The effect of social interactions on the establishment of an antibiotic resistant Escherichia coli mutant

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1. Abstract

Currently, the increase in antibiotic resistance in bacteria is becoming a major global concern. Before an antibiotic resistant mutant is fixated in a population, it needs to go through an establishment phase. Understanding the process of establishment entails the survival of the initial rare mutant until the mutant reaches a frequency where it is no longer vulnerable to extinction due to random mortality. The chance of successful establishment is expected to be low, even with a selective advantage in the environment. The knowledge of the factors influencing the mutant establishment remains limited. Understanding how these factors impact the establishment is key to understanding the spread of antibiotic resistance.

TEM-1 is a commonly encountered β-lactamase found in gram negative bacteria. It confers resistance to some β-lactam antibiotics such as penicillin. A previous study by Arno Hagenbeek showed that the presence of *Escherichia coli* with TEM-1 significantly increased the establishment of a more resistant mutant under exposure of the antibiotic cefotaxime.

In this study, three potential mechanisms were investigated: (i) removal of antibiotic, by either binding to cellular components or (ii) by TEM and (iii) the addition of nutrients through cell death. The three mechanisms were investigated using a bioassay and an interaction experiment. In the "B-lactamase interaction experiment", a low number of mutants (150 cells) were plated with a 10^6 of wildtype, single mutant, heat-killed wildtype or heat-killed single mutant cells at two cefotaxime concentrations, above the minimal inhibitory concentration (MIC) of the wildtype or single mutant. It was found that one potential mechanism, removal of the antibiotic by the single mutant enzyme, significantly increased the chance of mutant establishment compared to the wildtype treatment. The increase in the mutant establishment has implications to the expected interactions between a wildtype or low resistant strain and more resistant mutant strain. The insight gained from this study could be used to improve the understanding of determining factors influencing the establishment of a more antibiotic resistant mutant.

2. Introduction

Nowadays, the emergence of antibiotic resistance is a major challenge in modern medicine (Santos-Lopez et al. 2019). The rapid spread of resistance makes antibiotics less effective, for example in the USA annually around 99.000 deaths occur due to antibiotic-resistant pathogens-associated hospital-acquired infections (Aslam et al. 2018). The number of deaths attributed to antibiotic resistant pathogens is projected to rise to millions by 2050 (de Kraker, Stewardson, and Harbarth 2016).To combat the rise of antibiotic resistant infections, two strategies have been proposed. One of the strategies is based on the procurement of new antibiotics as alternatives to the existing ineffective antibiotics. This strategy has been criticised because, in the long term, resistance against the new antibiotics will arise while the antibiotic discovery rate is decreasing (Baym, Stone, and Kishony 2016; Aslam et al. 2018). Therefore, this strategy is seen as untenable and unrealistic in the long run. The second strategy is to slow or halt the emergence of antibiotic resistance in the first place, so that the existing antibiotics and new antibiotics remain effective longer. This would ensure that resistant pathogens are less likely to occur and lower the deaths associated with antibiotic-resistant pathogens. However for this strategy to be developed, it is necessary to better understand the evolution of drug resistance (Furusawa, Horinouchi, and Maeda 2018).

The first step of the antibiotic resistance emergence is for a susceptible strain to acquire a mutation or gene that will confer resistance to the antibiotic in the environment. The acquirement of these genes can occur through horizontal gene transfer or mutations. Horizontal gene transfer is the transmission of a gene from one bacterium to another through various processes, like transduction and conjugation. Mutations can appear in a few individuals. Therefore, it is possible that the mutants go extinct due to random mortality. Once an allele is established in the population, the influence of genetic drift on the allele is lower. This effect occurs approximately at 1 divided by the selection coefficient of a beneficial allele (1/s) (Kimura 1968; Patwa and Wahl 2008). After the establishment of the mutant, the selection for a beneficial allele is theoretically expected to proceed deterministically (Patwa and Wahl 2008) until it is present in the entire population, at which point the allele is fixated. The selective benefit of alleles that provide antibiotic resistance is high in a clinical environment (Alonso, Sanchez, and Martinez 2001; Patwa and Wahl 2008; Munita and Arias 2016). Therefore, the fixation could be almost guaranteed when the antibiotic resistance is established (Alonso, Sanchez, and Martinez 2001).

Only a few empirical studies exist that focus on establishment under influence of antibiotics. For example, in populations of rifampicin-resistant *Pseudomonas aeruginosa*, the majority of *de novo* lineages susceptible to antibiotics were lost to genetic drift (Gifford and MacLean 2013). This decreased the likelihood of the population becoming susceptible again, showing that the reversibility of antibiotic resistance is very low due to the process of establishment (Gifford and MacLean 2013). Another study found that the establishment of a single streptomycin resistant *Pseudomonas aeruginosa* cell was less than 5% in concentrations of $1/8^{th}$ below the lethal dose (Alexander and MacLean 2019). The dose had the effect of extending the lag-time and an increase in the mortality rate of the resistant strain, reducing the likelihood of outgrowth from a single cell to a whole population of cells. This showed that stochastic population dynamics dominated the emergence of a resistant strain and frequent loss of resistant strains under sub-lethal doses of antibiotics. These studies show that the establishment probability can be very low.

A common resistance mechanism is the enzymatic breakdown of antibiotics. Beta-lactamase enzymes can breakdown beta-lactam antibiotics by cleaving their beta-lactam ring, thereby conferring resistance against these compounds (Palzkill and Botstein 1992; Søndergaard and Nørskov-Lauritsen 2016). TEM-1 is one of the most widespread enzymes to confer beta-lactam antibiotic resistance (Salverda, de Visser, and Barlow 2010). TEM is active in between the outer membrane and inner cytoplasmic membrane of the cell. It has been found that β-lactamases can leak into the environment inactivating the antibiotic (Rojo-Molinero, Macià, and Oliver 2019). Furthermore, in biofilm, resistant mutants have been found to protect the susceptible cells from antibiotic treatment. This effect has not been seen in planktonic growth, where the growth of resistant mutants with susceptible cells did not show any protective effect of the mutant (Rojo-Molinero, Macià, and Oliver 2019). This cooperative behaviour of the resistant mutants has been explained by the detoxification of environment by the β-lactamase. The efficiency of TEM-1 to degrade some of antibiotics can be lower or higher depending on the structure of the antibiotic. For example, the efficiency of TEM-1 to degrade ampicillin is higher than to degrade cefotaxime (Raquet et al. 1994). The TEM-1 gene can adapt to several antibiotics, like cefotaxime, through mutation and selection. Therefore, it is a commonly used beta-lactamase for research into the evolution of antibiotic resistance (Salverda, de Visser, and Barlow 2010). Cefotaxime is an antibiotic that disrupts the peptidoglycan synthesis by binding on penicillin binding protein 3 (PBP3) (LeFrock, Prince, and Left 1982). This protein catalyses the peptidoglycan formation, which is crucial for the cell wall formation (Kohner et al. 2009;

