3$), ± standard error of the mean.

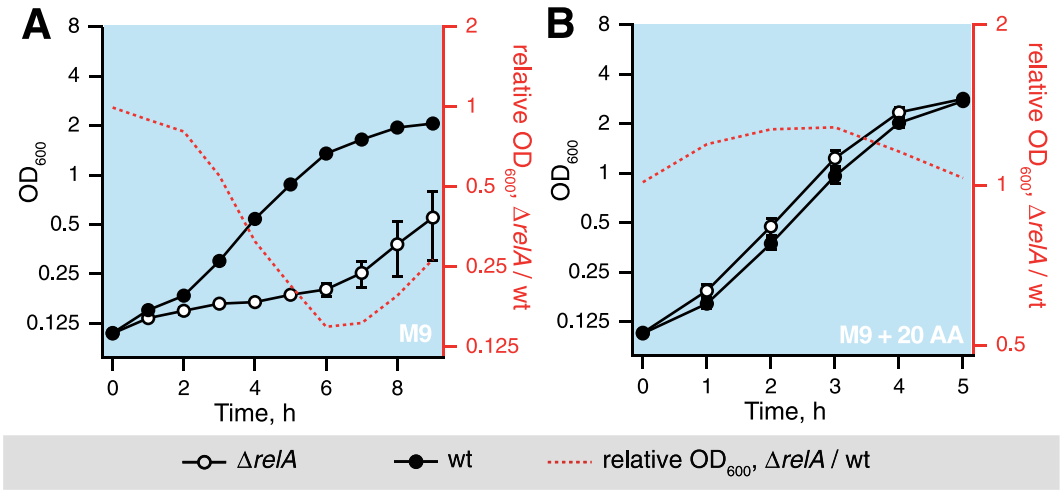

Figure 2. Eventual growth resumption of the Δ*relA* strain in M9 is not due to compensatory mutations and its regrowth delay can be relieved by the addition of the full set of 20 amino acids. (**A**) After 15 hours in stationary phase in M9 supplemented with 0.4% glucose (hereafter M9), cells were diluted into fresh M9 and grown until the stationary phase. After 15 hours in the second stationary phase cells were diluted into fresh M9 and the OD_{600} of the second growth resumption of wild type (filled circles) and isogenic relaxed strain (empty circles) was followed. (**B**) The growth resumption delay of the Δ*relA* culture disappeared upon addition of the full set of 20 amino acids (each at 100 μg/ml) to the outgrowth M9 medium. The ratio of OD_{600} for Δ*relA* to OD_{600} of the wild type strain (red dotted line) serves as a numerical measure of the difference in growth resumption kinetics between the two. Results are shown as mean values of biological replicates (**A**, n = 2; **B**, n = 3) and error bars (too small to be seen for some of the points) indicate standard error of the mean.

Additionally, the relaxed strain showed a specific growth delay when leucine is omitted. Tyrosine omission does not result in lower stationary phase OD_{600} when we use exponential phase culture inoculum, but does with the use of stationary phase inoculum (compare Fig. 3A,B and Fig. 4A,B).

Addition of individual amino acids does not rescue the growth resumption delay of the Δ*relA* strain. Next, we set out to determine if the addition of any specific amino acid rescues the relative delay in growth resumption of the Δ*relA* strain by testing the effects of addition of individual amino acids at final concentration of 100 μg/ml. None of the amino acids reversed the defect; conversely, several amino acids exacerbated it for both strains (Fig. 3D–F). Addition of valine and cysteine strongly inhibited the regrowth of both wild type and relaxed strain; serine completely inhibited the regrowth of Δ*relA*, but not wild type (Fig. 3D,E). Growth inhibition

