The effects of antibiotic concentration and social interactions on the establishment of antibiotic resistant *Escherichia coli* strains

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1. Abstract

The continuing increase in antibiotic resistance in bacteria is a growing global problem, that as of yet has no satisfactory solution. As the evolution of resistance is proving difficult to reverse, efforts should be made to avoid the emergence of resistance. A promising strategy would be diminishing the chances of successful establishment for antibiotic resistant mutants. The process of establishment entails the survival of the initially rare mutant until it reaches a frequency no longer vulnerable to extinction due to random drift. The chance of successful establishment is often low, even for traits with a selective advantage. This makes establishment a promising process for potentially halting or slowing the evolution of antibiotic resistance.

As of yet, knowledge on the factors influencing successful mutant establishment remains limited, and is mostly based on theoretical models. In this study, by utilizing *Escherichia coli* expressing the TEM-1 enzyme, or cefotaxime resistant mutants derived thereof, the influence of cefotaxime concentration, cell density, and wildtype presence on establishment probability is tested. To this end, cells were spread over agar plates, and the growth of visible colonies was taken as indicator of their successful establishment. It was found that all three factors, cefotaxime concentration, cell density, and wildtype presence, were capable of significantly affecting establishment. Increased cefotaxime concentration, and increased cell density, were found to decrease establishment probability. Strikingly, presence of wildtype cells was found to increase the likelihood of mutant establishment, even while the used cefotaxime concentrations were far above the minimum inhibitory concentration (MIC) of the wildtype. This finding could have implications to our expectations regarding mutant establishment under antibiotic exposure, as currently the influence of wildtype on mutant survival is largely disregarded once antibiotic concentrations approach wildtype MIC.

2. Introduction

2.1. Rise in antibiotic resistance

The continuous increase in antibiotic resistance in pathogenic bacteria is a growing problem on a global scale. Antibiotic resistance is estimated to cause 700,000 deaths annually worldwide, and if left unchecked is projected to cause 10 millions of deaths annually by 2050 (European Commission. 2016). This daunting prospect has sparked great scientific interest in solving the problem. Among the many approaches are attempts to acquire new types of antibiotics (Ling et al. 2015, Kealey et al. 2017), finding ways to prolong antibiotic effectivity (Wright. 2016, Baym et al. 2016), or elucidating the evolutionary mechanism of resistance emergence (Gullberg et al. 2011, Alexander and MacLean. 2018).

Initially, it was expected that the fitness costs associated with antibiotic resistance would select against it in the absence of antibiotics, eventually reversing the resistance. However, older antibiotics still remain ineffective, even decades after resistance has been encountered (Centers for Disease Control and Prevention. 2013). Furthermore, several studies have reported that antibiotic resistance can persist even in the absence of antibiotics (Sjölund et al. 2003, Andersson and Hughes. 2010, Gifford and Maclean. 2012, Händel et al. 2013). Generally the consensus in these studies is that this persistence of antibiotic resistance is due to compensatory mutations ameliorating the fitness costs of resistance. In this way resistant cells are not outcompeted, even if the population is no longer exposed to antibiotics. The persistence of antibiotic resistance means that once clinical efficacy of a specific antibiotic is lost, it will likely remain unusable for that purpose indefinitely, and a replacement has to be found.

This makes the procurement of new antibiotics vital for their continued use, as it provides the necessary alternatives when resistance against existing antibiotics arises. However, this approach is proving unsustainable in the long term, as the rate of resistance emergence is increasing, while the rate of antibiotic discovery is decreasing (Baym et al. 2016, Laxminarayan. 2014). Therefore, it is imperative to find a way to halt the emergence of antibiotic resistance.

2.2. Establishment

Among the evolutionary processes leading up to antibiotic resistance, the establishment of the initially rare resistant mutant is a particularly interesting subject for the purpose of stopping the evolution of resistance. Establishment entails a situation in evolution where a new allele is introduced into a population, and manages to survive past its initially low frequency, reaching an allele frequency no longer vulnerable to extinction due to random mortality (Patwa and Wahl. 2008, Wiesch et al. 2011). The probability of establishment is often expected to be low, as the initially small number of individuals that carry the new allele make it particularly vulnerable to extinction by genetic drift (Patwa and Wahl. 2008, Alexander and MacLean. 2018). In other words, a low frequency allele is likely to go extinct as only few individuals carry it, and thus the allele can easily be lost if the carrying individuals perish due to random mortality or lack of reproduction.

Once an allele is established at a decent frequency, approximately 1 divided by the selection coefficient of the allele (1/s), it is no longer vulnerable to genetic drift, and selection will proceed deterministically (Kimura. 1968, Patwa and Wahl. 2008). Once this occurs, assuming it confers a selective advantage, the allele will increase in frequency until it is eventually present in the entire population (fixed). In other words, once an advantageous allele establishes, fixation

probability is theoretically 100% (Patwa and Wahl. 2008). As the use of antibiotics confers a huge selective advantage towards resistance, attempting to manipulate this selection process will likely not yield favourable results. However, establishment is mostly affected by genetic drift, and remains a stochastic process even for highly beneficial alleles. This makes establishment a promising process to potentially halt the evolution of antibiotic resistance.

In population genetics, the matter of establishment probability and the subsequent fixation is a classical subject, with work dating back almost a 100 years (Haldane. 1990, Patwa and Wahl. 2008). Despite this, only few empirical studies exist that focus on establishment (Chelo et al. 2013, Gifford and Maclean. 2013, Alexander and Maclean. 2018). These studies have reported several conditions affecting establishment of adaptive mutants in various organisms.

In *Caenorhabditis elegans* it has been reported that higher initial frequencies, and beneficial alleles, increased the likelihood of successful establishment of a low frequency invading strain (Chelo et al. 2013). More relevant to the subject of antibiotic resistance, it was found that in rifampicin-resistant *Pseudomonas aeruginosa* populations the majority of *de novo* lineages susceptible to antibiotics were lost to genetic drift, decreasing the likelihood of the population becoming susceptible again (Gifford and Maclean. 2013). Additionally, in *P. aeruginosa* it was found that even at antibiotic concentrations that inhibit the wildtype while allowing mutant survival (above wiltype MIC but below resistant mutant MIC), emergence of resistance remained unlikely (Alexander and Maclean. 2018). Such concentrations are within the “mutant selection window”, and were theorised to favour the emergence of resistance (Drlica and Zhao. 2007). However, while the antibiotic concentration favoured resistance selection, it also caused an increase in resistant mutant mortality and lag-time, which hampered establishment of the *de novo* resistant strain (Alexander and Maclean. 2018).

This conclusion is in accordance with findings of a different study, reporting that low sub-MIC (minimum inhibitory concentration) antibiotic concentrations can stochastically clear even large bacterial populations (Coates et al. 2018). This stochastic clearance has been attributed to the random nature of cell death and survival under (bactericidal) antibiotic exposure. The cell death induced by bactericidal antibiotics is not constant, but occurs at random in the population, at one time killing a negligible number of cells, and at other times killing large fractions of the population. These random periods of high mortality can add to the stochastic fluctuations inherent to the population, causing severe fluctuations and potentially resulting in the extinction of the population. Increases in bactericidal antibiotic concentration result in increases in mortality, and thus increase the intensity of these fluctuations. Therefore, Coates et al. (2018) concluded that even low sub-MIC concentrations can stochastically cause the extinction of

bacterial populations, and that increasing the antibiotic concentration increases the chance of this stochastic extinction occurring.
Thus, while some empirical studies exist on the topic of establishment, only one (Alexander and Maclean. 2018) has previously investigated establishment within the context of resistance emergence, and this study focussed solely on the effect of antibiotic concentration on the likelihood of establishment.