Kjeldsen, Sommer, and Olsen 2015). The exposure to cefotaxime leads to lysis of bacterial cells, causing the contents of the cells to leak into the environment (Pitout, Sanders, and Sanders 1997; Kjeldsen, Sommer, and Olsen 2015).

Previous work by a former Master student of the Laboratory of Genetics (Wageningen University), Arno Hagenbeek, was focussed on the factors affecting establishment of antibiotic resistant bacteria. One of the factors researched was the effect of the presence of *Escherichia coli* cells, expressing TEM-1, on multiple mutants under cefotaxime exposure. The "Wildtype interaction assay" was designed to simulate an environment where a low number of mutants (100 cells) arise from a wildtype population at various levels of cefotaxime, all of which are above the minimal inhibitory concentration (MIC) of the wildtype. The chosen YFP labelled TEM mutants were a single mutant (G238S), a double mutant (E104K/G238S) and a triple mutant (E104K/M182T/G238S) and were inoculated with two different densities of BFP labelled wildtype cells (TEM-1). This experiment showed that the addition of 10^6 wildtype cells increased colony number, i.e. establishment, of all three tested mutants. Multiple possible mechanisms could have caused this increase. One was the increase of nutrients through cell death. The surrounding surviving cells of the mutant could scavenge the cell contents of the susceptible cells, which can enhance the establishment of the mutant (Nioh and Furusaka 1968). The influx of nutrients from the lysis could increase mutant establishment rate (Hibbing et al. 2010). Another cause could have been the removal of antibiotic by either β-lactamase or covalent binding by cellular components. This covalent binding could then lower the cefotaxime concentration, which would again enhance the establishment of the mutant.

3. Objectives

The main aim of the current study was to research the possible mechanisms behind the previous observation of a wildtype population increasing in establishment of a TEM mutant in *Escherichia coli*. The first objective was to confirm the results gained by Arno Hagenbeek and if whether the choice of fluorochromes affected the results. To this end, the "Wildtype interaction experiment" was conducted. In this experiment a high resistant mutant was introduced at low numbers into a population of wildtype cells. To control for a possible fluorochrome effect, the two fluorochromes of the strains were switched.

After the first objective was researched, the possible mechanisms that were investigated in this study were (i) the addition of nutrients through cell death and (ii) the removal of antibiotic through either binding of the antibiotic to cellular components or through active β-lactamase

breakdown. The “B-lactamase interaction experiment” was similar to “Wildtype interaction experiment”, but the treatments used were either heat-killed wildtype, heat-killed low resistant mutant cells, alive wildtype or alive low resistant mutant. The heat-killed cells were used to investigate the role of nutrient addition through cell death, heat killed cells are used in the “B-lactamase interaction experiment”. The use of a low resistant mutant would allow for the mechanism of beta-lactamase to be investigated. The single mutant has a higher efficiency than the wildtype β-lactamase. This efficiency might lead to higher establishment for the mutant compared to the treatment with wildtype. An increase of establishment of the triple mutant with the single mutant compared to the treatment with wildtype could indicate the use of a more efficient β-lactamase influences the mutant establishment.

As an additional test, an assay was developed to see how long the single mutant, or the wildtype could survive the 1.76 µg/ml cefotaxime concentration. This time was important for the “Cefotaxime bioassay”, because in the bioassay alive and active TEM expression is necessary. During the antibiotic incubation step, the wildtype and single mutant are expected to produce TEM. The time available for TEM expression is therefore important.

To investigate the removal of antibiotic by binding to cellular components, like PBP3, an ancestor of the wildtype strain was used in the “Cefotaxime bioassay”. The ancestor strain does not have β-lactamase. Therefore, the ancestor might only lower the antibiotic concentration by binding the antibiotic to cellular components. The “Cefotaxime bioassay” was an assay in which either wildtype, a low resistant mutant or the ancestor strain was incubated in medium with 1.76 µg/ml cefotaxime. This concentration is above minimal inhibitory concentration (MIC) of the single mutant, wildtype and ancestor. After which the medium was used for a MIC assay to check if the antibiotic concentration had decreased. As a control to check the antibiotic concentration, medium with cefotaxime was incubated without cells.

4. Materials and Methods

4.1 Bacterial strains and culturing

The experiments in this study were performed using *Escherichia coli* strains derived from the MG1655 strain, which was modified with yellow (SYFP2) or blue (mTagBFP2) fluorescent markers integrated into the chromosomal DNA (Gullberg et al. 2014). The BFP MG1655 strain is used in this study as the ancestor strain to the TEM-1 wildtype strain. This ancestor strain does not have TEM and therefore no beta-lactamase activity. Additionally, these strains were modified with 3 different TEM beta-lactamase alleles with either TEM-1 wildtype, and TEM with one- or three-point mutations (MG1655 - G238S, from here on referred to as single mutant; and MG1655 - E104K/M182T/G238S, from here on referred to as triple mutant). The increasing number of point mutations were shown to increase the cefotaxime resistance of the strains (Palzkill 2018; Wals, Doucet, and Pelletier 2009; Salverda, de Visser, and Barlow 2010). The TEM alleles were both present in a YFP version and BFP version. The variant used will be stated per experiment. All strains have TEM under a *lacz* promoter and expression of the allele was induced using 50µM IPTG (isopropyl β-D-1-thiogalactopyranoside) in all experiments using cefotaxime.