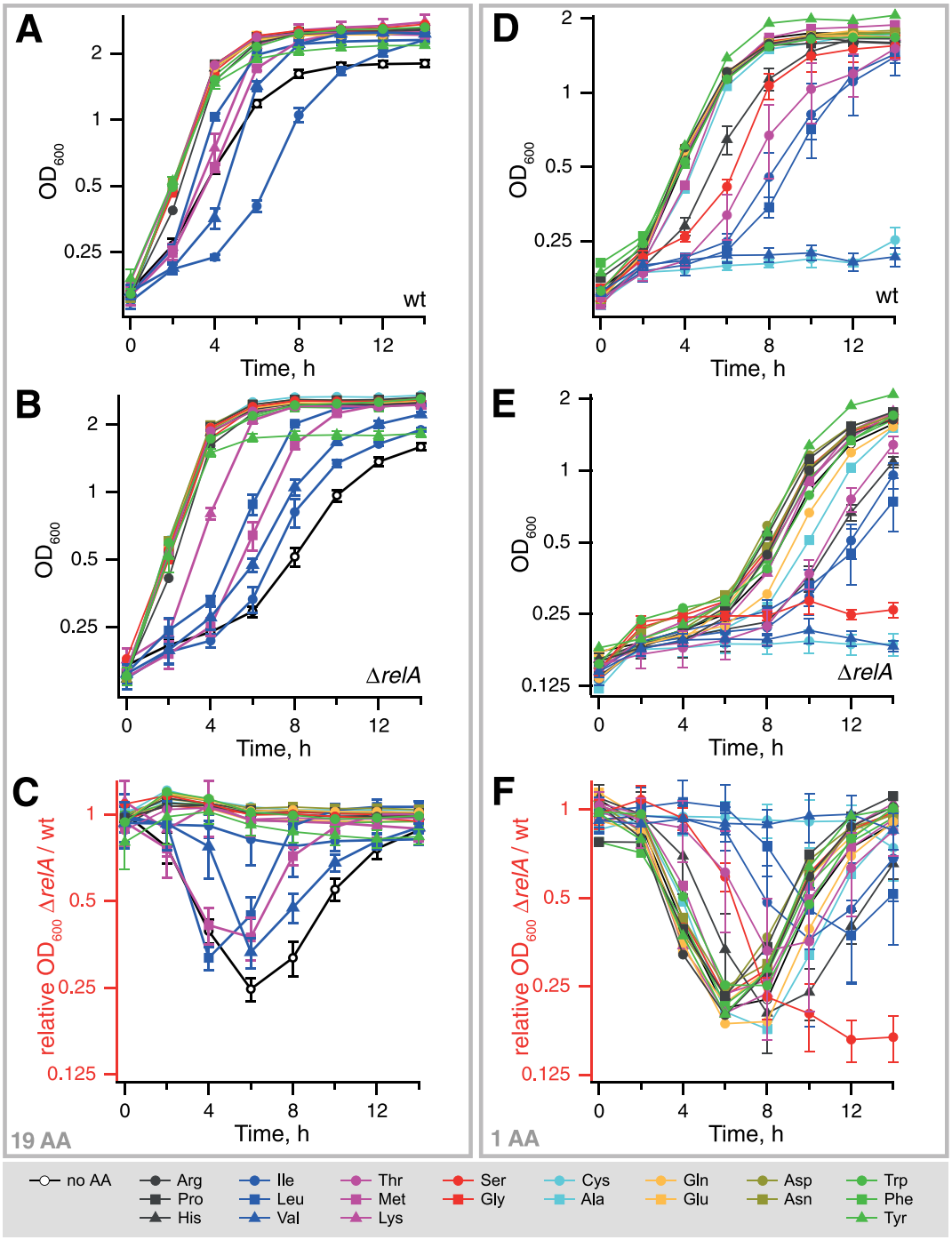

Figure 3. The effects of amino acid composition on transition of wild type and relaxed BW25113 *E. coli* strains from stationary phase to new exponential growth. After 15 hours in the stationary phase in M9 medium supplemented with 0.4% glucose, cells were gently pelleted, washed with M9 and diluted into fresh M9 medium supplemented with either 19 amino acids (**A–C**) or with one amino acid (**D–F**). Omitted (**A–C**) or added (**D–F**) amino acids are indicated by standard three-letter abbreviations with colours grouping amino acids to their biosynthesis pathways as per Keseler and colleagues[55] with an exception of grey symbols for Arg, Pro and His which are synthesized via unrelated *ad hoc* pathways. Empty symbols designate the M9 medium without the addition of any amino acids. Growth resumption was followed for *E. coli* wild type (**A,D**) and Δ*relA* (**B,E**) cultures. The ratio of OD_{600} for Δ*relA* to OD_{600} of the wild type strain (**C,F**) serves as a numerical measure of the difference in growth resumption kinetics between the two. The results are shown as mean values of biological replicates ($n \geq 3$). The error bars (too small to be seen for some of the points) indicate standard error of the mean and for the sake of clarity are omitted on traces lacking specific effects.