2.3 TEM β-lactamase

In order to investigate establishment chance in the context of antibiotics resistance, this study utilizes the well-studied TEM β-lactamase enzyme. Wildtype TEM-1 β-lactamase is an enzyme that hydrolyses several β-lactam antibiotics, such as penicillin, breaking them down and rendering them non-effective. This antibiotic breakdown effectively makes the TEM-carrying strain resistant against the targeted antibiotics (Raquet et al. 1994, Schenk et al. 2012, Mira et al. 2015). While wildtype TEM-1 breaks down a wide variety of β-lactam antibiotics, it is inefficient at hydrolysing the β-lactam cefotaxime (Raquet et al. 1994, Salverda et al. 2011, Schenk et al. 2012), a bactericidal antibiotic that functions by disrupting the final process of peptidoglycan synthesis, crucial in cell wall formation (Johann et al. 1997, Kjeldsen et al. 2015). However, as has been observed in several studies (Barlow and Hall. 2002, Salverda et al. 2011, Schenk et al. 2012), the TEM-1 gene has great potential to adapt to cefotaxime through mutation and selection.

This study aims to provide further insight into the factors affecting the establishment of antibiotic resistant lineages, which might eventually yield knowledge on how to minimize the chances of establishment for these undesirable strains. The current study investigates the effect of sub-MIC antibiotic concentrations on establishment, which has been investigated in another study (Alexander and Maclean 2018). However, by using *Escherichia coli* strains expressing TEM-1, or one of three cefotaxime resistant TEM alleles, the current study investigates this effect in multiple strains with varying antibiotic resistance. Additionally, the impact of two social factors, namely cell density, and the presence of wildtype cells were also studied in the various strains under different antibiotic concentrations.

The main goal of this study was to investigate whether cefotaxime concentration, cell density, or the presence of wildtype (TEM-1), affected the establishment chance of several *E. coli* strains with varying cefotaxime resistance when grown on agar plates.
As has been previously reported (Alexander and Maclean. 2018, Coates et al. 2018), it can be expected that increasing antibiotic concentration would increase random mortality, causing an

increase in extinction probability. Therefore, the hypothesis of the current study is that that increasing antibiotic concentration would decrease the chance of successful establishment.

In structured environments, such as biofilms, β-lactamase enzymes break down the targeted antibiotic locally (Frost et al. 2018, Medaney et al. 2015). Thus, while one would expect that increasing cell density would increase resource competition, it also puts multiple resistant colonies in close proximity to each other. Consequently due to their close proximity resistant colonies would reduce the local cefotaxime concentration faster, possibly aiding their growth and establishment. Therefore, it was hypothesised that increased cell density would result in increased establishment probability.

For the wildtype-presence experiment, it was expected that increasing the wildtype (TEM-1) cell density would decrease establishment probability of the mutants. This was expected because the TEM-1 strain has poor cefotaxime activity, and thus would not benefit the mutants by breaking down the antibiotic. Therefore, wildtype presence was expected to cause resource competition, but without significantly decreasing cefotaxime, thus hampering establishment of the mutant.

3. Materials and methods

3.1. Bacterial strains and culturing

The experiments were performed using several *E. coli* strains derived from the MG1655 strain. These strains were previously modified to have yellow (SYFP2) or blue (mTagBFP2) fluorescent markers integrated into the chromosomal DNA (Gullberg et al. 2014). Additionally 4 different TEM β-lactamase alleles were integrated, including wildtype TEM-1, and 3 TEM alleles containing 1 (G238S), 2 (E104K/G238S), or 3 (E104K/M182T/G238S) point mutations. Each additional point mutation was shown to increase cefotaxime resistance (Farr et al. unpublished). Unless stated otherwise, the experiments were conducted using the YFP labelled strains.

Before each experiment, the strains were retrieved from -80°C glycerol stocks. Each strain was streaked out on a 1.5% LB agar plate, and incubated overnight at 37°C. A single colony of each strain was taken from the plate and inoculated into 1 ml liquid M9-cas medium (Supplement table 1) in a 14 ml plastic tube. The tubes were incubated overnight at 37°C, shaking at 250 rpm. The overnight cultures were diluted to the density specified by the assay (see below) using PBS. Unless stated otherwise, all experiments were conducted on 92 mm plates filled with 25 ml 1.5% M9-cas agar with varying cefotaxime concentrations.

3.2. Cefotaxime concentration assay

Inocula were made by serially diluting an overnight culture (approximately $2.5*10^9$ cells/ml) of each strain to 4000 cells/ml in 4 steps. Using a cell spreader, 50 µl inocula (approximately 200

cells) were spread on M9-cas plates with different cefotaxime concentrations. Each strain was tested on incrementally increasing concentrations (Table 1). The used cefotaxime concentrations for each strain were intended to result in a detectable decrease in establishment, while still allowing for colony survival. Small scale preliminary experiments with exponentially increasing cefotaxime concentrations were performed to find the concentrations meeting these criteria. After inoculation the plates were incubated at 37°C. In order to prevent excessive growth resulting in colonies overlapping, plates were only incubated until colonies were clearly visible (20-48h). As growth was slower in high cefotaxime treatments, this resulted in incubation time varying between treatments. However, as establishment takes place early in colony growth, different incubation times are not expected to affect the results. After incubation, the number of colonies on each plate was counted. For each treatment 8 replicates were used.

Table 1. The cefotaxime concentrations (µg/ml) used for each strain during the cefotaxime concentration assay.

Strain	**Used cefotaxime concentrations (µg/ml)**							
MG1655galK::YFPΔcat-TEM-1	0	0.015	0.02	0.025	0.03	-	-	-
MG1655galK::YFPΔcat-G238s	0	0.12	0.14	0.16	0.18	0.2	0.22	0.24
MG1655galK::YFPΔcat-E104K/G238S	0	0.96	1.12	1.28	1.44	1.6	1.76	
MG1655galK::YFPΔcat-E104K/M182T/G238S	0	1.44	1.6	1.76	1.92	2.08	2.24	-

3.3. Density effect assay

Inocula were made by serially diluting overnight cultures (approximately $2.5*10^9$ cells/ml) for each strain to 3000 cells/ml in 4 steps. Using a cell spreader, 50 µl inocula (approximately 150 cells) were spread on either 92 mm plates containing 25 ml M9-cas agar, or 60 mm plates containing 10 ml M9 agar. This resulted in density treatments of approximately 2.3 cells/mm^2 and 5.3 cells/mm^2 respectively. Several cefotaxime concentrations were also used, chosen based on the results of the cefotaxime concentration assay (Table 2). For each strain concentrations were chosen with approximately 75% or 50% of the survival found in 0 µg/ml cefotaxime. After inoculation, the plates were incubated at 37°C until the colonies were clearly visible (20h-26h), followed by counting the number of colonies on each plate. For each treatment 10 replicates were used.

Table 2. The cefotaxime concentrations (µg/ml) used for each strain during the density effect assay.

Strain	**Used cefotaxime concentrations (µg/ml)**		
MG1655galK::YFPΔcat-TEM-1	0	0.02	0.025
MG1655galK::YFPΔcat-G238S	0	0.14	0.2
MG1655galK::YFPΔcat-E104K/G238S	0	1.12	1.28
MG1655galK::YFPΔcat-E104K/M182T/G238S	0	1.44	1.76

3.4. Wildtype interaction assay

To simulate a situation where resistant mutants arise from a wildtype population the TEM-1 strain with poor cefotaxime activity was taken as a susceptible wildtype and mixed with a low number of resistant mutants. Overnight cultures (approximately $2.5*10^9$ cells/ml) of the mutants were serially diluted to 2000 cells/ml in 4 steps, and the TEM-1 overnight culture was serially diluted to either $2*10^4$ cells/ml or $2*10^7$ cells/ml in 4 steps. The mutant dilutions were then mixed 1:1 with either PBS, $2*10^4$ cells/ml TEM-1 dilution, or $2*10^7$ cells/ml TEM-1 dilution, resulting in dilutions with 1000 mutant cells/ml and 0, 10^4, or 10^7 wildtype cells/ml respectively. Inocula of 100 µl were spread on M9 plates with a cell spreader, resulting in plates containing 0, 10^3, or 10^6 wildtype cells, and 100 cells of their respective mutant strains. The same cefotaxime concentrations used in the density effect assay (Table 2) were used, with the exception of 0 µg/ml. All used concentrations were above the wildtype MIC, and should not allow for wildtype colony growth. After inoculation, the plates were incubated at 37°C until the colonies were clearly visible (20h-40h). After incubation the number of surviving colonies on each plate were counted.