Before each experiment, the strains were retrieved from -80 ºC glycerol stocks. These glycerol stocks were then used to inoculate 2 ml of liquid M9-cas minimal medium (Supplement 9.1) in a 12 ml tube. The tubes were incubated overnight at 37 ºC, while shaking at 250 RPM. These overnight cultures where then diluted to the needed density specified in the assays (see below), using phosphate buffered saline (PBS). The cell density of the used strains was first determined by colony count on LB-agar plates in triplicates. This was done as a pilot experiment before any experiment was conducted.

4.2 Wildtype interaction experiment

The aim of this experiment was to confirm the results gained by Arno Hagenbeek's "Wildtype interaction assay" and to exclude a possible effect of the fluorochromes. To this end, the experimental setup used by Arno was repeated. In this experiment a low amount of 100 mutant cells was mixed with a population of wildtype. Both tested cefotaxime concentrations were above the MIC of the wildtype thus, this strain would not survive the treatment. Only the triple mutant was used as the mutant in this experiment. In the "Wildtype interaction assay" by Arno Hagenbeek, the YFP marker was used for the mutant and the BFP marker for the wildtype.

Therefore, in the current experiment both marker combinations for the wildtype and mutant were used vice versa in two separate setups.

The overnight culture of the wildtype (approximately $2.5x10^9$ cells/ml) was serially diluted to either $2x10^4$ or $2x10^7$ cells/ml. The triple mutant (approximately $2.2x10^9$ cells/ml) was diluted to $2x10^3$ cells/ml in multiple steps. The mutant dilution was then mixed 1:1 with either PBS, $2x10^4$ cells/ml or $2x10^7$ cells/ml wildtype. This results in a dilution with 1000 mutant cells/ml and 0, 10^4 or 10^7 wildtype cells/ml. 100 µl of these dilutions was then spread on M9-$cas_{50\mu M\ IPTG}$ plates, containing either 1.76 or 1.44 µg/ml cefotaxime, using glass 3 mm beads. This then resulted in a final dilution of approximately 150 cells of the mutant and either 0, 10^3 or 10^6 wildtype cells to be tested. After spreading the dilutions, the plates were incubated at 37 ºC until colonies were clearly visible (16-20 h) and were then counted. To ensure that the counted colonies were mutant colonies, a fluorescence microscope (LEICA M165 FC) was used to detect the YFP or BFP signal. GFP and CFP filters were used to distinguish between YFP and BFP colonies, respectively. If colonies were found to have the wildtype fluorochrome, the number of wildtype colonies were subtracted from the mutant colony count. Each treatment was replicated 15 times. To control for the correct dilution of the wildtype and triple mutant, the dilutions of cells before the mixing were diluted to $1x10^3$ cells/ml and then plated on LB-agar with 50 cells, 100 cells or 200 cells on LB- agar in triplicate. These plates were then incubated at 37 C° and counted the next day.

4.3 B-lactamase interaction experiment

Lastly an experiment was made to distinguish between the possible mechanisms of increasing mutant colony count when the mutant is grown with wildtype cells, as seen in "Wildtype interaction experiment". To test this, an experiment was performed like the wildtype interaction experiment. The experiment was conducted by mixing a low number of triple mutants with 10^6 cells of heat-killed or alive TEM-1 wildtype, and 10^6 cells of heat-killed or alive single mutant. The use of YFP wildtype or single mutant allowed to see their colonies clearly, because the YFP marker has a higher fluorescence expression. This higher fluorescence expression could allow for the easy detection of single mutant or wildtype colonies. The 10^6 cells were chosen because it produced the largest effect in the "Wildtype interaction experiment". The use of heat-killed cells was to indicate if nutrients through cell death could increase the triple mutant establishment. The alive wildtype and alive single mutant were to see if the removal of antibiotic, through either binding or β-lactamase, could cause an increase of triple mutant

establishment. In this experiment, the addition of single mutant treatment was to see if this would have an additional impact due to the more efficient β-lactamase.

An overnight culture of the YFP wildtype (approximately $2.5x10^9$ cells/ml) and an overnight culture of YFP single mutant (approximately 2.0 $x10^9$ cells/ml) were each diluted to $2x10^7$ cells/ml. The BFP triple mutant (approximately 2.0 $x10^9$ cells/ml) was diluted to $3x10^3$ cells/ml in multiple steps. The mutant dilution was then mixed 1:1 with either PBS, $2x10^7$ cells/ml heat-killed wildtype, $2x10^7$ cells/ml alive wildtype, $2x10^7$ cells/ml heat-killed single mutant or $2x10^7$ alive cells/ml single mutant. The heat-killed wildtype and heat-killed single mutant were prepared using the heat-kill protocol as described above. This resulted in a dilution with 1500 mutant cells/ml and 0 or 10^7 cells/ml of either the wildtype or single mutant cells (killed or alive). 100 µl of these dilutions containing 150 mutant cells and 0 or 10^6 cells of the wildtype or single mutant, were then spread on $M9_{50\mu M\ IPTG}$ plates with glass 3 mm beads. The plates had either 1.44 or 1.76 µg/ml cefotaxime. After spreading the dilutions, the plates were incubated at 37 ºC until colonies were clearly visible (16-20 h). To control for the added number of mutant cells, 150 triple mutant cells were incubated on $M9_{50\mu M\ IPTG}$ plates without cefotaxime, with the same dilution as used for the other plates. The colonies were then counted; to ensure that the counted colonies were mutant colonies, a fluorescence microscope was used as described above. If colonies were found to have the wildtype fluorochrome, the number of wildtype or single mutant colonies were subtracted from the count. In addition to testing the amount triple mutant by colony count, the single mutant and wildtype cells were also plated on LB-agar for counting. Each treatment was replicated 15 times. To control for the correct dilution of the wildtype, single mutant and triple mutant, the dilutions of cells before the mixing were diluted to $1x10^3$ cells/ml and then plated on LB-agar with 50 cells, 100 cells or 200 cells on LB- agar in triplicate. These plates were then incubated at 37 C° and counted the next day.