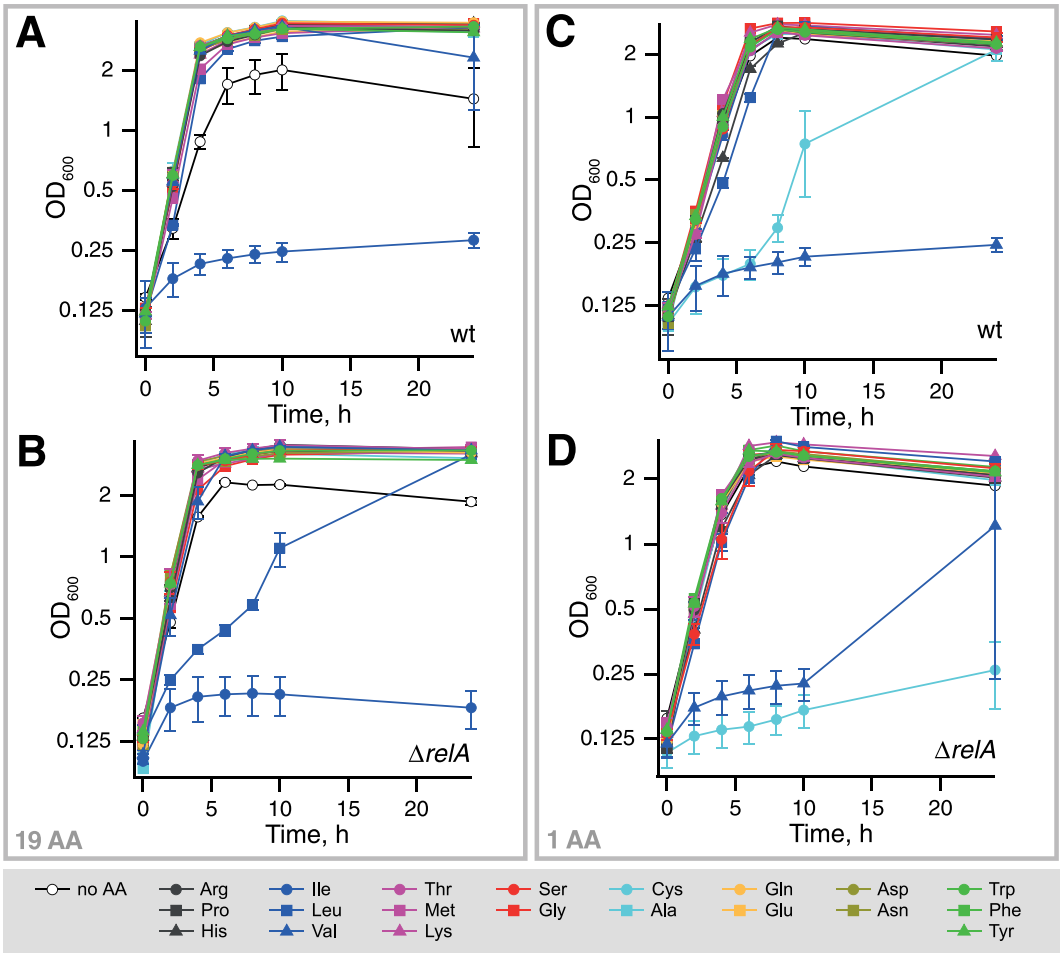

Figure 4. The effects of amino acid composition on exponential growth of wild type and relaxed BW25113 *E. coli* strains. Individual cultures in M9 medium were started from a single colony, grown up to OD_{600} of 0.8, diluted to OD_{600} of 0.1 and grown to 0.5. After that cells were gently pelleted, washed with M9 and resuspended in fresh M9 medium supplemented with either 19 amino acids (**A**,**B**) or with one amino acid (**C**,**D**). The results are shown as mean values of biological replicates (n = 2). The error bars (too small to be seen for some of the points) indicate standard error of the mean and for the sake of clarity are omitted on traces lacking specific effects.

by valine and cysteine is present when we use exponentially growing inoculum, suggesting that the effect is not specific for growth resumption but rather bacterial growth per se (Fig. 4). While the addition of histidine, serine, threonine, isoleucine and leucine caused a prolonged lag phase after stationary phase in both of the two strains (Fig. 3D,E), the severity of the effect was somewhat different, with serine causing a more pronounced growth resumption delay in the relaxed strain (Fig. 3E). The inhibitory effect of serine was absent in the case of exponentially growing cells (Fig. 4C,D).