To ensure that all counted colonies were indeed the result of mutant establishment, the TEM-1 strain expressing BFP, and mutants expressing YFP were used. After counting, all plates were viewed under a fluorescence microscope (LEICA M165 FC) to control that all colonies indeed expressed YFP. GFP and CFP filters were used for detecting YFP and BFP signals, respectively. Colonies found to express BFP were subtracted from the colony count. For each treatment 15 replicates were used.

3.5. Statistical procedures

In the statistical tests of each assay, the different strains were tested separately.

In order to test whether increasing cefotaxime concentration affected colony number, linear regressions were performed on the results of the cefotaxime concentration assay.

To find whether the relevant treatments had any significant effect, the data of both the density effect and wildtype interaction assays were tested using two-way ANOVA's. The colony counts of both assays were transformed by taking the square root, in order to acquire normal

distributions and equal variance. In both assays a significant interaction was found for at least one strain. Therefore two-way ANOVA's with interaction were used in all samples for consistency. For both assays, treatments were compared using a Tukey multiple comparison. All statistical tests were performed in R version 3.6.0 (https://www.r-project.org/)

4. Results

4.1. Cefotaxime concentration assay

To test whether increasing cefotaxime concentration affected establishment, the 4 *E. coli* strains were spread over agar plates with increasing cefotaxime concentrations. Figure 1 shows a linear regression of the results, indicating that increasing cefotaxime concentrations resulted in decreasing colony numbers for all tested strains. Table 3 shows the test statistics of the corresponding linear regression analyses. Since the primary goal of the experiment was to investigate a possible negative relationship between establishment probability and cefotaxime concentration, a simple linear regression was chosen to infer if increasing cefotaxime concentrations indeed lowered total colony number. The linear model could be considered to not fit the data perfectly. Especially in the more resistant strains, E104K/G238S and E104K/M182T/G238S, a quadratic model would likely provide a better fit. However, for the purposes of this experiment a linear model was deemed satisfactory, as the negative slope clearly and intuitively shows the negative relationship between concentration and colony count. Furthermore, the difference in intensity for the slopes of the different strains convincingly illustrates the differences in resistance between the different strains.

During the experiment, one replicate within the 1.28 µg/ml cefotaxime treatment of the E104K/G238S strain was discarded due to excessive colony overlap precluding accurate counting.

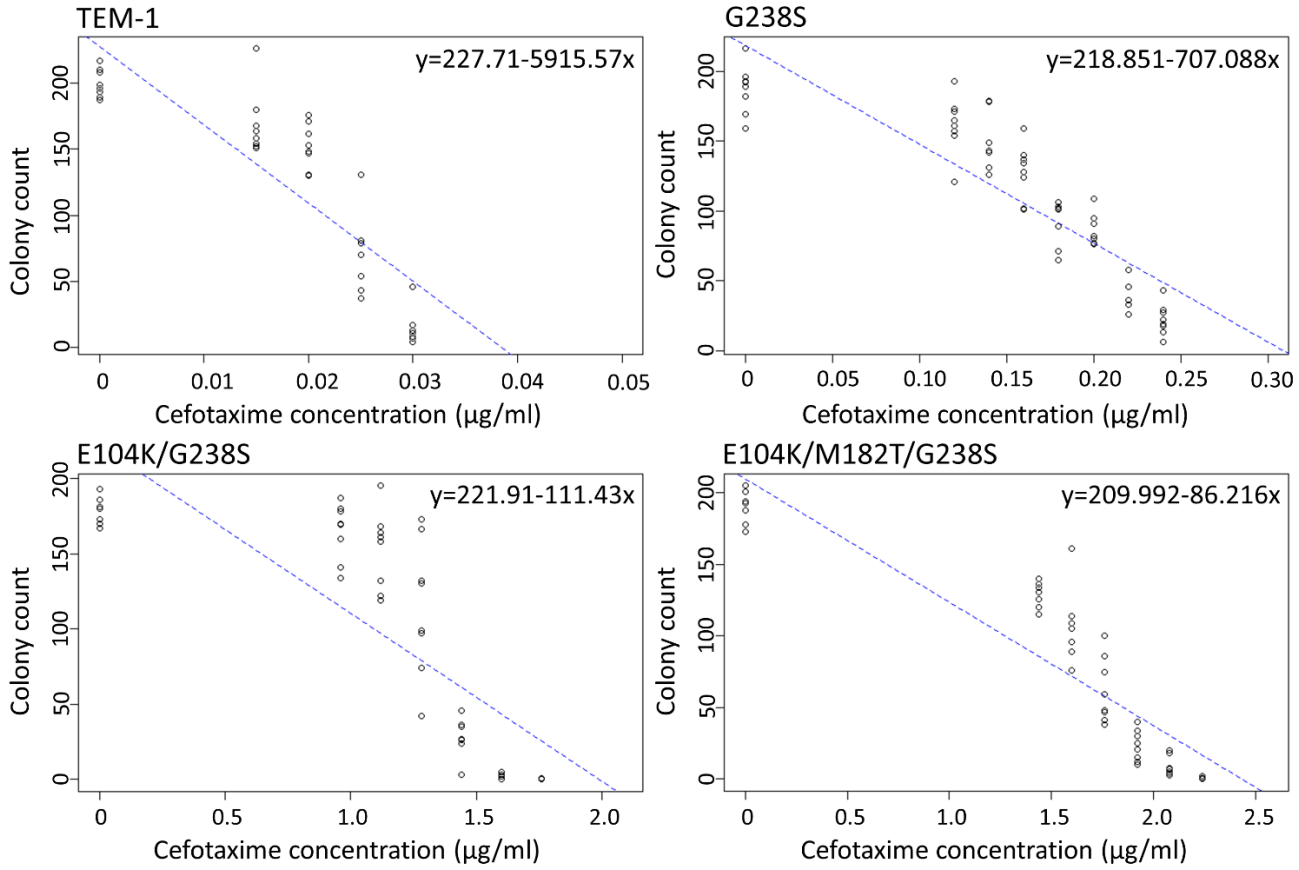

Figure 1. Number of colonies per plate for each tested strain plotted against cefotaxime concentration. The blue line indicates the linear regression. The formula of the line is noted on the right side of the graphs.

Table 3. Test statistics of the linear regressions on the cefotaxime concentration

Strain	Adjusted R-squared	F-statistic	p-value
TEM-1	0.7275	105.1	1.69e-09
G238S	0.7496	189.6	< 2.2e-16
E104K/G238S	0.6171	88.02	7.46e-13
E104K/M182T/G238S	0.8022	224.1	< 2.2e-16

4.2 Density effect assay

To investigate if cell density affected establishment, the 4 *E. coli* strains were spread over agar plates with a smaller or larger surface area, resulting in a high (5.3 cells/mm^2) or low (2.3 cells/mm^2) cell density respectively. Figure 2 shows that generally there is a tendency for colony numbers to be lower in the 5.3 cells/mm^2 treatment when compared to the 2.3 cells/mm^2 treatment. Tukey multiple comparison shows that the decrease is not significant in all cases. However, the decrease in colonies for the high cell density is consistent in all tested strains and concentrations, with the exception of the 0.025 µg/ml cefotaxime plates with the TEM strain. A significant interaction was found between plate size and cefotaxime concentration in

all strains, except for E104K/G238S (Supplement table 5-8). Within the 0 µg/ml cefotaxime and 5.3 cells/mm^2 treatment of the E104K/M182T/G238S strain, one replicate was discarded due to excessive colony overlap.