4.4 Cefotaxime lethality assay

During the "Cefotaxime bioassay" it was necessary to have alive cells actively expressing TEM. Therefore, the aim of the cefotaxime lethality assay was to test how long the wildtype, or the single mutant could survive in the highest cefotaxime concentration (i.e. 1.76 µg/ml). In the Cefotaxime lethality assay, the wildtype and the single mutant were incubated, in the same density as in de "B-lactamase interaction assay" of cells ($4x10^4$ cells/ml), in triplicates with 1.76 µg/ml cefotaxime in 4 ml M9-$cas_{50\mu M\ IPTG}$ medium. The tubes were incubated for 7 hours at 37°C and 250 rpm. Every 30 minutes (starting with T=0), 100 µl were taken from each tube and spread on an LB-plate to check for growth. The plates were incubated for 16 hours at 37

ºC. After incubation the colonies were counted and the time where all cells died was noted for the next assay.

4.5 Cefotaxime bioassay

The cefotaxime bioassay was performed to distinguish between the removal of antibiotic by active TEM and binding of the antibiotic to cellular components. The following strains were used: MG1655 YFP-TEM (wildtype), MG1655 YFP (ancestor strain, i.e. without β-lactamase activity) and MG1655 YFP-G238S (single mutant). The treatments used were incubation treatments with alive wildtype, heat-killed wildtype, alive ancestor, heat-killed ancestor alive single mutant, heat-killed single mutant, and no cells. Both single mutant and wildtype were used to inoculate the wells after the treatment medium was added. The heat-killed or alive wildtype wells were inoculated with wildtype and the heat-killed or alive single mutant wells were inoculated with single mutant. The ancestor, either heat-killed or alive, and no cells were both used in the either plates inoculated with wildtype or plates inoculated with single mutant. The antibiotic concentration in the medium without cells is not expected to change so therefore it is a control of the antibiotic concentration.

From each of the overnight cultures, half of the liquid was used for the heat-killing the cells and the other halve was used as alive cells. After heat killing of half of the liquid, the cells of both the alive and heat-killed were diluted and incubated in $M9_{50\mu M\ IPTG}$ medium with 1.76 µg/ml cefotaxime in triplicates. This was done by incubating halve of the overnight culture at 80 ºC for 3 hours then put on ice for 20 minutes. The heat-killed bacteria were then diluted and incubated in $M9_{50\mu M\ IPTG}$ medium with 1.76 µg/ml cefotaxime in triplicates. Furthermore, a blank of just $M9_{50\mu M\ IPTG}$ medium with 1.76 µg/ml cefotaxime was used in triplicate. All treatments were incubated for 3.5 hours at 37 ºC and shaking at 250 RPM. The medium without cells was also incubated to ensure no changes observed were occurring due to the time of the antibiotic in the medium.

After the incubation, the cultures were centrifuged at 4000 x g for 15 minutes to spin down any bacterial cells and components. After centrifugation, the supernatant was collected and filtrated through a 0.2 µm sterile filter to remove all potential remaining bacterial components. This supernatant was then serially diluted with M9-cas medium. The dilution range was from 0.66 µg/ml to 0.055 µg/ml putative (i.e. assuming no antibiotic breakdown has happened during incubation) cefotaxime concentration in 11 steps, to cover concentrations below and above the MIC of the wildtype and single mutant strains. Every step was 20% less putative cefotaxime

concentration. This Cefotaxime bioassay was then inoculated with a final cell density of 10^5 cells/well in a 96 well plate with 200 µl volume per well. Wells were after the addition of the cefotaxime medium either inoculated with wildtype of the single mutant strain, this depended on the strain used like in the medium incubation step. The incubation of the 96 well plate was at 37 ºC for 24 hours. Bacterial growth was accessed by OD600 measurement with a plate reader, where higher absorption then the medium control, fresh medium without antibiotics (200 µl medium without cells), were considered as growth. The absorption of cut-off point for observed growth was around 0.1.

4.6 Statistical analysis

In the statistical tests of the "Wildtype interaction experiment", the different strains (YFP triple mutant and BFP triple mutant) were tested separately. In order to test whether the wildtype interaction experiment and the "B-lactamase interaction experiment" treatments had any significant effect on the establishment of the triple mutant, the data were tested using two-way ANOVA's. The colony counts of both assays were transformed to get a normal distribution and equal variance. In the wildtype interaction experiment, the colony count of YFP mutant was transformed to the power of 2 and BFP mutant colony count was untransformed. In the "B-lactamase interaction experiment" all colony counts were transformed to the power of 2. In the assays, a significant interaction of the treatments was found for nearly all strains. Therefore, two-way ANOVA with an interaction term for cefotaxime concentration and treatment were used in all samples consistently. For both assays, the treatments were compared using Tukey multiple comparison. All tests were performed in R version 3.6.1 (https://. r-project.org/)

5. Results

5.1 Wildtype interaction experiment

To test whether the effect of the presence of susceptible wildtype on the establishment of triple mutant could be reproduced, the wildtype interaction assay was repeated. This experiment was performed with 2 quantities of wildtype cells that were added to plates containing a low number of mutant cells. Figure 1 shows that the addition of wildtype cells increased the mutant colony numbers. According to the Tukey multiple comparison, the increase was in all instances significant. The choice of fluorochrome of the mutant versus the wildtype did not influence the establishment effect seen earlier in the experiment of Arno Hagenbeek. The plates were studied under the fluorescence microscope after incubation and generally did not show any wildtype colonies surviving.