Switching the carbon source of the outgrowth medium from glucose to glycerol abolishes the growth resumption delay of the relaxed strain. Rich LB and poor M9 minimal media dramatically differ in amino acid content: while in LB medium amino acids and peptides serve both as building blocks for protein as well as a source of carbon, ammonium and energy[27], M9 usually lacks amino acids altogether and the most commonly used carbon source is glucose, as was used in the experiments described above (Figs 1–4). As we have shown, the addition of 20 amino acids set to M9 supplemented with 0.4% glucose abolishes the delay in growth resumption of the Δ*relA* strain (Fig. 2). Importantly, addition of amino acids also decreases the doubling time of both the wild type and the relaxed strain almost twice (from 70 ± 3 to 44 ± 1 and from 82 ± 7 to 43 ± 0.3 minutes, respectively, Table 1). One could argue the relative growth delay of the relaxed strain in the absence of amino acids is merely a consequence of the necessity of *relA* functionality during slow growth *per se*, rather then a specific effect of the lack of amino acids.

To probe this conjecture, we have performed the regrowth experiments while reducing the growth rate in M9 lacking amino acids by substituting the glucose, a preferred carbon source for *E. coli*, for less optimal carbon source, glycerol. This further reduction of the growth rate can be counteracted by the addition of 20 amino acid set, which allows us to probe the connection amongst amino acid and carbon source composition, growth rate and growth resumption delay in the Δ*relA* strain. While the doubling time increases to 109 ± 5 (Δ*relA*) and