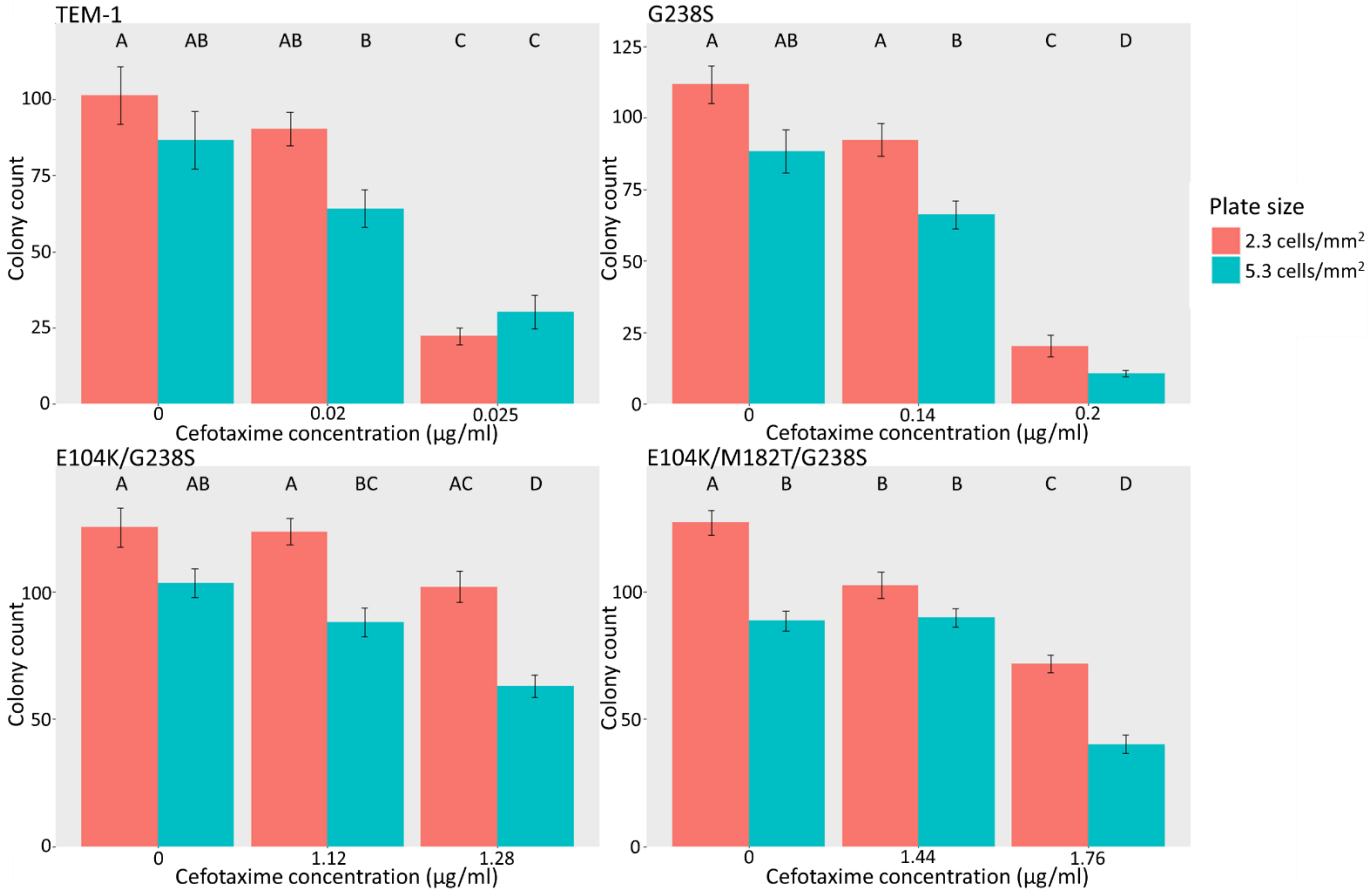

Figure 2. The average amount of colonies per plate for each strain for different cefotaxime concentrations and densities. The error bars indicate the standard error across replicates (N=10). Different letters signify that the treatments within strains were shown to be significantly different using a Tukey multiple comparison (p<0.05).

4.3. Wildtype interaction assay

To test whether the presence of susceptible wildtype affected the chance of successful establishment, different quantities of susceptible wildtype cells were added to plates containing resistant mutants.

Figure 3 shows that the addition of wildtype cells increased mutant colony number. According to Tukey multiple comparison, this increase was generally not significant for the addition of 1000 wildtype cells. However, in all instances adding 10^6 wildtype cells significantly increased mutant colony numbers compared to plates without wildtype. Though not always significant, increased wildtype cell density appears to generally increase mutant colony numbers. This effect is most apparent in the G238S on 0.2 µg/ml cefotaxime plates, where both treatments

increasing wildtype density resulted in higher mutant colony numbers compared to the lower wildtype densities.

When the plates were studied under the fluorescence microscope after incubation, generally there were no wildtype colonies found. The only exceptions were the 0.14 µg/ml cefotaxime treatment for the G238S strain, and the 1.76 µg/ml cefotaxime treatment for the E104K/M182T/G238S strain. In both cases there were wildtype colonies found in the 10^6 wildtype cells treatments. For the 1.76 µg/ml cefotaxime treatment containing the E104K/M182T/G238S strain, numerous small wildtype colonies were found. These colonies were small and not observed or included during the initial colony count. They were only observed during subsequent microscopy (Supplement figure 4). For the 0.14 µg/ml cefotaxime treatment with the G238S strain, a few larger wildtype colonies were found (Supplement figure 4). These were large enough to be perceived and counted as mutant colonies, but were subtracted from the colony count after microscopy confirmed them as wildtype colonies.

Within the 0.14 µg/ml treatment of G238S, one replicate of each wildtype density was discarded due to excessive colony overlap.