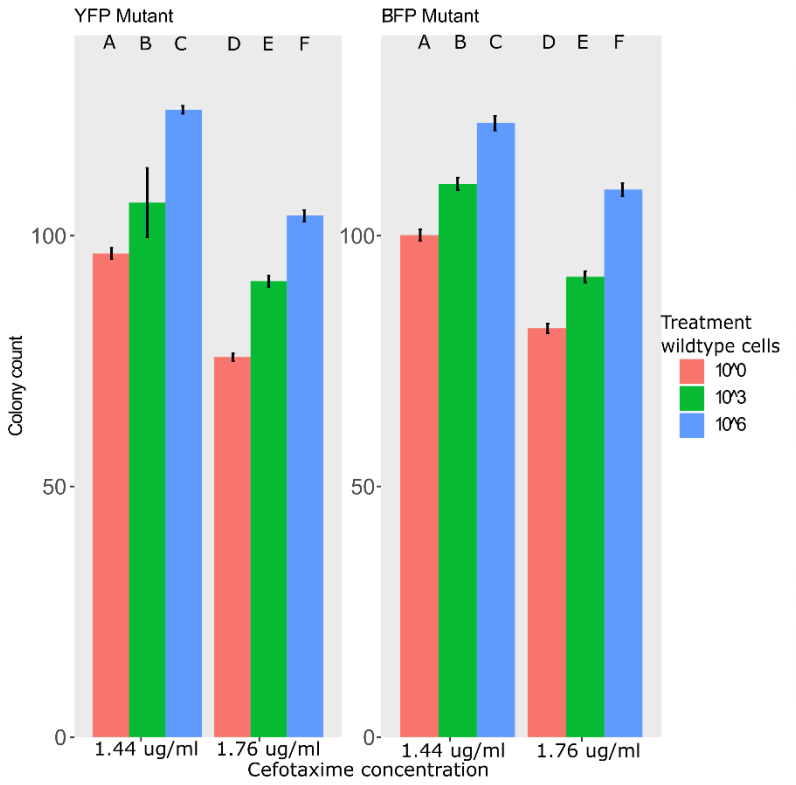

Figure 1: Wildtype interaction assay. The average amount of mutant colonies per plate under two different cefotaxime concentrations and total plated wildtype cells (treatment wildtype cells). The error bars indicate the standard error with 15 replicates tested per treatment. The significance is indicated with the letters above the columns. The significance was found using ANOVA and Tukey multiple comparison. The fluorochrome of the mutant is given in the title of the graph. The wildtype had YFP when the mutant was BFP, and vice versa.

5.2 B-lactamase interaction experiment

The next step was to test whether the increase of mutant establishment is due to the removal of antibiotic or due to addition of nutrients by lysed cells. To see if the addition of single mutant would have an additional impact due to the more efficient β-lactamase, the triple mutant was tested either alone or together with 10^6 cells of single mutant or wildtype. The use of alive wildtype and alive single mutant could give an indication if the more efficient beta-lactamase could increase the triple mutant establishment. The triple mutant was also tested either alone or together with 10^6 heat-killed wildtype or heat-killed single mutant. The addition of heat-killed cells was taken to see if the addition of nutrients caused the increase of mutant establishment.

Figure 2 shows that the addition of heat-killed wildtype or heat-killed single mutant did not significantly change the colony count of the triple mutant under both tested cefotaxime concentrations compared to the triple mutant alone. The addition of alive wildtype did significantly increase the establishment of the triple mutant. This increase was further pronounced by the addition of alive single mutant, as seen in Table 1, the addition of 1.44 µg/ml

cefotaxime reduced the establishment of the triple mutant by 34% compared to the 0 µg/ml cefotaxime treatment. Similar results occurred with the highest cefotaxime concentration of 1.76 µg/ml. In the subsequent study of the plates under the fluorescence microscope there were no wildtype or single mutant colonies found. The colony count of the added wildtype and single mutant were $1.05x10^6$ cells and $1.01x10^6$ cells respectively on the plates with the appropriate treatment.

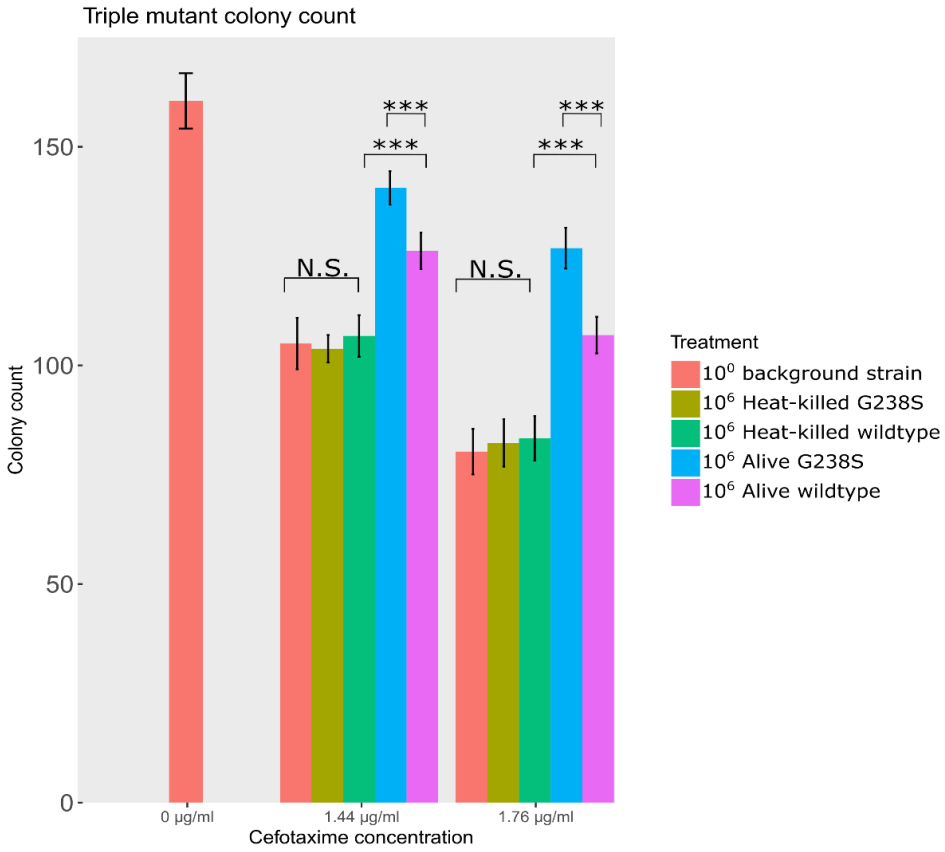

*Figure 2: B-lactamase interaction experiment. The average amount of mutant colonies per plate for the different treatments with two different cefotaxime concentrations and control plates with no cefotaxime. 10^0 background strain indicates that there no other strain on the plate than the triple mutant. The error bars indicate the standard deviation of each treatment with 15 replicates. N.S. = not significant, *** = p<0.001). The significance was found using ANOVA and Tukey multiple comparison.*

Table 1: Table showing how much the treatment has restored the establishment of the triple mutant and the percentage establishment compared to the 0 µg/ml triple mutant.

