Beneficial mutations in TEM-1 beta-lactamase and bacterial stress responses: Teasing apart cause and consequence

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Antibiotic resistance in bacteria forms a serious threat to human health and occurs because of spontaneous beneficial mutations in antibiotic resistance genes. Within a single gene, a variety of different beneficial mutations could occur. Though we can keep track of the mutations, the reason a mutation is beneficial remains largely unknown, especially when the mutation is synonymous. In this thesis I study the relationship between the expression of the antibiotic resistance gene TEM-1 β-lactamase, the expression of the stress response regulator transcription factor rpoS and the antibiotic resistance against cefotaxime for a variety of different beneficial single-nucleotide mutations in the TEM-1 β-lactamase gene. I find that when present, rpoS appears to have a positive effect on the antibiotic resistance. Changes in rpoS expression caused by the beneficial mutations, however, do not cause any variation in antibiotic resistance. Furthermore, I find that for the synonymous mutations, the increase in rpoS expression is probably a pleiotropic effect of the increased TEM expression.

Introduction

Since 1929, when Alexander Fleming discovered penicillin, antibiotics have revolutionised the world of medicine and therapeutic treatment. Recently however, due to the intensive use of antibiotics, pathogens are becoming more resistant to antibiotics, hereby posing a serious threat to public health. The high adaptive potential of these pathogens, thus evolution itself, is the basis of this threat and resistance therefore seems to be inevitable. In addition, transfer of antibiotic resistance to other bacteria through mobile genetic elements like plasmids is helping the spread of antibiotic resistance. As such, understanding the genomics and evolutionary biology of antibiotic resistance is key to finding solutions to reverse or slow down the rate of evolution of antibiotic resistance [1].

Resistance to antibiotics is often associated with reduced fitness in the absence of the antibiotic. This assumption is not without reason as resistance mutations often target important biological functions in the cell [2]. Indeed, resistant mutants generally have a reduced fitness in absence of antibiotics in comparison with the parental strain [2]. Reducing the use of antibiotics could therefore result in selection for susceptible strains. This concept suggests there is a possibility to reverse the resistance of antibiotics. Experiments and theoretic models suggests this is possible [3]. In practice however, the available data suggests the reversal of antibiotic resistance will be very slow to non-existent [4]. Once resistance has emerged and if it has a fitness cost, it is more likely that there is compensatory evolution (mitigation of fitness costs) then reversion to susceptibility.

Even though antibiotic resistance in pathogens cannot be easily reversed, knowledge about antibiotic resistance and its evolution can give us insight into how to prevent further resistance development in pathogens. A lot of research therefore has been done to investigate the mechanisms behind antibiotic resistance [5] and on the evolution of antibiotic resistance genes like the mapping of mutational pathways [6] and investigating the role of epistasis and pleiotropy [7, 8] on the evolution of these genes.

Epistasis occurs when a phenotypic trait is dependent on multiple genes and when the effect of the two genes are non-additive. The phenotype can be fitter than expected (synergistic epistasis) or less fitt (antagonistic epistasis). Furthermore, Deleterious mutations can enhance beneficial mutations (sign epistasis) and two deleterious mutations can have a beneficial effect when combined and vice versa (reciprocal sign epistasis) [7]. Pleiotropy occurs when one gene or allele has an influence on different phenotypic traits. Analogous to sign epistasis, sign pleiotropy occurs when the different phenotypic effects of the mutation have an opposed effect on the fitness of the organism.

Antibiotic resistance of a pathogen is often hereditary, although non-hereditary resistance also occurs. Bacteria can obtain these genes in various ways, such as due to beneficial mutations in the chromosome that are subsequently selected for (i.e. evolution) [9]. Bacteria can also acquire antibiotic resistance genes by horizontal gene transfer [10]. The resistance of bacteria can also come from intrinsic resistance [11, 12] like having an extra outer membrane in gram-negative bacteria, through which some antibiotics simply cannot enter [13]. Another innate ability gram-negative bacteria have is the expression of efflux pumps that are situated in the membrane and can effectively reduce drug concentration in the cell by active transport of these drugs out of the cell [13]. In addition to intrinsic resistance, other mechanisms include altering the target of the antibiotic [12, 14] or modification of the antibiotic itself. For example, a class of antibiotic resistance enzymes called β-lactamases are able to target β-lactam antibiotics (such as penicillin and ampicillin) by hydrolyzing the β-lactam ring, thereby destroying the action of the antibiotic [15].

In fact, one of these β-lactamases is also the subject of our investigation, namely TEM-1 β-lactamase, from now on abbreviated as TEM-1. TEM-1 is a β-lactamase found in gram-negative bacteria [16] and has been used as a model to study antibiotic resistance for much evolutionary research [16, 17] like the roles of epistasis [7] and pleiotropy [8].

Back in 2002, Barlow et al created the vector pACSE3, which carries a p15A origin of replication (5-10 copies/cell), a gene for tetracyclin resistance, the $lacI^{Q}$ gene that encodes the Lac repressor, and a regulatory region that includes the Lac-repressor-regulated pTac promoter [18]. They used the pACSE3 to clone in TEM-1 creating the pACTEM1 vector. A follow-up study by Schenk

et al involving pACTEM-1 found 48 unique beneficial mutations (of which 10 are synonymous mutations) after random mutagenesis and screening for increases cefotaxime (CTX) resistance. Furthermore, they estimated that around 87 single-nucleotide mutations in the whole TEM-1 allele will have an increased CTX resistance [17]. From these 48 mutations most of the mutations had a small (positive) effect including these 10 synonymous mutations and a few mutations that had a large effect on the antibiotic resistance against CTX.

My thesis is a follow-up study on these 48 beneficial single-point mutations. In particular I am interested in the synonymous mutations and the mechanisms in which they increase the Ctx resistance.

Beneficial mutations that influence Ctx resistance are likely to either change the amount of the enzyme in the cell, in particular the periplasm, or they change the enzymatic activity, or both [19, 20]. TEM-1 confers low resistance to Ctx, even though is is a β-lactam antibiotic. The reason for this is that Ctx, like other modern cephalosporins, has bulky side groups which cause steric hindrance [21]. Mutations that change the catalytic site of TEM-1, like influencing the cavity space or causing a change in the interaction between TEM and the Ctx substrate, are bound to have an impact on the resistance, as has been suggested for G238S and R164S [21, 22, 23]. In fact, most of the beneficial mutations found were located in the vicinity to the catalytic site in the oxyanion cavity or on loops near the entrance of this cavity [17].

Synonymous mutations do not change the amino acid sequence. Instead, they are likely to increase the enzyme levels. To do this, some mutations might prolong mRNA half-life [24], promote the correct folding and export of the protein [25] or affect mRNA stability in order to promote translation [26]. Preliminary research on these mutations show that generally an increased TEM expression is obtained. This is a plausible explanation for the increase in antibiotic resistance, however, it might not represent the total increase in resistance. Other factors also need to be considered. As a matter of fact, further research found that having more TEM expression also correlates with an increase in expression of the RNA polymerase subunit S (rpoS) as well (figure 1).