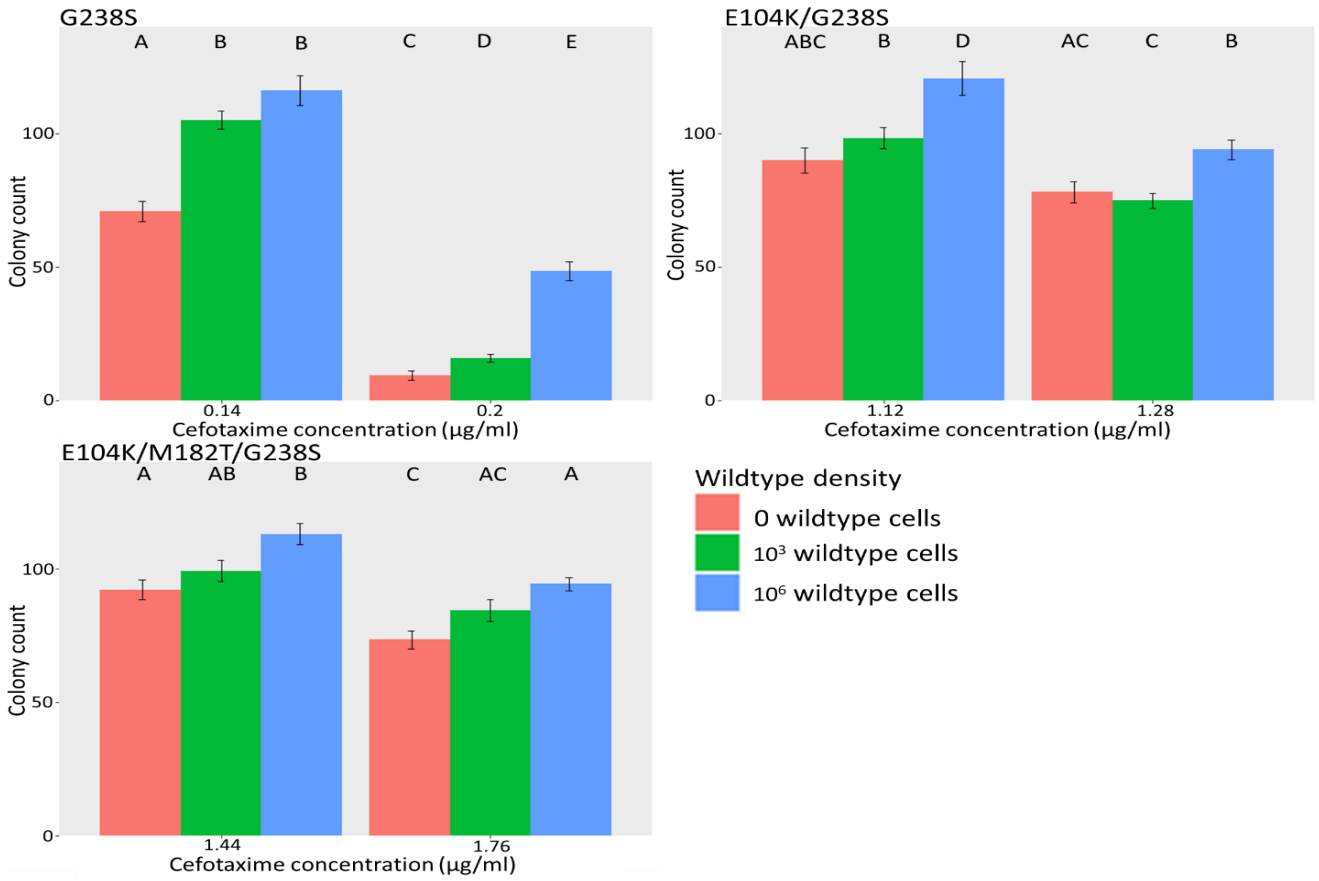

Figure 3. The average amount of mutant colonies per plate for each strain for different cefotaxime concentrations and wildtype densities. The error bars indicate the standard error of the treatment. Different letters signify that the treatments within strains were shown to be significantly different using Tukey multiple comparison.

5. Discussion

In this study the effects of cefotaxime concentration, cell density, and wildtype presence, on establishment were tested for different *E. coli* strains with varying cefotaxime resistances due to the expression of different mutant alleles of TEM β-lactamase. The growth of visible colonies, derived from single cells, was taken as indicator of successful establishment. As the colonies originated from a single cell, they would initially have a low number of cells, and would be vulnerable to extinction due random mortality. Thus the colonies could be considered as non-established populations during their initial growth. However, once the colonies reach a size that is observable during colony counting, they will easily number millions of cells, and will vastly exceed the population size vulnerable to extinction by random mortality. Colonies that were counted could thus be considered to have successfully survived through the establishment phase.

5.1. Cefotaxime concentration effect

Regression analyses confirm that there exists a negative relation between cefotaxime concentration and the number of observable colonies. Similar observations have been reported in liquid experiments (Alexander and Maclean 2018). Cefotaxime acts as a bactericidal antibiotic (Kjeldsen et al. 2015), and it has been observed that even sub-MIC cefotaxime concentrations lead to increases in cell death both for *Streptococcus pneumoniae* (George and Morrissey. 1997) and *E. coli* (Postek et al. 2018). Therefore, it is likely that cefotaxime causes random mortality and intensified population fluctuations, similar to what has been reported in *E. coli* by Coates et al. (2018). Such random mortality is likely to increase extinction chance (Coates et al. 2018), and consequently lowers the odds of success for establishing *de novo* mutants (Alexander and Maclean 2018). As mentioned previously, when antibiotic concentrations are increased, the antibiotic-induced random mortality also increases, resulting in an increased probability of population extinction (Coates et al. 2018). This would explain the negative relationship between colony number and cefotaxime concentration observed in the current study.

Furthermore, the results of the current experiment show that this observed negative relationship is less severe for strains with a higher resistance against cefotaxime, with these strains requiring higher cefotaxime concentrations in order to observe significant decreases in colony numbers.

In summary, the results of the cefotaxime concentration assay confirm the conclusion of Alexander and Maclean (2018) that sub-MIC antibiotic concentrations can significantly lower establishment chances of an antibiotic resistant strain. Furthermore, the magnitude of this establishment decrease, and the antibiotic concentrations required to achieve it, were shown

to be heavily dependent on the resistance of the strain in question, which makes intuitive sense.

5.2. Cell density effect

Initially it was expected that increased cell density would result in an increase in establishment. More specifically, since the β-lactamase enzymes break down the targeted antibiotic in their local area (Frost et al. 2018, Medaney et al. 2015), it was expected that many resistant colonies existing in close proximity would collectively break down local cefotaxime. This would effectively result in lower antibiotic concentrations, which has been proven to be beneficial for establishment and colony growth (Alexander and Maclean. 2018, Postek et al. 2018). However, instead the opposite was observed, increased cell density, decreased observable colony numbers.

A possible explanation could be that an increase in resource competition resulting from the increased density (Friesen et al. 2003, Ghoul and Mitri. 2016) was more impactful than the expected increase in cooperation. It has been reported that despite beneficial cooperation, competition often remains the dominant factor between different populations (Foster and Bell, 2012), and it can be expected that this would be especially likely under low resource conditions, such as the used M9 minimal medium. Additionally, it has been shown that an increased cell density can lead to an increased intercolony competition, causing slower, less stable, growth and smaller colonies (Malakar et al. 2000, Tack et al. 2015). This slow and unstable growth could decrease the odds of successful establishment, as populations would spend a prolonged time at a size vulnerable to extinction, and would have more difficulty overcoming any sudden mortality.

Another possibility is that the use of smaller plates interfered with accurate colony counting. Smaller plates make it possible to achieve higher cell densities using the same culture dilution, thus minimising the effects of pipetting errors. However, smaller plates also increase the likelihood that colonies start to overlap, or grow in the rim of the plate, both of which make accurately distinguishing individual colonies more difficult. This could result in multiple colonies being counted as only one, and thus provide a reduced number of counted colonies. If such errors occurred consistently, they could have caused the lower colony counts observed for the high density treatment.

5.3. Wildtype effect

The presence of wildtype cells was found to increase the number of visible mutant colonies. This result was unexpected, as the wildtype cells were expected to hamper establishment by

increasing competition, without significantly breaking down cefotaxime. Though the observed increase in colony numbers was initially unexpected, it could be argued to be in accordance with the frequently observed “inoculum effect” (Queenan et al. 2003, Udekwu et al. 2009, Lenhard and Bulman. 2019). The inoculum effect describes the phenomenon of increased initial cell number resulting in a decrease in antibiotic susceptibility (Lenhard and Bulman. 2019). The conditions of the wildtype assay could be described as an increase in cell number, and would thus be prone to the inoculum effect.

Here, several possible mechanisms will be given that could potentially explain the observed increase in mutant colonies due to the increase in wildtype number.
The first possible explanation could be the activity of the TEM-1 enzyme present in the wildtype reducing cefotaxime concentration. TEM-1 is inefficient at breaking down cefotaxime (Raquet et al. 1994, Schenk et al. 2012). But, since the wildtype was present in high density, this might have compensated for their relative inefficiency, and the high amount of TEM-1 enzyme present might still have significantly reduce the cefotaxime concentration. A lower antibiotic concentration would in turn result in an enhanced mutant establishment chance and colony growth (Alexander and Maclean. 2018, Coates et al. 2018, Postek et al. 2018). However, it has been reported that TEM-1 hyperproduction does not alter the resistance of *E. coli* against cefotaxime (Wu et al. 1994). Even if the strain in question produces 120-fold more TEM-1, there is no observable change in cefotaxime resistance (Wu et al. 1994). This suggests that the cefotaxime activity of TEM-1 is sufficiently low that, regardless of the amount of TEM-1 present, it has minimal effect on the cefotaxime concentration. Considering this, it seems unlikely that an increased density of TEM-1 expressing wildtype would significantly lower cefotaxime concentration.