Treatment	Average colony count	Percentage establishment compared to 0 µg/ml cefotaxime	Percentage establishment restored
0 µg/ml cefotaxime			
Triple mutant	160	-	-
1.44 µg/ml cefotaxime			

Triple mutant	105	65.6	-
10^6 Heat-killed wildtype	107	66.3	1.3
10^6 Heat-killed Single mutant	104	65	0
10^6 Wildtype	126	78.8	13.2
10^6 Single mutant	141	88.1	22.5
1.76 µg/ml cefotaxime			
Triple mutant	80	50	-
10^6 Heat-killed wildtype	83	51.9	1.9
10^6 Heat-killed Single mutant	82	51.3	1.3
10^6 Wildtype	107	66.9	16.9
10^6 Single mutant	127	79.4	29.4

5.3 Cefotaxime lethality assay

To test how long the wildtype or single mutant survive in a high cefotaxime concentration (above the MIC of the wildtype and mutant), both strains were incubated in 1.76 µg/ml cefotaxime. The addition of cefotaxime above the MIC (MIC wildtype = 0.07 µg/ml, MIC single mutant = 0.32 µg/ml cefotaxime) caused a reduction of wildtype colonies from uncountable colonies to 20 colonies in 30 minutes. After this reduction the colonies stay around 10-20 colonies until 150 minutes when the population had completely died. For the single mutant it took 30 minutes longer to completely die.

5.4 Cefotaxime bioassay

In this experiment, alive YFP wildtype TEM-1, alive the single mutant, alive ancestor strain, heat-killed wildtype, heat-killed single mutant and heat-killed ancestor strain were incubated for 3 hours to see whether the possible effect of the alive cells was also occurring with heat-killed cells. By incubation of TEM-1 wildtype heat-killed or alive and ancestor strain heat-killed or alive a possible decrease of the cefotaxime could occur. The assay was designed to distinguish between binding and degradation through active β-lactamase. The lowering of the actual antibiotic concentration by the alive ancestor would be taken as an indicator of removal via binding of the antibiotic. The decrease of antibiotic concentration by alive wildtype is an indicator of the removal of antibiotic by beta-lactamase or binding of the antibiotic. If only the alive wildtype, and not the ancestor, gives a decrease in the actual antibiotic concentration, this would be taken as an indicator of the removal by active beta-lactamase. Also, the use of alive single mutant as second background strain could make this distinction clearer by a possible

larger decrease in the antibiotic concentration by the more efficient beta-lactamase compared to the ancestor strain.

Table 2 shows the putative cefotaxime concentration at which no growth was found per tested strain. The table shows that there was no growth in the wells that had a higher putative cefotaxime concentration than the MIC with no evidence of a lower cefotaxime concentration following the incubation step. This means that there was evidence of binding or removal of antibiotics from the environment

Table 2: Cefotaxime bioassay. In the table the putative cefotaxime concentration is shown in µg/ml, where no growth was observed. WT = TEM-1 wildtype, SM = single mutant. The cells used for the initial culturing treatments are given in the top. The WT and SM on the left are the strains the used to inoculate the MIC assay.

	Alive WT	Heat-killed WT	Alive Ancestor	Heat-killed Ancestor	Alive SM	Heat-killed SM	CTX
WT	0.07	0.07	0.07	0.07	-	-	0.07
SM	-	-	0.31	0.31	0.31	0.31	0.31

6. Discussion

In this study, the effect of interactions on the establishment of an antibiotic-resistant mutant strain was tested under different cefotaxime concentrations. The tested social interactions were (i) the addition of nutrients through cell death and (ii) removal of the antibiotic. Removal of antibiotics can happen through both the degradation of the antibiotic by active β-lactamase or through binding of antibiotic by cellular components like PBP3. Successful establishment of the mutant was indicated by the growth of visible colonies on agar plates. These colonies are derived from single cells, thereby making them initially vulnerable to extinction due to random mortality. However, the colonies that have the size to be observable during colony counting are numbered in the millions of cells and will exceed the population size where they are vulnerable to extinction by random mortality (Shapiro and Trubatch 1991). Therefore, the colonies that were counted have successfully survived the establishment phase.

The first objective was to see if the results gained by Arno Hagenbeek could be reproduced repeatable. The presence of wildtype cells on agar plates was found to increase the number of visible mutant colonies. There are some differences between the results gained here and Arno's results. The results gained in this study were significant. This is contrary to Arno's results, where he got no significant increase in triple mutant establishment in the 10^3 wildtype cells treatment. The overall effect, the addition of wildtype increasing the triple mutant establishment, is shown to be reproducible by this study. Furthermore, the results show that the fluorochrome did not influence the outcome of this experiment. It has been described that the incorporation of fluorescent proteins can affect the growth and physiology of the bacteria. The incorporation of GFP, for example, could lead to increased sensitivity to antimicrobial agents compared to the wildtype cells (Allison and Sattenstall 2007). How this occurred is yet unknown and there has been no research on the effect of YFP or BFP. If the fluorochromes affected the fitness of the strains, this could have had a significant effect on the interactions. A less fit wildtype could have had less of an effect on the mutant establishment or no effect on it. For example, if the wildtype dies easier with BFP, then the triple mutant has potentially easier access to the additional nutrients from the wildtype cells. When the YFP wildtype then dies later the effect of the mutant could have been less. Similarly, the effect of a potential β-lactamase could have been reduced or increased when the fitness of the strains was significantly affected by the fluorochrome. Therefore, the absence of such differences

between the fluorochromes lead to the conclusion that there is indeed an effect of the wildtype cells on the mutant establishment and not the fluorochromes.

In the next step, the possible mechanisms behind the observed effect of increase of triple mutant establishment were tested. To test whether the addition of nutrients via cell death, had an impact on the mutant establishment, heat-killed cells were added as a treatment in the "B-lactamase interaction experiment". In the results there was no significant difference between the treatments with heat-killed cells added and no cells added to the triple mutant. This indicates the absence of a significant effect of the heat-killed cells on the establishment of the triple mutant. In other studies, it was shown that bacterial growth can be increased by cell death of other cells. This has been shown to occur after cell lysis via sonication, due to nutrient starvation and other factors (Koch 1959; Mason and Hamer 1987; Banks and Bryers 1990; Bruggen, Semenov, and Zelenev 2000). It has been found that the addition of heat-killed cells to medium could increase the growth of bacteria (Nioh and Furusaka 1968; Mason and Hamer 1987). The establishment of the triple mutant could have been increased by addition of these heat- killed cells, but it did not. Therefore, the mechanism of addition of nutrients due to cell death can be excluded as a mechanism to increase the mutant establishment.