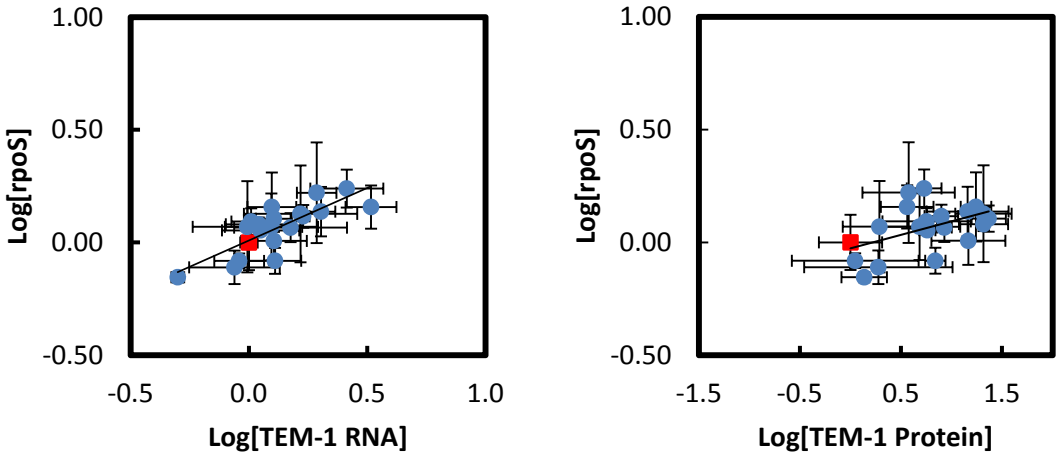

Figure 1: Dependence of rpoS RNA expression on the TEM-1 RNA expression (A) and TEM-1 protein expression (B). The expression values shown are from a panel of 20 (out of the 48) beneficial TEM alleles as found in Schenk et al, 2012 [17], ten synonymous mutations and 10 non-synonymous mutations. The values have been normalized to the expression values of the wt cell carrying the original pACTEM1 plasmid (the red dot). The error bars represent the SEM.

RpoS is a stress response regulator transcription factor that is upregulated by *E. coli* when bacteria are subjected to different stress conditions like osmotic shock, high temperature, UV radiation, or depletion of nutrients [27]. It is usually expressed during entry in the stationary phase and is mostly known for regulating genes induced by the stationary phase [28]. It is able to control

the expression of various stress response genes in categories like DNA repair [29, 30], removal of reactive oxygen species [31, 32], cell morphology and cell division [33, 34], cell membrane permeability [35], metabolism [36], virulence [37] and cell lysis [38]. Furthermore, it has been shown that rpoS has an influence on the resistance against antibiotics like Tetracyclin [39] and Penicillin [40].

In this thesis I will investigate whether the increase in rpoS expression, in addition to the increased TEM expression, has an effect on Ctx resistance or whether the increase in rpoS expression is just a pleiotropic effect of the increased TEM-1 expression. To visualize these hypotheses more clearly, figure 2 gives an overview.

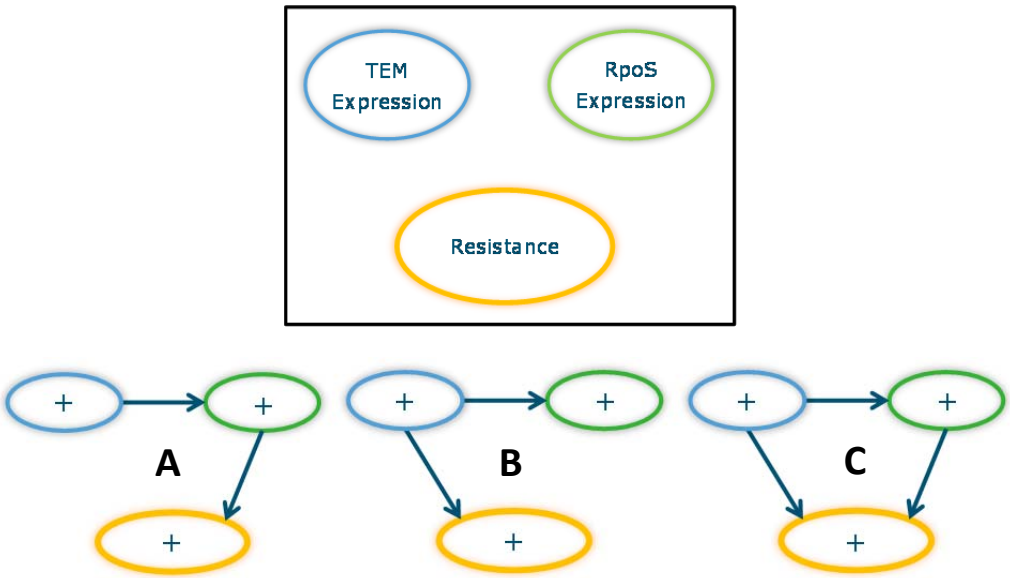

Figure 2: A Schematic overview of the possible relations between the TEM expression, rpoS expression and resistance. A) An increased TEM-1 expression causes the increased rpoS expression which contributes to a higher resistance. B) An increased TEM-1 expression causes the increased resistance and as a pleiotropic effect, increases rpoS expression. C) An increased TEM-1 expression causes the increased rpoS expression. Both contribute to a higher resistance.

To test which hypothesis has the most experimental support, an rpoS knockout (ΔrpoS) *E. coli* cell was created from the wild type strain (wt) using ET/Red recombination. After that, plasmids carrying a few selected TEM alleles were transformed into the ΔrpoS cells after which resistance assays and mRNA and protein expression assays were performed on both the ΔrpoS cells and the wt cells. To characterize the ΔrpoS cells, in addition to the other assays, growth experiments were performed to find the growth rate in exponential phase and survival in stationary phase. The mutations that were selected are 2 synonymous mutations, G87* and E89*; 2 other small effect mutations, A135T and F72V; and a large effect mutation G238S.

Materials and Methods

bacterial strains and plasmids

Escherichia coli strain DH5αe (Invitrogen, Carlsbad, CA, USA) was used as our wt control strain. RpoS was deleted using ET recombination [41, 42] to obtain our ΔrpoS strain (for method, see section rpoS knockout experiment). These strains were used as host cells for the pACTEM plasmids. These plasmids were previously obtained by random mutagenesis on the pACTEM1 plasmid (created by Barlow et al, [18]) followed by selection for increased resistance to ctx. After sequencing only the single point mutations were selected for further analysis [17].

All overnight cultures were grown in LB medium at 37°C, shaking at 250rpm and in the presence of 15 µg tetracycline/mL to ensure the plasmids preservation, unless otherwise stated. Expression. Expression of TEM alleles was induced by adding 50 µM isopropyl-β-D-thiogalactopyranoside (IPTG).

RpoS knockout experiment

the rpoS gene was knocked out of the DH5αe strain using Red/ET recombination [41, 42] and the primers ΔrpoS_ET_F (AGGCTTTTGCTTGAATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTAAATC-CTGATGTTACATTGCAC) and ΔrpoS_ET_R (ACAGAAAAGGCCAGCCTCGCTTGAGACTGGCCTTTCTGA-CAGATGCTTACCTCTGCCAGTGTTACAACCA) . The kanamycin resistance gene KanR2, obtained by amplification using the ΔrpoSF (AAATCTCTGATGTTACATTGCAC) and ΔrpoSR (CTCTGCCAGTGTTACAACCA) primers out of the pENTR – D Topo vector, was used as the substitution for rpoS. Transformation of the DH5α strain with pRedET expression plasmid, Red/ET expression, transformation of the cells with the KanR2 PCR product and Red/ET recombination was performed following the protocol 'Quick & Easy BAC Modification kit, by Red/ET Recombination' by Gene Bridges [43]. After recombination, the bacteria were selected for kanamycin resistance by growing on LB Agar containing 50 µg/mL kanamycin. 3 colonies were picked and grown in LB medium before stored as a glycerol stock at -80°C.