Another possible explanation could be the covalent binding of cefotaxime to penicillin-binding proteins. This covalent binding disrupts the final process of peptidoglycan synthesis, crucial in bacterial cell wall formation (Johann et al. 1997, Kjeldsen et al. 2015). It might be possible that if a sufficiently large amount of wildtype cells are present, the local cefotaxime concentration is lowered due to covalently binding to the large number of penicillin-binding proteins. The relation between cell density and unbound antibiotic concentration has not been studied. However, the covalent binding of β-lactams to penicillin-binding proteins has been investigated through the use of fluorescent probes (Tiyanont et al. 2006, Kocaoglu and Carlson. 2013, Kocaoglu and Carlson. 2015). Using hypersusceptible *E. coli* (a strain with increased cell envelope β-lactam permeability), and a fluorescent probe specific for certain penicillin-binding proteins, Kocaoglu and Carlson (2013) demonstrated that when the cells were exposed to higher concentrations of β-lactam antibiotics, a smaller fraction of the probe would bind to the

penicillin-binding protein. This decrease in probe binding was attributed to the β-lactam antibiotic covalently binding to the penicillin-binding protein, excluding the probe from binding with the same penicillin-binding protein. The findings of Kocaoglu and Carlson (2013) confirm several of the assumptions required for cefotaxime binding to be a likely explanation. Firstly, it was found that within the range of cefotaxime concentrations used within the wildtype assay (0.14-1.76 µg/ml), binding of the probe on several penicillin-binding proteins was significantly reduced, confirming that at these concentrations binding of cefotaxime to these proteins takes place. Secondly, the findings suggest that this binding is not reversed, despite the cell lysis that likely occurred at the used cefotaxime concentrations. In other words, the findings of Kocaoglu and Carlson (2013) suggest that lowering the cefotaxime concentration using covalent binding in susceptible cells is possible, regardless of cell survival. However, it remains untested whether the extent of the resulting reduction in cefotaxime concentration would be sufficient to have a significant effect on establishment, and further study on this would be required.

Another possible cause for the observed increased establishment rate could be an increase in nutrients due to wildtype lysis. Exposure to cefotaxime can lead to the lysis of bacterial cells, causing their cell content to leak into the environment (Johann et al. 1997, Kjeldsen et al. 2015). The content of the lysed cell can subsequently be scavenged by surrounding surviving cells, enabling or enhancing cell growth (cryptic growth). To the best of my knowledge, no studies have specifically investigated cryptic growth in cells lysed by antibiotics. However, several studies report the occurrence of cryptic growth in cells lysed by sonication (Mason and Hamer. 1987, Banks and Bryers. 1990), nutrient starvation, and other factors (Koch. 1959, van Bruggen et al. 2000). Assuming lysis by cefotaxime is comparable to lysis by sonication, wildtype death would result in an influx of nutrients for the mutant, similar to the observations of Mason and Hamer (1987) and Banks and Bryers (1990). If the resulting influx of nutrients increased the mutant growth rate, then the benefitting mutant would reach an established population size faster, and would be more resistant to random mortality, thus increasing the chance of successful establishment.

Regardless of the underlying mechanism, the finding that non-resistant wildtype cells can benefit resistant mutant cells could have significant implications for current antibiotic resistance management concepts. For example, the idea of a “mutant selection window” (Drlica and Zhao. 2007, Firsov et al. 2017, Alexander and Maclean. 2018) posits that resistant mutants evolve within a predictable “window” of antibiotic concentrations. Mutant evolution is said to take place in concentrations between the wildtype MIC, and the concentration that effectively inhibits the mutant. Another concept, “competitive release” (Huijben et al. 2013, Birger et al. 2015), posits

that the clearance of wildtype cells by the antibiotic alleviates competition and thus the addition of antibiotic actually benefits the resistant mutant. Both these theories reflect a consensus that views wildtype cells only as a competitive force inhibiting the evolution of resistance. This consensus is contradicted by the findings of the wildtype assay, which suggest that the presence of wildtype cells can also significantly benefit the resistant mutants.

Within the wildtype interaction assay, several wildtype colonies were found during fluorescence microscopy in the 0.14 µg/ml cefotaxime treatment of the G238S strain and the 1.76 µg/ml cefotaxime treatment of the E104K/M182T/G238S strain (Supplement figure 4). It is a frequently observed phenomenon that antibiotic-sensitive strains can survive in the presence of many resistant strains, due to the antibiotic clearing activity of these resistant strains (Clark et al. 2009, Perlin et al. 2009, Medaney et al. 2016, Frost et al 2018). Such survival of susceptible cells is often attributed to persister cells, cells that lie dormant, enabling them to survive the exposure to antibiotics until it is cleared (Shah et al. 2006, Amato et al. 2013, Medaney et al. 2016). It seems likely that the observed wildtype colonies were the result of the growth of such persister cells. The fact that these wildtype colonies were only found in two treatments could be attributed to the longer incubation time of these treatments. This increased incubation time could have given more opportunity for cefotaxime breakdown and subsequent wildtype growth, which could explain the absence of wildtype in the majority of other treatments. However, the 0.2 µg/ml cefotaxime treatment also suffered from slow growth and was incubated equally long, yet no wildtype colonies were observed within this treatment. A possible explanation for the absence of persister colonies in the 0.2 µg/ml treatment could be that that the corresponding strain, G238S, degrades cefotaxime slower than the E104K/M182T/G238S strain used in the 1.76 µg/ml cefotaxime treatment. Thus, as the E104K/M182T/G238S strain is far more efficient at breaking down cefotaxime, it might have been able to reduce the concentration to levels tolerable to the wildtype faster, even if the initial concentration is far higher.

5.4. Density- and wildtype experiment contradiction

In the wildtype experiment, the increase in the number of wildtype cells, while maintaining the same surface area, could be considered an increase in total cell density. Considering this, there exists a striking contradiction between the wildtype experiment, where increasing cell density by adding wildtype increased establishment chance, and the density experiment, where increasing cell density by decreasing plate size reduced establishment chance. Here some possible explanations are given that could explain these contradicting result.

One potential explanation could be the covalent cefotaxime binding discussed previously. Even though cell density is increased, total cell numbers remain unchanged in the density

experiment treatments. Thus, there would be no change in the amount of cefotaxime that can be bound. In contrast, in the wildtype experiment, where the number of cells ranges from 100 to 10^6, there could be a large difference in the total cefotaxime bound.
However, it should also be considered that for the density experiment decreased plate size and medium volume were used to create the high cell density treatment. Therefore, as there is less medium, there also exists less total cefotaxime in the high cell density treatment.

Alternatively, the contrasting results could potentially be explained due to the influx of nutrients by wildtype cell death, and the resulting cryptic growth discussed previously. Cell density in the wildtype experiment is increased by the addition of a large number of wildtype cells. Furthermore, during the wildtype experiment, cefotaxime concentrations above the wildtype MIC were used in all treatments. In contrast, cell numbers in the density experiment remained the same between treatments, and cefotaxime concentrations were always below the MIC of the used strain. The cefotaxime concentrations being survivable for all present cells, and the lower total number of cells, would likely result in a smaller number of cell deaths in the density experiment, compared to the wildtype experiment. Therefore, there would likely be a far smaller influx of freed nutrients to be scavenged by surviving cells, which could explain the contrasting results of the wildtype experiment and the density experiment.