Another mechanism studied in this thesis was the removal of antibiotic through binding of the antibiotic to cellular components, like PBP3. As described in the introduction, PBP3 is the main target for cefotaxime (LeFrock, Prince, and Left 1982). In the "Cefotaxime bioassay" the binding of antibiotic was tested by including the ancestor cells in the assay. As seen in the results section, there was no evidence of removal of antibiotics by binding or by β-lactamase. This result is contrary to the "B-lactamase interaction experiment", where the single mutant significantly increased the triple mutant establishment compared to the wildtype. This contradiction could be explained by the "Cefotaxime bioassay" not being sensitive enough. The dilutions of the antibiotic were 20% steps. There is a chance that the removal by either binding or the β-lactamase occurred, but to such a small amount that the decrease was in between two steps causing the result to go unnoticed. Especially the TEM-1 β-lactamase of the wildtype has low efficiency in breaking down cefotaxime (Raquet et al. 1994; Kohner et al. 2009; Schenk et al. 2012). This inefficiency can lead to such very small differences in the antibiotic concentration, that it could be lower than the tested steps. Furthermore, because alive wildtype as culture treatment with single mutant as the MIC inoculum was not done, there is no comparison between the alive wildtype and the alive single mutant as culture

treatment. This difference in active β-lactamase might have caused a difference that would be interesting.

The significant increase, seen in the "B-lactamase interaction experiment", compared to the triple mutant without cell added, suggests some removal occurring. Because the wildtype still has β-lactamase, the binding cannot be excluded from influencing the triple mutant establishment in either the "Wildtype interaction experiment" or "B-lactamase interaction assay". In other studies, the effect of removal of antibiotic by binding has been shown (McDermott, Walker, and White 2003; Manning and Kuehn 2011). This removal is also seen by PBP3 proteins in *E. coli* (Tormo et al. 1986). The removal made it possible for other cells to grow. Therefore, in future experiments, a strain without β-lactamase could be added as an additional control in an experiment like "B-lactamase interaction experiment" to include the role of binding of antibiotic to cellular components.

The last mechanism studied was the removal of antibiotics through β-lactamase. In the "B-lactamase interaction experiment", there was a significant difference between the single mutant treatment and the wildtype treatment. The addition of the more efficient single mutant strain did significantly increase the number of triple mutant colonies compared to the treatment with the wildtype strain. A possible mechanism behind this increase could be the increased TEM activity or expression of the single mutant or the longer survival of the single mutant compared to the wildtype. The longer survival time is found by the "Cefotaxime lethality assay". In the assay the single mutant did survive 30 minutes longer than the wildtype did. At above lethal concentration the susceptible strain will eventually go extinct, but not all at once. The time can be highly variable and may take some time to eradicate the bacteria as earlier studied (Coates et al. 2018). The additional time could have been used by the single mutant to produce more β-lactamase than the wildtype.

Another mechanism to affect the increased establishment of the triple mutant under influence of the single mutant could be the differences in expression between wildtype and single mutant. It has been shown that synonymous mutations could cause higher antibiotic resistance by increasing the expression of TEM-1 (Zwart et al. 2018). Further studies have shown that the resistance level of bacteria to β-lactam antibiotics is linearly correlated to the production of beta-lactamase (Song et al. 2009). To the best of my knowledge, the expression benefit of the G238S mutant compared to the TEM-1 has not been researched. In another study, it was found that the increase in resistance with G238S substitution is primarily due to the increased catalytic efficiency, increasing the activity of the enzyme (Gniadkowski 2008; Palzkill 2018).

Therefore, it might be assumed that an increase in expression is negligible to the increase in activity compared with the wildtype. Therefore, the expression benefit can be excluded from influencing the triple mutant establishment with the single mutant compared to the wildtype.

The β-lactamase affecting the establishment of the triple mutant could be considered a social interaction between the triple mutant and the single mutant. Social interactions are believed to shape community through cooperative integration and competitive exclusion (Madsen, Sørensen, and Burmølle 2018). Previously, it has been found that sensitive bacteria can survive an antibiotic treatment in the presence of resistant mutants through the inactivation of beta-lactam antibiotics in the environment (Clark et al. 2009; Yurtsev et al. 2013; Rojo-Molinero, Macià, and Oliver 2019). A new possible social interaction might have occurred here where the triple mutant did benefit from the wildtype or single mutant being present.

The observations made in this study, may have implications for the understanding of the factors influencing establishment. As seen in Arno's work the interaction of wildtype and triple mutant does increase the establishment of the triple mutant. The further increase of establishment, as seen in the "B-lactamase interaction experiment", by a low resistant strain could indicate that the level of antibiotic resistance of a wildtype population might be of importance to the probability of establishment of a more antibiotic resistant mutant. This could, in turn, lead to a better understanding of the emergence and spread of antibiotic resistance.

In conclusion, TEM β-lactamase of the single mutant could be involved in addition to binding of antibiotic in the increase of the establishment of a mutant. Although the β-lactamase of the single mutant is shown to have a positive effect on the establishment of the triple mutant, there might be several mechanisms involved. Future research might be focussed on the inclusion of the ancestor strain of the wildtype into a similar experiment as the "B-lactamase interaction experiment". The ancestor should not have β-lactamase. The inclusion of ancestor could determine if the binding of antibiotic by cellular components does influence the establishment of the mutant compared to the wildtype. Thereby, the significance of the influence of TEM-1 can be determined compared to the binding of antibiotic by cellular components.

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9. Supplement

9.1 M9 medium and agar protocol per litre

Table 1: General recipe used for making a liter of M9-cas medium or agar. For the recipe of 5x M9 salts see Table 2. If agar was used, this was first weighed and ddH_2O was added until the volume was 755ml; this was thereafter autoclaved. After cooling down (~55 C^0), the other components were added.