Verification of successful rpoS deletion was done using a colony PCR on the ΔrpoS cells using the primers rpoS_F (CGGGAACAACAAGAAGTTAAGG) and rpoS_R (GCGTCACATATTAATGACTCAG). There primers complement a ~20 bp region that flank the rpoS gene in DH5α. Both regions should not have not been deleted in the rpoS gene deletion experiment. A gel electrophoresis on the resulting PCR products was performed to check for correct fragment size. As a marker, the digestion of λ-phage with EcoRI, BamHI and HindI was used. As an extra check, the PCR product was purified using a NucleoSpin Gel & PCR Cleanup kit from Machery-Nagel and sent for sequencing by Eurofins Genomics Sequencing with primers rpoS_F and rpoS_R.

Transformation of ΔrpoS cells with TEM plasmids

Cells were made competent for transformation as follow, first a culture was grown overnight in YENB medium at 37°C. The next day, the cell culture is diluted 100x in 500 ml YENB medium and grown until the OD_{600} value (measured by a Smartspec 3000 spectrophotometer from BioRad) was just a little under 0.6. After cooling on ice for 5 min, the solution was centrifuged using a JA-14 rotor in a Beckmann centrifuge. All Centrifuge steps are done the same, i.e. 5 min at 3500 RPM and 4°C. After discarding the supernatant the pellet was resuspended in 100 ml cold sterile water and centrifuged again. This washing and centrifuging cycle is repeated again with 100 ml cold sterile water, then with 10 ml cold 10% glycerol solution and finally resuspended in 1 ml 20% glycerol solution. At this stage, the cells were competent for transformation. The cell solution (50 µl) was then mixed with 2 µl purified plasmid (~100 ng/µl) before electroporation (1350V, 10 µF, 600 Ohms) in an Eppendorf Electroprator 2510) and recovery at 37°C in SOC medium. After recovery, the bacteria were selected for tetracyclin resistance by growing on LB Agar containing 15 µg/mL tetracyclin. After growing overnight, 3 colonies were picked and grown in LB medium before stored as a glycerol stock at -80°C.

Verification of successful transformation was done by Sanger Sequencing purified plasmids at Eurofins Genomics Sequencing using P3 (TCATCCGGCTCGTATAATGTGTGGA) and P4 (ACTCTCTTCCGGGCGCTATCAT) primers. Plasmid isolation and purification was done using a Nucleospin Plasmid EasyPure kit (Machery-Nagel) on an overnight culture.

Growth kinetics

To estimate the growth speed of wt and ΔrpoS cells in exponential phase, 3 replicate overnight cultures were diluted a thousand times in 25 ml LB medium with no antibiotic or IPTG and grown at 37°C in 100 ml Erlenmeyer flasks while shaking. At regular intervals of 1 hour the absorption at wavelength of 600 nm (OD_{600}) was measured. In addition, at regular intervals of 2 hours, 50 µl of a $8*10^3$ cells/ml dilution (diluted according to the OD_{600} value) was plated out on LB agar plates containing no antibiotic or IPTG and grown at 37°C overnight. From the colony count we could estimate the amount of viable cells present in the cell culture at the different time points and calculate the cell concentration. By plotting the cell concentration as a function of time in the exponential growth phase, we found the exponential growth formula of the cells, in the form of $x_t = x_0 e^{rt}$ where t is time passed for the initial cell concentration (x_0) to reach the cell concentration at time t (x_t) and r is the growth rate. The doubling time (t_d) was estimated using the following formula: $t_d = \frac{t*\log(2)}{\log(x_t)-\log(x_0)}$.

To determine the number of viable cells in the stationary phase over time, the same experiment was performed where the grown cells were diluted a thousand times in the evening and grown overnight at 37°C. The cells were plated out at different time intervals over the next two days. The viable cell concentration in the cultures could be estimated using colony counts after growth on LB agar for ~20 hours at 37°C .

Resistance Assays

Resistance levels of the wt cells and ΔrpoS cells against cefotaxime (Sigma, St. Louis, MO, USA), Ampicillin (Amp) and Tetracycline (Tet) were estimated using a standard Minimum Inhibitory Concentration (MIC) assay. Three to six replicates of overnight cultures of the strains were diluted to ~10^4 cells/mL in LB media with two-fold increasing CTX, Amp and Tet concentrations ranging from 0.001875-2 µg/ml, 0.125-128 µg/ml and 0.06-64 µg/ml respectively. After ~48 hours the OD_{600} was measured in a VICTOR^3 multilabel platereader (PerkinElmer) to check for growth. The minimum inhibitory concentration (MIC) is estimated as the lowest concentration of antibiotic where no growth was found.

In addition to the MIC, Ctx resistance levels of the wt cells and ΔrpoS cells carrying pACTEM plasmids were estimated by determining survival on agar plates in the presence of Ctx ($IC_{99.99}$ assay). Two to ten replicate overnight cultures were diluted and regrown to log-phase (OD_{600} value between 0.3 and 0.5). After growth, the cultures were diluted to cells densities of $4*10^7$ cells/ml, $4*10^5$ cells/ml and $4*10^3$ cells/ml. 50 µl cell solution was then plated onto a series of LB agar plates with two-fold increases in Ctx concentrations ranging from 0.015625 - 4 µg/ml and incubated for ~40 hours. The amount of cells able to grow under these conditions was determined using colony counts. These were then compared to the colony counts of the same cells grown without Ctx. The resistance level ($IC_{99.99}$) was defined as the Ctx concentration at which one in ten thousand cells produces a visible colony. This value was calculated by fitting all data points to a general dose response model based on the following relationship: $S = ae^{-pD^k}$ where S is survival, D is the antibiotic concentration, p is the probability of death per unit concentration of Ctx, k is a constant for dose-dependent interactions between molecules and a is a constant for scaling the response (i.e., taking into account that the actual units of CFU plated vary per experiment).

Protein Expression

To measure the protein expression levels, a western blot using a monoclonal antibody was performed. 3 replicates of each sample (*E. coli* cells containing no plasmid, pACTEM1 and G87* in

both the wt and ΔrpoS strains) were used in this assay. The cells were grown overnight and lysed by short sonification. After centrifugation and collecting the supernatant the protein content was measured using the Bradford method (BioRad Industries). After separating using electrophoresis (SDS-PAGE at 60 mA (for 2 gels) for 2h) the gels are washed in MQ before transferring to a nitrocellulose membrane overnight at 30V. The membrane was then stained using Ponceau S and after imaging the membrane was destained again in mQ water. After rinsing in TBS solution (1x TBS; 0.3% Tween-20) the membrane was blocked overnight in blocking solution (1x TBS; 0.3% Tween-20; 3% BSA). The membrane was then incubated for 1 hour with the primary mouse monoclonal antibody to TEM β-lactamase (ab 12252, Abcam, Cambridge) (140.000x diluted in 1x TBS; 0.3%Tween-20; 1% BSA), washed in TBS solution and incubated for 1 hour with the secondary antibody GAR-HRP (3000x diluted in 1x TBS; 0.3%Tween-20; 1% BSA). The HRP was then developed with the Immune Star Western C kit (BioRad) and a chemiluminescent picture was taken using a molecular imager Gel Doc XR+ system using ImageLab (BioRad) after which the relative TEM signal was calculated.