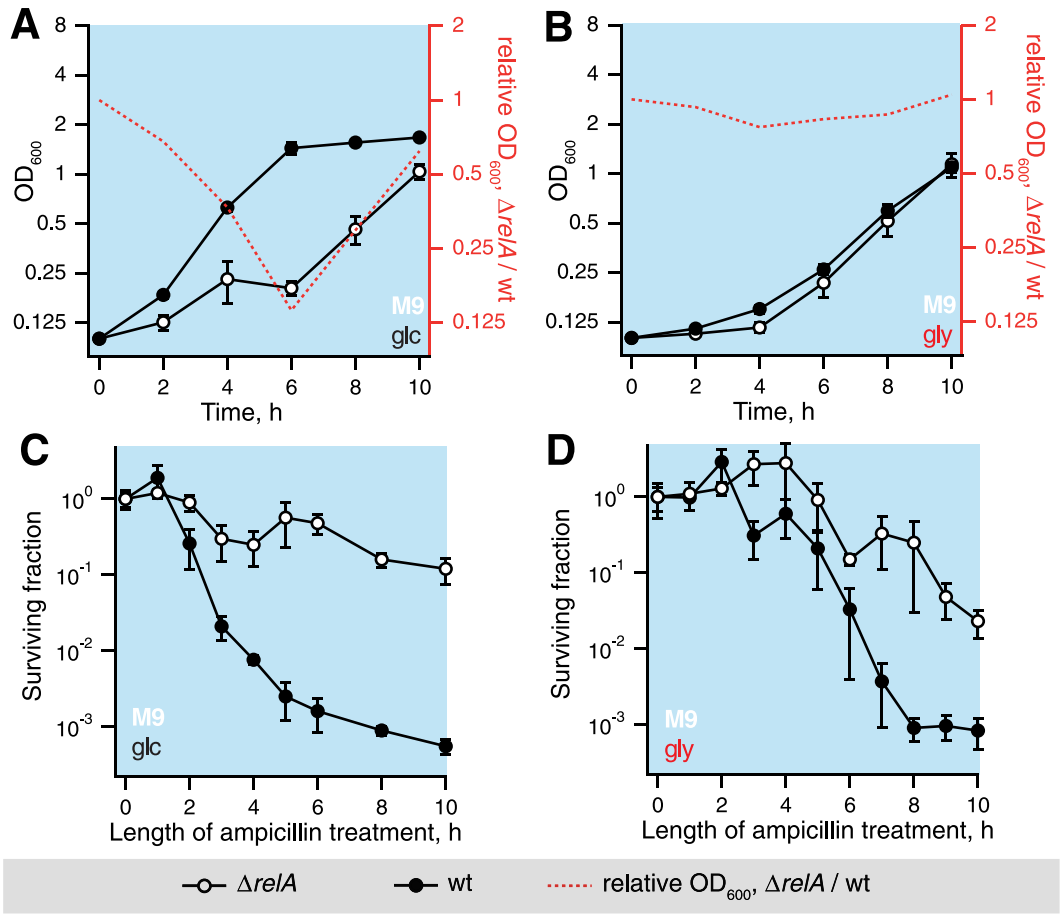

Figure 5. The effects of the carbon source composition of the outgrowth media on regrowth kinetics and ampicillin sensitivity upon transition of wild type and relaxed BW25113 *E. coli* strains from stationary phase to fresh M9 media. After 15 hours in the stationary phase in M9 medium, cells were gently pelleted, washed with M9 and diluted into fresh M9 medium supplemented with 0.4% glucose (**A**) or glycerol (**B**), and the ratio of OD_{600} for Δ*relA* to OD_{600} of wild type strain was plotted as a numerical measure of the differences in growth resumption between the two strains. To follow the ampicillin tolerance during *E. coli* regrowth in the presence of 0.4% glucose (**C**) or glycerol (**D**), the bacterial cultures were treated as described above but the regrowth medium was supplemented with ampicillin at 200 μg/ml and cell viability (colony forming units, CFU) was measured instead of OD_{600}. Results are shown as mean values of biological replicates ($n \geq 3$) and error bars indicate standard error of the mean.

123 ± 3 minutes (wt) in M9 supplemented with glycerol instead of glucose, the relaxed cells initiate the regrowth almost early as the wild type (Fig. 5A,B, Table 1). Addition of the 20 amino acids set to M9 medium supplemented with glycerol increases the growth rates to the levels similar to that in M9 supplemented with glucose. However, the growth resumption kinetics of the relaxed and wild type strains remain unchanged, i.e. Δ*relA* and the wild type regrow similarly (Supplementary Figure 3, Table 1). Taken together, these results demonstrate that the relative growth delay of the relaxed strain is modulated by both carbon source and amino acid composition of the outgrowth media.