Another possible explanation would be that the use of smaller plates indeed led to measurement errors in the density experiment, causing lower colony counts. Since the wildtype experiment was performed using a single plate size, it would not be prone to these measurement errors. Furthermore, as the cell density within the wildtype experiment was increased 10^4-fold, as opposed to the 2-fold increase in the density experiment, any potential density effect would likely be far more pronounced in the wildtype experiment. Combining this, it might be possible that density experiment exhibited a similar, albeit smaller, positive density effect, but that this effect was lost due to measurement errors caused by using different plate sizes.

5.5. Conclusions

In summary, all three tested factors, i.e. cefotaxime concentration, cell density, and wildtype presence, significantly affected the establishment of antibiotic resistant *E. coli* cells. Antibiotic-induced mortality reducing establishment has previously been reported (Coates et al. 2018, Alexander and Maclean. 2018). The fact that antibiotic concentration has a negative relationship with establishment chance is of significant note when considering the evolution of resistance. As specified by the mutant selection window concept, antibiotic concentration should be high enough to allow for the selection of resistance, yet low enough to not completely

inhibit the strain. Alexander and Maclean (2018) added to this, by reporting that even at antibiotic concentrations far below the resistant MIC, effective inhibition of *de novo* resistant mutants is still achieved, as the establishment of these mutants is severely affected even at these relatively low concentrations. This means that the range of concentrations that favour the evolution of resistance is more narrow than is mostly assumed. The current study confirms the findings of Alexander and Maclean (2018), by showing that sub-MIC concentrations indeed severely diminishes resistant mutant establishment. Additionally, the current study utilized a different model organism (*E. coli* as opposed to *P. aeruginosa*), solid medium instead of liquid, and multiple strains with varying degrees of resistance. Thus showing that this reduction of resistance establishment when exposed to sub-MIC antibiotic concentrations, is also observable under completely different conditions.

Furthermore, the current study also shows that social factors, such as cell density and wildtype presence, also affect establishment. The finding that wildtype presence affects establishment is especially notable. The phenomenon of non-resistant cells benefiting from the breakdown of antibiotics by resistant cells is well documented (Clark et al. 2009, Perlin et al. 2009, Yurtsev et al. 2013, Medaney et al. 2016, Frost et al 2018). But the presence of wildtype cells being in any way beneficial to resistant cells is generally not considered. Furthermore, this benefit was even observed far above the wildtype MIC.

The findings of this study suggest that the presence of wildtype cells can potentially be of significant benefit to the establishment of resistant mutants. This contradicts the general idea within antibiotic resistance management that susceptible wildtype cells are primarily a competitive, and thus inhibiting, entity in the evolution of antibiotic resistance.

6. References

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7. Supplement

7.1. M9-cas general recipe per liter

Table 1. General recipe used for making a liter of M9-cas medium. For the recipe of the 5X M9 salts see table 2. 0.2 mg/ml cefotaxime solution was added to reach the desired final concentration of cefotaxime. If the desired concentration would require less than 10 µl of added 0.2 mg/ml solution, then 0.02 mg/ml solution was used instead. The total volume of cefotaxime solution was subtracted from the added water to avoid dilution of the medium.

Component	**Volume**
5X M9 salts	200 ml
1M $MgSO_4$	2 ml
20% Glucose	20 ml
0.1 M $CaCl_2.2H_2O$	1 ml
10% Casaminoacids	20 ml
2 mg/ml Uracil	1 ml
1 mg/ml Thiamin	1 ml
ddH_2O	755 ml - volume of cefotaxime solution
Agar	15g
50 mM IPTG	1 ml
0.02 mg/ml cefotaxime	Determined by desired cefotaxime concentration
0.2 mg/ml cefotaxime	Determined by desired cefotaxime concentration

Table 2. Recipe used for making 1 liter of 5X M9 salts for use in the M9-cas medium. After adding the specified salts, water was added until a total volume of 1 liter was reached.

Component	**Mass**
$Na_2HPO_4.2H_2O$	42.5g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5g

7.2. Statistical data

Table 3. Statistical data on the results of the cefotaxime concentration assay.

Strain	Cefotaxime concentration (µg/ml)	Number of Samples	Average colony count	Standard deviation	Standard error
TEM-1	0	8	199.9	10.7	3.8
TEM-1	0.015	8	169.1	24.9	8.8
TEM-1	0.02	8	152.3	16.9	6.0
TEM-1	0.025	8	70.6	29.3	10.3
TEM-1	0.03	8	14.3	13.4	4.8
G238S	0	8	187.0	17.4	6.2
G238S	0.12	8	161.9	20.5	7.2
G238S	0.14	8	148.9	19.7	7.0
G238S	0.16	8	128.1	19.4	6.9
G238S	0.18	8	87.8	18.0	6.4
G238S	0.2	8	85.8	11.8	4.2
G238S	0.22	8	38.4	9.6	3.4
G238S	0.24	8	22.1	11.2	4.0
E104K/G238S	0	8	179.5	8.9	3.2
E104K/G238S	0.96	8	164.8	18.8	6.7
E104K/G238S	1.12	8	152.3	26.0	9.2
E104K/G238S	1.28	7	124.4	36.8	13.9
E104K/G238S	1.44	8	32.7	8.3	2.9
E104K/G238S	1.6	8	23.8	1.4	0.50
E104K/G238S	1.76	8	0.125	0.35	0.13
E104K/M182T/G238S	0	8	188.8	11.6	4.1
E104K/M182T/G238S	1.44	8	129.1	8.4	3.0
E104K/M182T/G238S	1.6	8	105.8	25.3	9.0
E104K/M182T/G238S	1.76	8	61.8	22.8	8.1
E104K/M182T/G238S	1.92	8	23.4	10.8	3.8
E104K/M182T/G238S	2.08	8	0.875	0.83	0.3

Table 4. Statistical data on the results of the density assay.

Strain	Cefotaxime concentration (µg/ml)	Plate size	Number of Samples	Average colony count	Standard deviation	Standard error
TEM-1	0	60mm	10	86.7	29.9	9.4
TEM-1	0	92mm	10	101.4	30.1	9.5
TEM-1	0.02	60mm	10	64.2	19.2	6.1
TEM-1	0.02	92mm	10	90.4	17.6	5.6
TEM-1	0.025	60mm	10	30.2	176	5.6
TEM-1	0.025	92mm	10	22.2	8.5	2.7
G238S	0	60mm	10	88.4	23.9	7.6
G238S	0	92mm	10	111.7	20.9	6.6
G238S	0.14	60mm	10	66.1	15.8	5.0
G238S	0.14	92mm	10	92.3	18.0	5.7
G238S	0.2	60mm	10	10.7	3.6	1.1
G238S	0.2	92mm	10	20.2	11.8	3.7
E104K/G238S	0	60mm	10	103.3	1.8	5.6
E104K/G238S	0	92mm	10	125.1	2.4	7.7
E104K/G238S	1.12	60mm	10	88.1	1.8	5.6
E104K/G238S	1.12	92mm	10	123.4	1.6	5.2
E104K/G238S	1.28	60mm	10	62.9	1.4	4.4
E104K/G238S	1.28	92mm	10	101.9	1.9	6.0
E104K/M182T/G238S	0	60mm	9	88.6	1.2	3.9
E104K/M182T/G238S	0	92mm	10	127.0	1.5	4.9
E104K/M182T/G238S	1.44	60mm	10	89.7	1.1	3.5
E104K/M182T/G238S	1.44	92mm	10	102.5	1.7	5.2
E104K/M182T/G238S	1.76	60mm	10	40.2	1.2	3.7
E104K/M182T/G238S	1.76	92mm	10	71.8	1.1	3.5

Table 5. Statistical data on the results of the wildtype interaction assay.