RNA Expression

The mRNA expression levels were measured using RT-qPCR. The same cultures as for the protein expression experiment were used. The RNA was isolated from an overnight *E. coli* culture using the NucleoSpin RNA kit (mini) from Machery Nagel. The RNA content was measured using the nanodrop spectrophotometer (Thermo Scientific). To synthesize cDNA a reverse transcriptase reaction was performed using the iScript™ cDNA Synthesis Kit (BioRad). 15 µl of RNA template (1 µg total RNA in 15 µl uclease-free water) was mixed with 5 µl of RT mastermix (4 µl 5x RT Buffer and 1 µl iScript RT polymerase) for a total reaction volume of 20 µl. The program used was 5 min at 25^{o}C, 30 min at 42^{o}C followed by a final reverse transcriptase inactivation at 85^{o}C for 5 min. The reactions were performed on a T-Gradient thermocycler from Biometra.

For the Real-time qPCR all reactions were performed on the C1000 Thermal Cycler system (BioRad). The cDNA samples obtained using the reverse transcriptase experiment, was diluted 10 times after which 1 µl was added to 9 µl of the q-PCR mastermix (3.4 µl mQ water, 5 µl of 2x SYBR Green Master Mix (BioRad) and 0.3 µl of primers (10 µM)). The q-PCR program was first 3 min at 95^{o}C after which 40 cycles were done of 10 s at 95^{o}C and 30 s at 60^{o}C, where the fluorescence is measured at the end of each 60^{o}C step. A melting curve measurement was performed afterwards using the standard melting curve protocol of the BioRad Software (65^{o}C – 95^{o}C with increments of 0.5^{o}C between each measurement of fluorescence).

Results

Wt and ΔrpoS cells

Verification of successful rpoS deletion

To verify whether the rpoS deletion experiment using Red/ET recombination had succeeded, a colony PCR was performed on the ΔrpoS cells to amplify the region where the rpoS gene was deleted and the kanR2 gene was inserted. The resulting PCR product yielded an approximate 1150 bp fragment on the agarose gel (see figure 3) A 1379 bp fragment was expected that included the kanR2 gene and a small region flanking the gene. As an extra check, the PCR products were sent for sequencing.

The sequence results showed that replica #2 contained a single point mutation. The other replicas had no errors. The stock of replica #2 was discarded as a consequence. Further experiments were performed using the other 2 stocks.

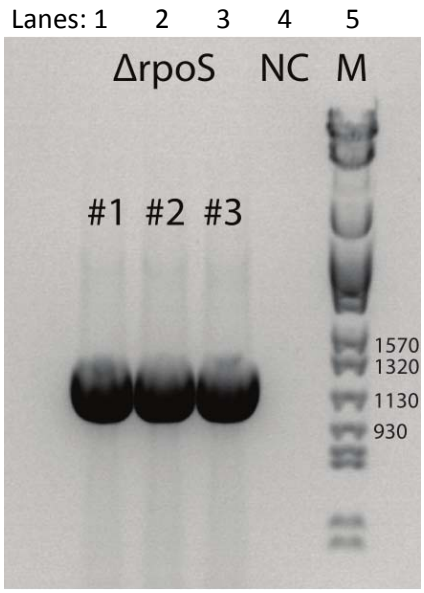

Figure 3: PCR amplification of the rpoS gene region for ΔrpoS cells. Lane 1-3: PCR products of the 3 ΔrpoS replicas amplified by rpoS_F and rpoS_R primers. Lane 4: Negative control. Lane 5: Marker containing Lambda-phage DNA cut by EcoRI, BamHI and HindI.

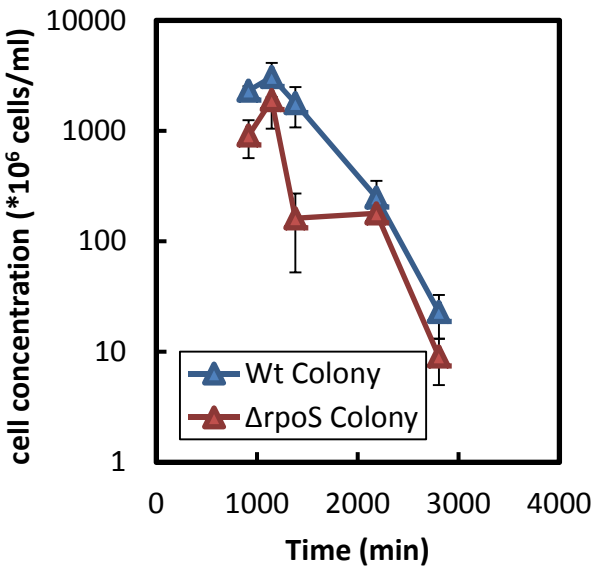

Figure 4: Growth curve in the stationary phase for the wt cells and the ΔrpoS cells. The cell concentration was determined by plating dilutions of the culture on LB agar plates and colony counts.

Verifying successful transformation with TEM-1 plasmids

Checking the successful transformation of the ΔrpoS cells with the TEM-1 plasmids was done by isolating the plasmids from the transformed cells and sending the plasmids for sequencing. The sequence results from Eurofins Genomics Sequencing showed no errors, verifying the transformation had succeeded.

Defining the growth kinetics of the wt and ΔrpoS cells

To define the growth kinetics of both strains, two growth experiments were performed. The first experiment was to find the growth rate and doubling time of our cells during exponential growth. The second experiment was to find the decline in viable cells over time in the stationary phase.

The cell concentration as a function of time in the exponential growth phase (Supplementary figure S1) gave us the exponential growth formula with a growth rate of 0.0103 (± 0.0013) min^{-1} for

the wt cells and a growth rate of 0.0114 (± 0.0015) min^{-1} for the ΔrpoS cells. The mean doubling times (± SD) are 68.23 (± 8.75) minutes and 60.99 (± 0.82) minutes respectively. The ΔrpoS cells seem to grow faster but an independent T-test showed that the differences between growth rates is not significant (t = -1.494, d.f. = 2.06, P = 0.27).

The viable cell concentration as a function of time in the stationary phase is plotted in figure 4. The results demonstrate that 1100 minutes after the start of growth, thus in stationary phase, a large portion of the ΔrpoS cells die off quickly, after which survival seems to stabilize. This is a big difference compared to the wt cells, where the mortality rate is more steady. In later stages of the stationary phase, there seem to be little difference in mortality of the ΔrpoS cells compared to the wt cells.

Resistance Assays

The introduction of antibiotics to the cell also causes stress and as such having rpoS or not is likely to have an influence on the resistance of these cells against these antibiotics. To study the influence of rpoS on the resistance of *E. coli* cells towards Ctx, Amp and Tet, a MIC assay was performed for both wt cells and ΔrpoS against these antibiotics (figure 5). A factor 2 difference in the MIC value was found between the ΔrpoS cells and the wt cells, the ΔrpoS cells being less resistant to the antibiotics Ctx, Amp and Tet.