Relaxed strain is killed by ampicillin considerably slower then the wild type during growth resumption in M9 supplemented with either glucose or glycerol. The bacterial growth rate is a key factor affecting antibiotic susceptibility. In the case of the antibiotic ampicillin the killing efficiency is believed to be directly proportional to the rate of growth[36]. Therefore, the effects of *relA*'s loss of functionality on growth resumption kinetics are expected to alter the antibiotic killing kinetics. To test this conjecture, we followed antibiotic killing by ampicillin after stationary phase cultures were diluted into M9 supplemented with either glucose (Fig. 5C) or glycerol (Fig. 5D). Surprisingly, the Δ*relA* strain was killed considerably slower then the isogenic wild type under both conditions. In the case of the wild type strain there is a correlation between the regrowth and ampicillin killing kinetics, i.e. the earlier bacteria start regrowth, the more efficiently they are killed by ampicillin. At the same time the relaxed strain is killed by ampicillin considerably less efficient then the wild type even in if the growth kinetics are very similar in M9 supplemented with glycerol (compare Fig. 5B,D). As a result, the effect of *relA* disruption on ampicillin tolerance is heavily dependent on medium composition: while in the presence of glucose after 5 hours of incubation with ampicillin – time point that is often used for end-point persister

measurements, e.g[37,38]. – the relaxed strain has approximately two orders of magnitude higher persister count, in M9 supplemented with glycerol persister frequencies for the two strains are nearly identical.

Discussion

***E. coli* growth, nutrient availability and RelA functionality.** We have systematically analyzed the effects of amino acid and carbon source availability, and RelA functionality in K-12 BW25113 *E. coli* strains during their transition from stationary phase to new exponential growth. The RelA-specific effects during this transition are confounded by two aspects that one has to consider. First are the defects in amino acid metabolism that are specific to K-12 *E. coli* strains, the workhorse of microbiology for almost a century[39]. Due to a frameshift mutation in one of the central isoenzymes of acetohydroxy acid synthase (AHAS)[34], addition of valine to minimal medium leads to cessation of growth that can be rescued by the addition of isoleucine, although the exact mechanism behind it is still matter of debate[8,9,33]. We clearly see the valine effect in our experiments (Figs 3 and 4). Second is the role of RelA and (p)ppGpp in amino acid biosynthesis. (p)ppGpp is crucial for amino acid synthesis as evidenced by both ppGpp0 (i.e. completely lacking the alarmone) *E. coli*[12] and *B. subtilis*[40] being auxotrophic for several amino acids including methionine and branched chain amino acids leucine, isoleucine and valine. The knock out strain used in the current work, while lacking RelA does have an intact copy of the second enzyme synthesizing (p)ppGpp in *E. coli* – SpoT[12]. While not directly causing auxotrophy, disruption of *relA* does lead to perturbed regulation of amino acid biosynthesis. Simultaneous addition of "one-carbon" amino acids (serine, glycine and methionine, SMG) suppresses bacterial growth, but while the wild type can overcome it, the relaxed can not[30]; and the effect is counteracted by addition of isoleucine[31,41]. The difference in the behaviors of wild type and relaxed strains is likely due the stringent response promoting biosynthesis of branched chain amino acids (BCAA), such as isoleucine[8,9]. We clearly see that omission of one of the BCAA results in RelA-specific retardation of growth resumption (Figs 3 and 4). Cysteine is known to cause transient amino acid starvation in the uropathogenic *E. coli* strain SP536[42]; the mechanism behind this phenomenon is not understood. We see manifestations of cysteine-induced starvation in our background: while inhibition of wild-type growth is transient, growth inhibition is near-complete in the course of 24 hours of observation of the relaxed strain (Fig. 4).