Strain	Wildtype density (number of cells)	Cefotaxime concentration (µg/ml)	Number of Samples	Average colony count	Standard deviation	Standard error
G238S	0	0.14	14	70.9	14.6	3.9
G238S	10^3	0.14	14	105.1	12.8	3.4
G238S	10^6	0.14	14	116.1	21.0	5.6
G238S	0	0.2	15	9.3	6.5	1.7
G238S	10^3	0.2	15	15.8	5.61	1.4
G238S	10^6	0.2	15	48.5	14.0	3.6
E104K/G238S	0	1.12	15	89.9	18.4	4.8
E104K/G238S	10^3	1.12	15	98.4	15.4	4.0
E104K/G238S	10^6	1.12	15	120.7	24.7	6.4
E104K/G238S	0	1.28	15	78.1	15.37	3.9
E104K/G238S	10^3	1.28	15	74.9	10.5	2.7
E104K/G238S	10^6	1.28	15	94.1	14.3	3.7
E104K/M182T/G238S	0	1.44	15	92.1	14.2	3.7
E104K/M182T/G238S	10^3	1.44	15	99.3	15.6	4.0
E104K/M182T/G238S	10^6	1.44	15	113.1	15.2	3.9
E104K/M182T/G238S	0	1.76	15	73.5	13.3	3.4
E104K/M182T/G238S	10^3	1.76	15	84.4	16.4	4.2
E104K/M182T/G238S	10^6	1.76	15	94.3	9.8	2.5

Table 6. Two-way ANOVA data of the density effect assay. An asterisk indicates significance (p<0.05).

TEM-1	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	2	240.7	120.4	68.3	1.61e-15*
Plate size	1	4.6	4.6	2.6	0.113
Cefotaxime concentration and plate size interaction	2	12.5	6.2	3.5	0.036*
Residuals	54	95.1	1.76		
G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	2	427.1	213.5	211.5	< 2e-16*
Plate size	1	24.5	24.5	24.3	8.26e-06*
Cefotaxime concentration and plate size interaction	2	0.3	0.2	0.2	0.843
Residuals	54	54.5	1.0		
E104K/G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	2	30.0	15.0	17.1	1.74e-6*
Plate size	1	40.4	40.4	46.2	8.78e-9*
Cefotaxime concentration and plate size interaction	2	3.5	1.7	2.0	0.148
Residuals	54	47.27	0.88		
E104K/M182T/G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	2	99.7	49.8	93.9	< 2e-16*
Plate size	1	35.4	35.4	66.6	6.13e-11*
Cefotaxime concentration and plate size interaction	2	6.5	3.2	6.1	4.13e-3*
Residuals	54	28.1	0.5		

Table 7. Two-way ANOVA data of the wildtype interaction assay. An asterisk indicates significance (p<0.05).

G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	1	587.6	587.6	788.5	< 2e-16*
Wildtype density	2	149	74.5	100.0	< 2e-16*
Cefotaxime concentration wildtype density interaction	2	23.2	11.6	15.6	1.91e-6*
Residuals	81	60.4	0.7		
E104K/G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	1	24.9	24.9	32.2	1.93e-7*
Wildtype density	2	25.2	12.6	16.3	1.05e-7*
Cefotaxime concentration and wildtype density interaction	2	1.9	1.0	1.2	0.295
Residuals	84	64.8	0.8		
E104K/M182T/G238S	**Df**	**Sum Sq**	**Mean Sq**	**F-value**	**p-value**
Cefotaxime concentration	1	18.7	18.7	33.0	1.42e-7*
Wildtype density	2	18.3	9.1	16.2	1.14E-06
Cefotaxime concentration and wildtype density interaction	2	0.2	0.1	0.2	0.813
Residuals	84	47.5	0.6		

7.3. Persister colony images

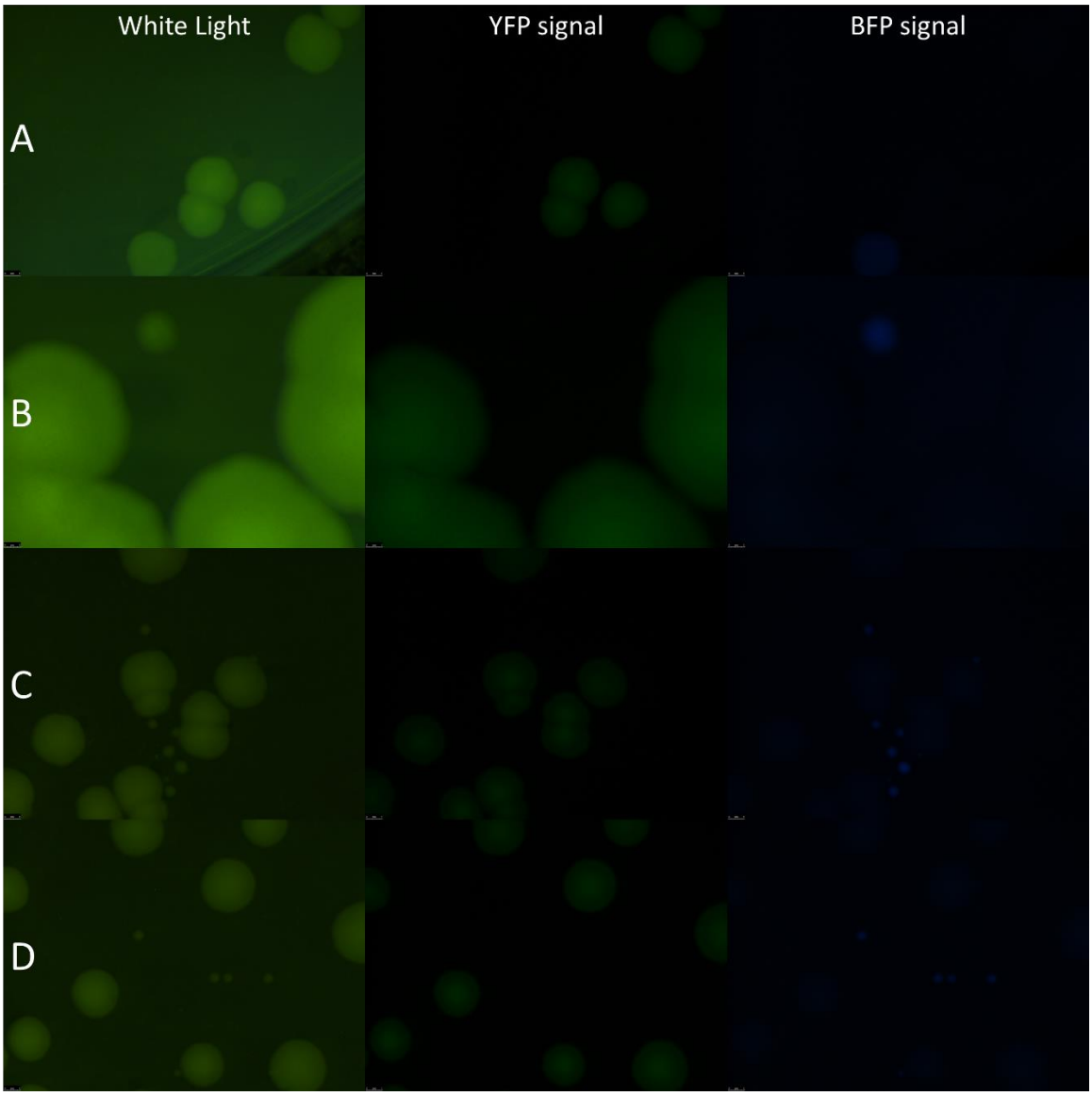

Figure 4. Examples of persister colonies observed in the G238S on 0.14 µg/ml cefotaxime (rows A and B) and the E104K/M182T/G238S on 1.76 µg/ml cefotaxime (rows C and D) treatments. Under white light all colonies are visible. For YFP signal only mutant colonies are visible, while for BFP signal only the TEM-1 wildtype is visible.