In addition to the MIC assay, the resistance level of a bacteria could be measured using an $IC_{99.99}$ assay as well. An $IC_{99.99}$ assay has a much higher accuracy than the MIC assay, though it is more labor-intensive. An $IC_{99.99}$ assay was performed for both the wt and the ΔrpoS cells to check for Ctx resistance in addition to the MIC. The obtained median values (± SD) are 0.031 (± 0.002) and 0.056 (± 0.005) for ΔrpoS cells and wt cells respectively. An independent T-test showed the wt cells having a significantly higher resistance to Ctx than the ΔrpoS cells (t = -5.091, d.f. = 10.194, P < 0.001). A bar chart comparing the median value of MIC to the Median $IC_{99.99}$ value is found in figure 5A. Similar results were found for the MIC and $IC_{99.99}$.

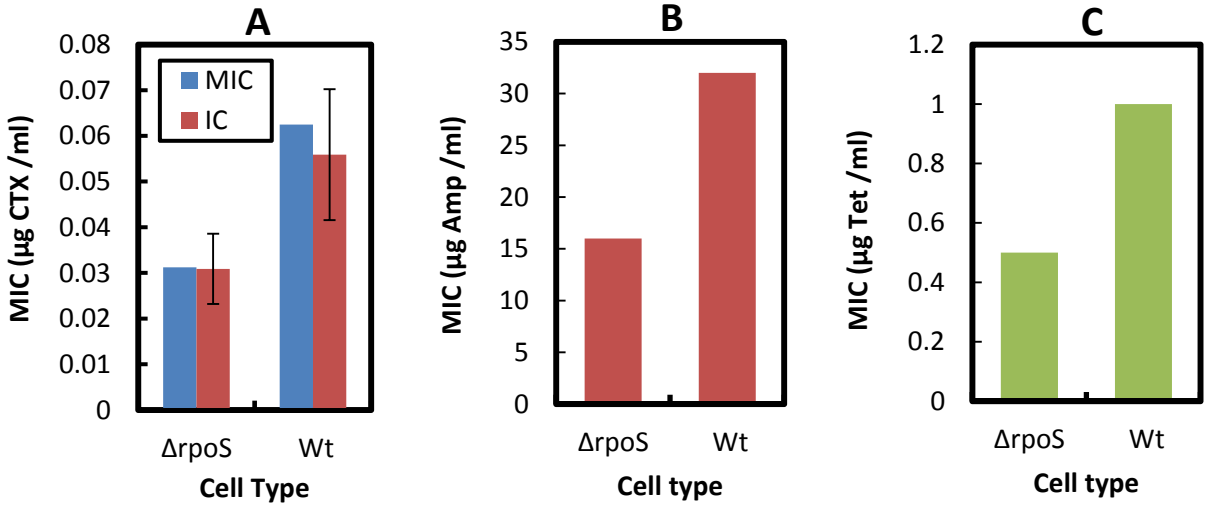

Figure 5: Bar Charts displaying the median of the resistance values of the ΔrpoS and wt cells. A) The MIC value and the $IC_{99.99}$ value for cefotaxime, B) The MIC value for ampicillin and C) The MIC value for Tetracyclin.

TEM alleles

Resistance assays

As previously mentioned, it was found that next to the increased resistance to Ctx for the single-nucleotide mutations, the TEM expression and rpoS expression both showed a general increase as well, the latter two being positively correlated (figure 1). To figure out whether the increase of rpoS expression has an effect on the improvement in Ctx resistance for the different TEM alleles, in particular the synonymous mutations, an $IC_{99.99}$ assay was performed on *E. coli* cells

containing different beneficial TEM alleles in both the wt and the ΔrpoS strain. The IC values can be found in table 1. The mutations that were not studied during this thesis are not shown. In addition to the actual IC resistance levels, the improvement in Ctx resistance relative to TEM-1 for each mutant was also calculated. The values found by Schenk et al. 2012, which were done in the wt background, can be found as well for comparison. The ranking is based on highest resistance to lowest resistance as found in Schenk et al. 2012 for all 48 beneficial mutations.

Independent T-tests on the improvement results were performed between the wt and ΔrpoS strains for the different TEM mutants, with the exception of A135T as only a single valid IC measurement was obtained. These results show that there were no significant differences for any of the mutants (for all tests d.f. = 2, G238S: t = -2.444, P = 0.134, G87: t = 1.164, P = 0.364; E89: t = 0.356, P =0.756; F72V: t = -2.210, P = 0.158).

Table 1: Ctx Resistance levels of *E. coli* cells containing different beneficial TEM alleles in both the wt and the ΔrpoS background. The mutations are ranked based on the Ctx resistance found in Schenk et al, 2012 for all 48 beneficial mutations. The improvement is calculated by dividing the $IC_{99.99}$ value of the mutants by the $IC_{99.99}$ value of pACTEM1.

			Schenk et al. 2012		Wild Type		ΔrpoS	
Rank	Mutation	Replacement	Resistance level IC99.99 (µg Ctx/mL) ± S.D.	Improvement	Resistance level IC99.99 (µg Ctx/mL) ± S.D.	Improvement	Resistance level IC99.99 (µg Ctx/mL) ± S.D.	Improvement
1	*g706a*	G238S	1.41 ± 0.15	27	3.59 ± 0.79	53	2.61 ± 0.086	74
18	*g255a*	G87*	0.121 ± 0.016	2.3	0.138 ± 0.010	2.1	0.064 ± 0.008	1.8
19	*g397a*	A135T	0.119 ± 0.005	2.3	0.082**	1.2	0.117 ± 0.044	3.3
23	*g261a*	E89*	0.096 ± 0.016	1.9	0.130 ± 0.040	1.9	0.063 ± 0.003	1.8
43	*t208g*	F72V	0.069 ± 0.003	1.3	0.105 ± 0.004	1.6	0.120 ± 0.041	3.4
-	none	pACTEM1	0.052 ± 0.002	1.0	0.067 ± 0.007	1.0	0.035 ± 0.001	1.0
-	no plasmid	-	-	-	0.056 ± 0.005	0.8	0.031 ± 0.002	0.9

*These mutations are synonymous mutations

**There is unfortunately only one measurement of the $IC_{99.99}$ value, therefore the Standard Deviation could not be established.

RNA Expression & Protein expression

As preliminary results found a correlation between the TEM expression and the rpoS expression for the different mutants (figure 1), it is interesting to find out the influence of having rpoS on the TEM-1 expression. All expression experiments were performed on the cells containing no plasmid, the original pACTEM1 plasmid and the G87 mutant in both the wt strain and the ΔrpoS strain. The mRNA expression of both rpoS and TEM-1 was measured using RT-qPCR. The TEM-1 protein expression was measured using a western blot.

We found no rpoS expression in the ΔrpoS strains, while the relative expression of rpoS in the wt cells is close to 1 for the different TEM alleles (figure 6A). A Brown-Forsythe robust test for the equality of means was performed to find whether the small differences in rpoS expression for the wt cells are significant. The results show it was not (d.f.= 2, 3.051, P = 0.68).