While the effects of amino acid composition on regrowth of the Δ*relA* strain were expected, the effects of substitution of the carbon source in M9 media from glucose to glycerol were surprising (Fig. 5). In the presence of glucose Δ*relA* strain regrows with a delay in comparison to the wild type, and in the presence of glycerol the two strains regrow equally well. The cause of this is not obvious, connections between (p)ppGpp and carbon metabolism are known; for example expression of the receptor protein of the global catabolic modulator cAMP (CRP) is under direct negative control of (p)ppGpp[43]. There are parallels between the effects on re-growth observed in this study and previous observations of the differential requirements for RelA in glycogen accumulation during amino acid starvation in the presence of different carbon sources[44,45]. The *relA* gene is needed when glucose is the carbon source, while the high cellular levels of cyclic AMP relieve the requirement for *relA* when glycerol is the carbon source[20,45]. Moreover, branched-chain amino acid biosynthesis is promoted by cAMP[46]. Since (p)ppGpp and amino acid metabolism are interconnected with carbon metabolism via many other pathways, such as tricarboxylic acid cycle[47] the connections among carbon source, RelA functionality and re-growth are far from simple.

Bacterial regrowth kinetics is intimately connected with bacterial sensitivity to bactericidal antibiotics: the frequency of persisters is reflecting the awakening kinetics[48]. Increased cellular (p)ppGpp level was suggested to be the ultimate driver of persister formation[49], and is implicated in antibiotic-specific tolerance mechanisms, i.e. protection from ampicillin acting via inhibition of cell wall biosynthesis[50]. Therefore, one could naively assume that the loss of RelA would result in, if anything, lower persister count, which is evidently not the case. Clearly, persistence is a multifaceted phenomenon, with media composition and growth conditions playing a major role via effects on metabolism[51] and growth rate[52].

Methods

Bacterial strains and plasmids. The *relA* deletion strain was constructed from strain BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) as described elsewhere[28] using primers relAF (CGATTTCGGCAGGTCTGGTCCCTAAAGGAGAGGACGGTGTAGGCTGGAGCTGCTTC) and relAR (CAATCTACATTGTAGATACGAGCAAATTTCGGCCTAATTCCGGGGATCCGTCGACC) for template PCR. Kanamycin resistance cassette was removed and Δ*relA* phenotype was confirmed on SMG plates[30] (Supplementary Figure 4).

Media and growth conditions. Cells were grown with vigorous agitation (200–220 rpm) at 37 °C in LB (Becton, Dickinson and Company) and M9 minimal medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 0.1 mM $CaCl_2$ and 2 mM $MgSO_4$)[25] or on LB agar plates (Becton, Dickinson and Company). M9 was supplemented with 0.4% (w/v) carbon source, which was glucose or glycerol. Amino acids were used at a concentration of 100 μg/ml, kanamycin at 25 μg/ml and ampicillin at 200 μg/ml. The data presented on Figs 3 and 4 were obtained using a 96-well plate reader Tecan Sunrise and the reset of the experiments were performed in flasks.

Growth recovery experiments. Bacterial cultures were started from single colonies on LB plate and grown until OD_{600} of 0.8. Resulting seeder culture was used to inoculate the experimental culture to starting OD_{600} of 0.1, which was grown aerobically into stationary phase (20 ml of medium in 125 ml flasks), kept in stationary phase for 15 h and directly diluted into fresh medium to OD_{600} of 0.1 or, during shift from LB to M9, harvested by centrifugation and, washed with M9 before transfer into fresh medium. Experiments with inclusion of 1 or 19 amino acids were conducted as follows: after 15 h in stationary phase, cells were harvested by centrifugation (in carbon source experiments washed with carbon source depleted M9),

resuspended to OD_{600} of 0.1 and grown aerobically in fresh medium on 96-well plates in a volume of 80 µl per well. OD_{600} readings of the 96-well plates (plate reader Tecan Sunrise) were converted to values for 1 cm path length (spectrophotometer Thermo Helios β) (Supplementary Figure 5). The length of the lag phase was determined by an intercept between the initial inoculum density ($OD_{600} = 0.1$) and the tangent of fastest exponential part of the growth curve that determines the doubling time. Lag and doubling times were calculated separately for individual growth curves ($n \geq 3$). Data analysis was performed in R[53] and the code is provided in the Supplementary Information.