	****for 1 L**	Final concentration
5x M9	200 ml	1x
$MgSO_4$ 1M	2 ml	0.002M
Glucose 20% w/v	20 ml	0.4%
$CaCl_2.2H_2O$ 0.1M	1 ml	0.0001M
Casamino acids 10%	20 ml	0.2%
Uracil 2mg/ml	1 ml	2 µg/ml
Thiamine 1mg/ml	1 ml	1 µg/ml
Cefotaxime	variable	Variable in tests
Agar	15 gram	<- first autoclave in 755 ml ddH2O then add components
ddH_2O	fill up to 1L	

Table 2: Recipe used for making 1 litre of 5x M9 salts for use in the M9-cas medium. After adding the salts, water was added until the total volume of 1 litre.

	5x M9 1 L
$Na_2HPO_4.2H_2O$ (di Sodium hydrogen Phosphate Dihydrate)	42.5g
KH_2PO_4 (Potassium dihydrogen phosphate)	15g
NaCl (Sodium chloride)	2.5g
NH_4Cl (Ammonium chloride)	5g

9.2 Statistics

Wildtype interaction assay

Table 3: Statistics of the wildtype interaction experiment with the YFP triple mutant. n = 15.

	Wildtype (cells)	Cefotaxime concentration (µg/ml)	Average colony count	Standard deviation	Standard error
YFP triple mutant					
10^0	0	1.44	96	4.3	1.11
10^3 Wildtype	10^3	1.44	107	26.8	6.9
10^6 Wildtype	10^6	1.44	125	3	0.8
10^0	0	1.76	76	3	0.8
10^3 Wildtype	10^3	1.76	91	4.4	1.1
10^6 Wildtype	10^6	1.76	104	4.35	1.1
BFP triple mutant					
10^0	0	1.44	100	4.5	1.2
10^3 Wildtype	10^3	1.44	110	4.9	1.3
10^6 Wildtype	10^6	1.44	122	5.6	1.5
10^0	0	1.76	82	3.8	1
10^3 Wildtype	10^3	1.76	92	4.3	1.1
10^6 Wildtype	10^6	1.76	109	4.9	1.3

Table 4: Two-way ANOVA data of the wildtype interaction experiment with the YFP and BFP triple mutant. An asterisk indicates significance (p<0.05)

	DF	Sum Sq	F-value	p-value
YFP triple mutant				
Cefotaxime concentration	1	8256	62.27	1.00e-11*
Treatment	2	12177	45.92	3.34e-14*z
Cefotaxime concentration x Treatment	2	138	0.52	0.597
Residuals	84	11136		
BFP triple mutant				
Cefotaxime concentration	1	260957506	254.973	<2e-16*
Treatment	2	402633351	196.700	<2e-16*
Cefotaxime concentration x Treatment	2	1694099	0.828	0.441
Residuals	84	85971666		

Table 5: Posthoc test of the two-way ANOVA. YFP triple mutant in the wildtype interaction experiment

Measure	Diff	Lower bound	Upper bound	P value
Concentration treatment (µg/ml)				
1.44-1.76	-19.16	-23.98	-14.32	0
Treatment				
10^0-10^3	12.63	5.54	19.73	1.62e-04
10^0-10^6	28.43	21.34	35.53	0
10^6-10^3	15.80	8.71	22.89	2.60e-06

Table 6: Posthoc of the two-way ANOVA. BFP triple mutant in the wildtype interaction experiment

Measure	Diff	Lower bound	Upper bound	P value
Concentration treatment (µg/ml)				
1.44-1.76	-3405.6	-3829.7	-2981.5	0
Treatment				
10^0-10^3	1967.7	1343.9	5757.6	0
10^0-10^6	5134.4	4511.2	5757.6	0
10^6-10^3	3167.3	2544.1	3790.5	0

B-lactamase interaction experiment

Table 7: Statistics of the B-lactamase interaction assay

Treatment	Average colony count	Standard deviation	Standard error
0 µg/ml cefotaxime			
10^0	160	6.32	1.63
1.44 µg/ml cefotaxime			
10^0	105	5.9	1.5
10^6 Heat-killed wildtype	107	4.6	1.2
10^6 Heat-killed Single mutant	104	3.1	0.8
10^6 Wildtype	126	4.2	1.1
10^6 Single mutant	141	3.8	1
1.76 µg/ml cefotaxime			
10^0	80	5.2	1.3
10^6 Heat-killed wildtype	83	5.1	1.3
10^6 Heat-killed Single mutant	82	5.4	1.4
10^6 Wildtype	107	4.2	1.1
10^6 Single mutant	127	4.7	1.2

Table 8: Two-way ANOVA data of the B-lactamase interaction assay. An asterisk indicates significance (p<0.05)

	DF	Sum Sq	F-value	p-value
Cefotaxime concentration	2	3.389e+09	1353.325	<2e-16*
Treatment	4	1.990e+09	397.228	<2e-16*
Cefotaxime concentration Treatment	4	4.501e+06	0.899	0.466
Residuals	154	1.928e+08		

Table 9: Two-way ANOVA data of the B-lactamase interaction assay. An asterisk indicates significance (p<0.05)

Measure	Diff	Lower bound	Upper bound	P value
Concentration treatment (µg/ml)				
0-1.44	1191	12740	11242	0
0-1.76	16230	16979	15481	0
1.44-1.76	4239	4672	3807	0
Treatment				
Triple mutant 0 CTX – Triple mutant	2913.40	-5077.22	3934.58	0
Triple mutant 0 CTX -- Heat-killed SM	2886.53	1865.32	3907.75	0

Triple mutant 0 CTX -- Heat-killed WT	2485.07	1463.85	3506.28	0
WT – Triple mutant 0 CTX	2020.80	99.58	3042.02	0.0000008
Triple mutant 0 CTX -- SM	-6264.20	-7285.42	-5242.98	0
WT – Triple mutant	4934.20	4100.38	5768.02	0
Triple mutant -- SM	-9177.60	-10011.42	-8343.78	0
SM-- Heat-killed WT	8749.27	7915.45	9583.09	0
WT-- Heat-killed WT	4505.87	3672.05	5339.69	0
WT--SM	-4243.40	-5077	-3409.58	0
Triple mutant-- Heat-killed WT	-428.33	-1262.15	405.49	0.676