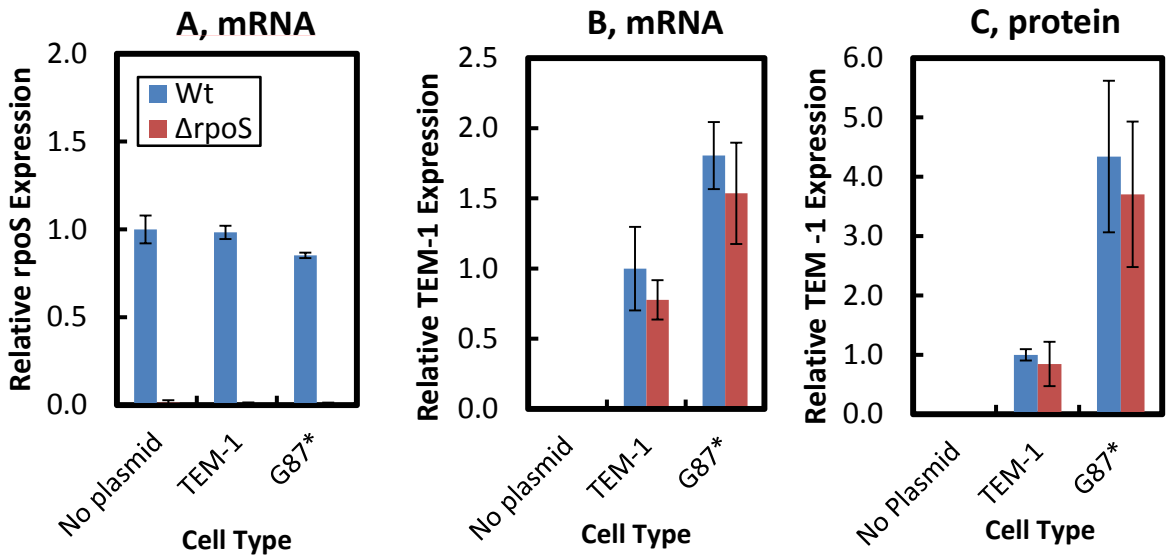

Figure 6: Bar charts showing the relative expression levels of rpoS mRNA (A), TEM-1 mRNA (B) and TEM-1 protein (C) for the different TEM alleles in both the Wt background (blue) and the ΔrpoS background (red). The rpoS expression was normalized to the expression level of the wt strain containing no plasmid. The TEM-1 expression was normalized to the expression levels of the wt strain carrying the original TEM-1 gene.

The relative mRNA TEM-1 expression levels did show differences among the mutants (figure 6B). The cells that do not contain a plasmid had no TEM-1 expression, the relative TEM-1 expression of the cells carrying the original pACTEM1 plasmid was close to 1, whereas the G87* mutants had a relative expression around 1.5. A two-way ANOVA test was done to check whether the differences between strains and between the different TEM alleles were relevant. Between strains this was not significant ($P = 0.156$) though it was significant between TEM alleles ($P = 0.001$). Furthermore, there was no significant interaction between the strains and the TEM-alleles ($P = 0.885$). An overview of the ANOVA results can be found in Supplementary table T1. In other words, no significant difference was obtained between the wt cells and the ΔrpoS cells, while the differences between the TEM alleles, in this case between the original TEM-1 and G87*, was significant. The interaction between the two showed no significance, thus the combination of strain and TEM allele did not have an effect. having rpoS present or not did not have an effect on the TEM-1 expression.

The relative protein expression levels of TEM-1 (figure 6C) showed an even clearer difference for the different TEM alleles. Again, no expression was found in the cells not containing a pACTEM1 plasmid. The relative expression of the G87* mutant gave a relative expression of around 4.0. The two-way ANOVA test showed similar results as for the mRNA expression levels (between TEM alleles: $P < 0.001$; between strains: $P = 0.302$; Interation: $P = 0.861$) (See Supplementary table T2). These results again reflect that there is a clear difference in TEM-1 expression between the TEM-alleles, but there is no difference between the wt strain and the ΔrpoS strain. Again, no interaction between strains and TEM alleles is found.

Discussion

Before we can start to unravel the possible relationship behind the TEM-1 expression, rpoS expression and Ctx resistance for the for the different TEM alleles in *E. coli* cells, we first need to characterize the wt and ΔrpoS strains individually. In particular, the ΔrpoS strains are interesting as there is no master regulator of the general stress response which makes is hard to predict how these cells will behave. Without the sigma factor rpoS, it is likely that the cell itself is less resistant to all kinds of stresses, like presence of antibiotics, DNA damage or extreme temperatures. Stresses to the cell are bound to happen eventually, if only by a lack of nutrients at the end of the exponential growth phase. As such, it is to be expected that having no rpoS present will have an influence on survival of the cell. At the exponential phase however, the cells are expected to grow at normal rates, unless rpoS affects growth rates or causes some cells to shut down growth spontaneously. This probably happens, but the effect is too small to be noticeable and will not cause differences in growth rates between the two strains.

No substantial differences are found in the growth rate in the exponential phase for the two strains (Supplementary figure S1). Both have a growth rate of ~0.11 which resulted in doubling times of a little over 1 hour. If anything, the growth speed of the ΔrpoS cells is a little higher, though the differences were not significant (see results, defining the growth kinetics section). This is as expected as in the exponential phase there is no need for a general stress response. Both strains are therefore expected to behave similarly. The cells however, grow very slow compared to normal growing *E. coli* cells which have a doubling time of ~20 minutes. This big of a difference in growth is unexpected and shows that the cells were perhaps grown in not ideal circumstances. A possible explanation is that the cells were grown in a slightly lower temperature than 37^{o}C, though the temperature in the incubation ovens are controlled and tested pretty regularly . Another, possibly more plausible, option would be that the pH in the medium used is not optimal for quick growth. In fact, making the LB medium, both agar and liquid, is done almost routinely and the pH of the medium was never checked or adjusted for that matter. Luckily, since both strains have been grown in the same buffer under the same conditions at the same time, the growth kinetics can still be compared.

The difference between the ΔrpoS cells and the wt cells is clearer in the second experiment where we looked at the viability of the cells over time in the stationary phase (figure 4). A large fraction (90-95%) of the ΔrpoS cells die off very quickly after reaching the stationary phase while the other fraction of the cells die a much later stage. The mortality of the wt cells seems to be more gradual. As rpoS plays a big role in stationary phase, especially in terms of survival, a quicker decline in survival of the ΔrpoS cells was expected and agrees with literature [44]. Why a small ΔrpoS subpopulation is able to survive much longer remains to be seen and requires more experiments. A possible explanation would be that these cells are persister cells. All bacterial cells produce a subpopulation of dormant cells within their own population. These dormant (non-dividing) cells are called persister cells and are highly resistant to physiological stresses compared to regular, fast-growing cells. Once the physiological stress is gone the surviving persister cells restore the population [45]. As these cells are highly resistant to stresses, it is to be expected that these cells are able to survive much longer during stationary phase. However, persister cells usually comprise a smaller percentage of the cell population compared to the cells that were able to survive in our survival assay (up to 1% in stationary phase). In addition, formation of these persister cells is linked to activation of the stringent response which in turn induces rpoS and the general stress response [46]. RpoS therefore is believed to have a role in the formation of persister cells. It is however not present in the ΔrpoS cells and the absence of rpoS might therefore disrupt the formation of persisters altogether.

A more elaborate experiment is necessary to more accurately determine the growth kinetics. For instance, there is a lot of time between data points and the actual growth and survival curves can therefore not be entirely represented.