Antibiotic killing. 15 h stationary phase cultures were prepared as described above for growth recovery experiments. The cells were then collected 10 min at 5000 g at room temperature, washed with M9 0.4% glucose or M9 0.4% glycerol, collected and resuspended again and diluted to OD_{600} of 0.1 in 20 ml medium in 125 ml flasks. The following ampicillin killing assays were performed essentially as described in[54]. A 10 µl aliquot was used for a CFU count at the zero hour time point, and then ampicillin was added to the remaining culture at 200 µg/ml. During following time course of ampicillin killing, flasks were incubated at 37 °C 200 rpm. Colony forming units were determined by series of tenfold dilutions out of which 5 µl was spotted on an LB plate. After overnight incubation of the plates at 37 °C, colonies were counted and CFU/ml was calculated.

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Author Contributions

V.V. and T.T. conceived the project. V.V., V.H. and T.T. designed the experiments. V.V. and S.O. performed experiments. V.H. and T.T. coordinated the study. V.V. and V.H. wrote the paper with contributions from T.T. and S.O.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Information sheet

Poomisvastuse roll *Escherichia coli* antibiootikumide taluvuses

Poomisvastus on bakterite seas laialt levinud kohanemismehhanism, mille puhul tõuseb rakus kahe guaniinnukleotiidi, ppGpp ja pppGpp ehk (p)ppGpp tase. Selle mehhanismi käivitavad muutused kasvukeskkonnas, nagu näiteks toitainete puudus ja kuumašokk; protsessi tulemusel seadistub rakufüsioloogia muutunud tingimustele vastupidavaks. *Escherichia coli*-s kontrollivad (p)ppGpp taset kaks ensüümi – RelA ja SpoT, mille järgi on nimetatud ka terve nende valguperekond: RelA ja SpoT-ga Homoloogsed (RSH) ensüümid. Poomisvastust seostatakse nii virulentsuse kui antibiootikumide resistentsusega. Lisaks on hiljuti pakutud välja, et poomisvastus võiks olla peamiseks käivitavaks jõuks nn persister-rakkude – antibiootikumi taluva (ehk tolerantse) fenotüübiga üksikrakud muidu antibiootikumi-tundlikkus rakupopulatsioonis – moodustumisel. Kuna nii antibiootikumide resistentsus kui tolerantsus kujutavad endast tõsist ohtu inimkonna tervishoiule, on äärmiselt oluline uurida (p)ppGpp ja antibiootikumi toime vahelisi seoseid.

Märksõnad: bakteriraku füsioloogia; poomisvastus; antibiootikumi taluvus; RelA ja SpoT-ga

Role of the stringent response in antibiotic tolerance of *Escherichia coli*

The stringent response is a near-universal bacterial adaptation system control mediated by accumulation of two guanine nucleotides ppGpp and pppGpp, collectively known as (p)ppGpp. The response monitors several environmental stress inputs, such as nutrient limitation and heat shock and remodels bacterial physiology in order to overcome the challenges. In *Echerichia coli* (p)ppGpp levels controlled by two enzymes – RelA and SpoT, the namesakes of RelA SpoT Homologue (RSH) protein family. The stringent response is associated to induction of virulence, antibiotic resistance and was recently suggested to be the driving force behind the formation of so-called persister cells – antibiotic-tolerant phenotypic variants in antibiotic-sensitive population. Since drug resistance and tolerance constitute a significant public health threat, understanding the connection amongst (p)ppGpp, antibiotic treatment and persistence is of great importance.

Key words: stringent response, antibiotic tolerance, bacteria, RelA SpoT Homologue enzymes

CERCS code: B230 Microbiology, bacteriology, virology, mycology

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