Another step in the characterization of the strains is measuring their inherent resistance. We are mostly interested in the resistance to Ctx, but resistance to other antibiotics like Amp and Tet is valuable as well. Previous research has already found that rpoS has an effect on Tet [38] and

penicillin [39]. Our results confirms the effect of rpoS on Tet, and in addition show that rpoS has an effect on Ctx and Amp resistances as well (Figure 5). The wt cells are approximately twice as resistant compared to the ΔrpoS strain. For Ctx resistance assays this is confirmed by both the MIC value and the IC value.

Compared to wt cells, rpoS knockout cells are more sensitive to stresses like antibiotics (Figure 5) and nutrient starvation (Figure 4), though when these stresses are not present, during exponential phase, the cells do not have substantial growth rate differences (Supplementary figure S1). The rpoS mRNA expression results (Figure 6A) show that the ΔrpoS cells do not express rpoS confirming the creation of a successful rpoS knockout strain and giving support that the missing rpoS protein is the cause for the differences in growth and resistance between the strains. With the characterization done, we can now properly evaluate the effect of the different TEM-alleles on ctx resistance and TEM and rpoS expression for both strains.

The improvement values for each TEM allele can be found in table 1. To more clearly visualize the differences in improvement of the Tem alleles excluding G238S, a bar chart was made (figure 7). From this chart it is clear that the relative improvements between the strains for the synonymous mutations E89* and G87* are comparable. The improvements of F72V and A135T seem to be higher in the ΔrpoS strain though the standard deviation in the ΔrpoS strains are very large and the T-tests show that the differences are not significant. Furthermore, all the values displayed here were done using only two replicas. More replicas are necessary to properly determine the resistance values. The expectation is that with more replicas the differences in improvement for F72V and A135T will become less substantial.

In both cases, whether the improvement in Ctx resistance for the ΔrpoS strain is higher or equal compared to the wt strain, it shows that an increase in rpoS expression is not the mechanism that causes the increase in Ctx resistance. An increase in rpoS expression therefore does not seem to play a role in the increased Ctx resistance for the different TEM alleles.

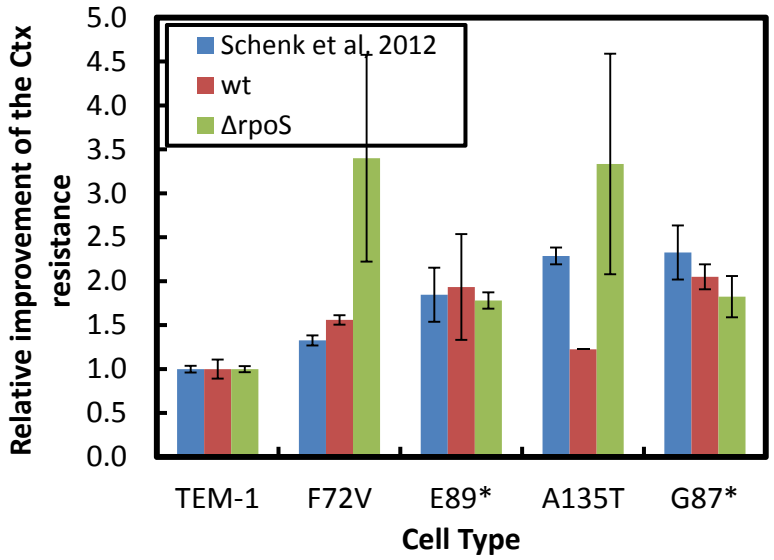

Figure 7: Relative improvement in Ctx resistance, measured by $IC_{99.99}$ for different TEM alleles compared to the original TEM-1 gene as measured by Schenk et al, 2012 (blue), measured in the wt background (red) and measured in the ΔrpoS background. The error bars show the standard deviation.

Although it seems that differences in rpoS expression is not the cause for the increase in Ctx resistance for the mutants, it could still have an effect on TEM expression. Our hypothesis is that rpoS expression is indeed affected by the amount of TEM expression. To confirm this, mRNA expression experiments for TEM and rpoS on both strains were performed. In addition, TEM protein expression was measured. Unfortunately the expression experiments could only be performed on the strains with no plasmid, containing the original pACTEM1 plasmid and containing the G87* TEM allele. As the main focus of this thesis was to find the relationship between TEM expression, rpoS expression and resistance for the synonymous mutations, G87* was chosen to be measured. Other TEM alleles might be just as interesting and relevant, however due to time restrictions, it was not possible to measure these.

As mentioned earlier, the rpoS mRNA expression levels show that the ΔrpoS cells do not express rpoS (Figure 6A). More interesting is the rpoS mRNA expression of the wt cells. These expression levels are not significantly different between the cell types, though it looks like that G87* has a slightly smaller rpoS mRNA expression. In the TEM expression experiments (Figure 6B and C) G87* clearly has a higher expression than the other cell types, for both mRNA and protein expression. This does not agree with the positive correlation of rpoS mRNA expression and TEM mRNA or TEM protein expression as shown in figure 1 and would suggest that there is no positive correlation. However, the error bars for both the values found in figure 1 and the expression values found in this thesis are large. Also the expression values in this thesis are obtained using only three replicas. More replicas might have given a higher expression value. Furthermore, as can be seen in figure 1, there are actually TEM alleles that have a decreased rpoS mRNA expression while having an increased TEM mRNA and protein expression in comparison with TEM-1. As such, G87* might not be representative for all TEM alleles, which is also not what we should expect. Increasing the scope of this experiment, thus performing both mRNA and protein expression experiments with more TEM alleles and more replicas is necessary. In addition I would suggest performing these expression experiments on cells that are not harvested during exponential phase as is the case for these results, but are harvested in early stationary phase. During exponential phase, rpoS mRNA expression for the various cells types is expected to be (equally) low, but once rpoS is actively expressed in early stationary phase, differences between rpoS mRNA expression will be higher. The correlation between rpoS expression and TEM expression might be more clear. It will be interesting to see the results.

Even though rpoS mRNA expression for G87* is not higher, as expected, TEM mRNA and protein expression could still be affected by the change in rpoS expression. However, two-way ANOVAs for both TEM mRNA expression as TEM protein expression show otherwise, see results. It shows that rpoS mRNA, present in the cell or not, does not have an influence on TEM expression. If rpoS would have an effect, one would expect the differences in TEM expression, both protein and mRNA, between the different strains to be larger. Futhermore, the two-way ANOVA would have shown a significant result of the interaction, but in this case the interaction is not significant by a large amount ($P = 0.885$ for TEM mRNA and TEM protein expression with rpoS mRNA expression and $P = 0.861$ for TEM protein expression with rpoS mRNA expression, see supplementary table T1 and T2).

To recap, rpoS itself has a large effect on the resistance of cells against multiple antibiotics like Ctx, Amp and Tet. However, differences in expression of rpoS are not the cause for the increased resistance against Ctx for the different beneficial mutations in TEM studied here. Furthermore, rpoS does not seem to have an appreciable effect on TEM expression. These results are in line with the hypothesis that the increased rpoS expression is a pleiotropic effect of the increased TEM-expression, but has no effect on the resistance as proposed in figure 2B.

That being said, most of the experiments done were performed with only 5 of the 48 beneficial mutations found, the expression experiments only with G87*, and might therefore not be completely representative for the other mutations. In addition, most experiments were performed with only 2 or 3 replicas. More replicas are necessary to properly evaluate the results. I therefor suggest to redo these experiments with more if not all beneficial mutations and to increase the amount of replicas.

Relationship TEM-expression with fitness

In addition to my thesis, Lion van de Pol, worked on his own thesis involving the beneficial point-mutations. He performed an experiment in which he let cells containing different TEM-alleles compete with each other in a single "bulk competition", and measured different selection rate constants (src) (a measure for fitness) for the different TEM alleles. Combining his data with the TEM and rpoS expression data found previous to my thesis, a negative correlation was found (Figure 8). That is, a higher TEM expression was correlated with a lower src. Especially synonymous mutation L139* (not analysed in my thesis) is a very interesting mutant as the fitness of this bacteria is much higher than the rest, see the datapoint in the upper left corner in figure 8. Why the fitness of this mutant is so much higher than the rest is not yet known. However, in addition to being very fit, this mutant has a very low TEM mRNA expression level. In light of the results of my thesis, where the increase in TEM-expression seems to induce the rpoS expression as a pleiotropic effect, one possible hypothesis is that the induced rpoS response actually represents the cause of the fitness cost of the cell. Further study is necessary to figure out this complex relationship between TEM expression, rpoS expression, resistance and growth and fitness.

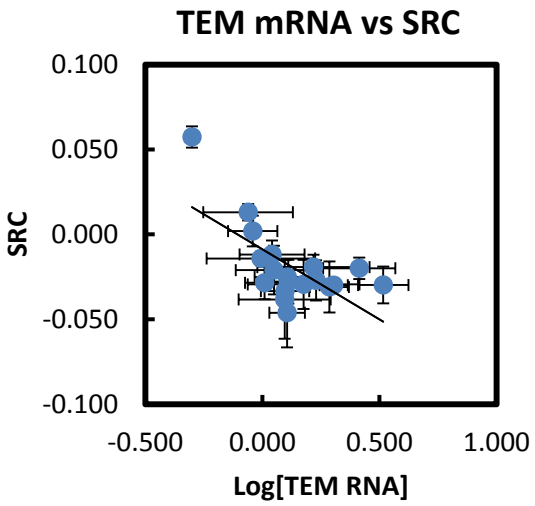

Figure 8: Dependence of the selection rate constants (src) on TEM mRNA expression. The expression values shown are from a panel of 20 (out of the 48) beneficial TEM alleles as found in Schenk et al, 2012 [17], ten synonymous mutations and 10 non-synonymous mutations. The values have been normalized to the expression values of the wt cell carrying the original pACTEM1 plasmid. The error bars represent the SEM.

Conclusion

In this thesis I have managed to successfully create an rpoS knockout cell from the wt *E. coli* strain DH5α. rpoS expression experiments confirm that rpoS is not expressed in the rpoS knockout cells. Characterization of the two strains (wt and ΔrpoS) found no substantial difference in terms of growth in the exponential phase. However, once stationary phase was reached, the ΔrpoS cells behaved different than the wt cells in that a large fraction of the ΔrpoS cells die off quickly in early stages of stationary phase compared to the wt cells. Further characterization in terms of resistance towards the antibiotics Cefotaxime, Ampicillin and Tetracyclin showed that the wt cells were twice as resistant towards these antibiotics compared to the ΔrpoS cells, implying that rpoS is indeed involved in the resistance against these antibiotics.

After characterization, both strains, after transformation with different alleles of the pACTEM1 plasmids, were used to find the relationship between rpoS expression, TEM-1 expression and resistance toward Ctx. It was previously found that an increased TEM-1 expression correlates with an increased rpoS expression. This work found support that the difference in rpoS expression is not the cause for the increase in Ctx resistance for the different mutations. In addition, differences in rpoS expression have no effect on the increased TEM expression. It seems that increased rpoS expression is a pleiotropic effect of the increased TEM expression. However, more experiments including more mutations and more replicas are necessary to properly support this hypothesis.

The relationship between TEM mRNA expression, TEM protein expression, rpoS expression, growth/fitness and resistance is a complex one. Based on the results obtained so far, the resistance of *E. coli* bacteria is mainly effected by the TEM expression levels. In addition, the TEM expression levels somehow inhibit growth and induce rpoS expression. Further study is necessary to figure out whether the growth is inhibited due to the increased rpoS expression.

I recommend a continuation of my experiments in both the wt and the ΔrpoS background to increase the amount of replicas. In addition I recommend to expand the scope of this thesis by increasing the amount of mutations tested. Fitness measurements of the mutations in both the wt and the ΔrpoS background should help in evaluating the relationship between fitness, TEM expression and rpoS expression. Furthermore, it can give the explanation why certain mutations like L139*, which have a very low TEM mRNA expression, have a high fitness level

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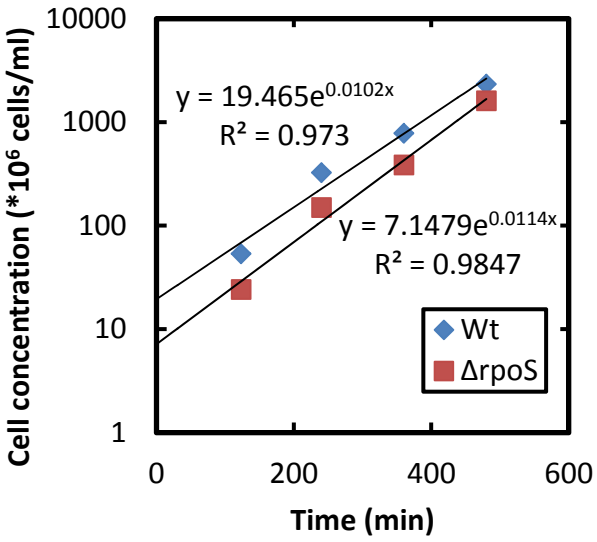
Supplementary Information



Supplementary Figure S1: Growth curve for the wt cells and ΔrpoS cells in the exponential phase including the calibration curve for both strains. The calibration curve gives the exponential growth formula.

Supplementary table T1: Two-way ANOVA of the mRNA expression levels of the TEM mutants in both the wt strain and ΔrpoS strains as shown in figure 6B.

Source	Sum of Squares	Df	Mean Square	F	P
Corrected model	2.019*	3	0.673	9.115	0.006
Intercept	19.656	1	19.656	266.178	< 0.001
Between strains	0.182	1	0.182	2.459	0.156
Between TEM-alleles	1.836	1	1.836	24.865	0.001
Strain*TEM-allele	0.002	1	0.002	0.022	0.885
Error	0.591	8	0.074		
Total	22.266	12			
Corrected total	2.610	11			

*R Squared = 0.774 (Adjusted R Squared = 0.689)

Supplementary table T2: Two-way ANOVA of the protein expression levels of the TEM mutants in both the wt strain and ΔrpoS strains as shown in figure 6C.

Source	Sum of Squares	Df	Mean Square	F	P
Corrected model	1.256*	3	0.419	23.089	< 0.001
Intercept	0.871	1	0.871	48.026	< 0.001
Between strains	0.022	1	0.022	1.216	0.302
Between TEM-alleles	1.234	1	1.234	68.017	< 0.001
Strain*TEM-allele	0.001	1	0.001	0.033	0.861
Error	0.145	8	0.018		
Total	2.272	12			
Corrected total	1.401	11			

* R Squared = 0.895 (Adjusted R Squared = 0.858)