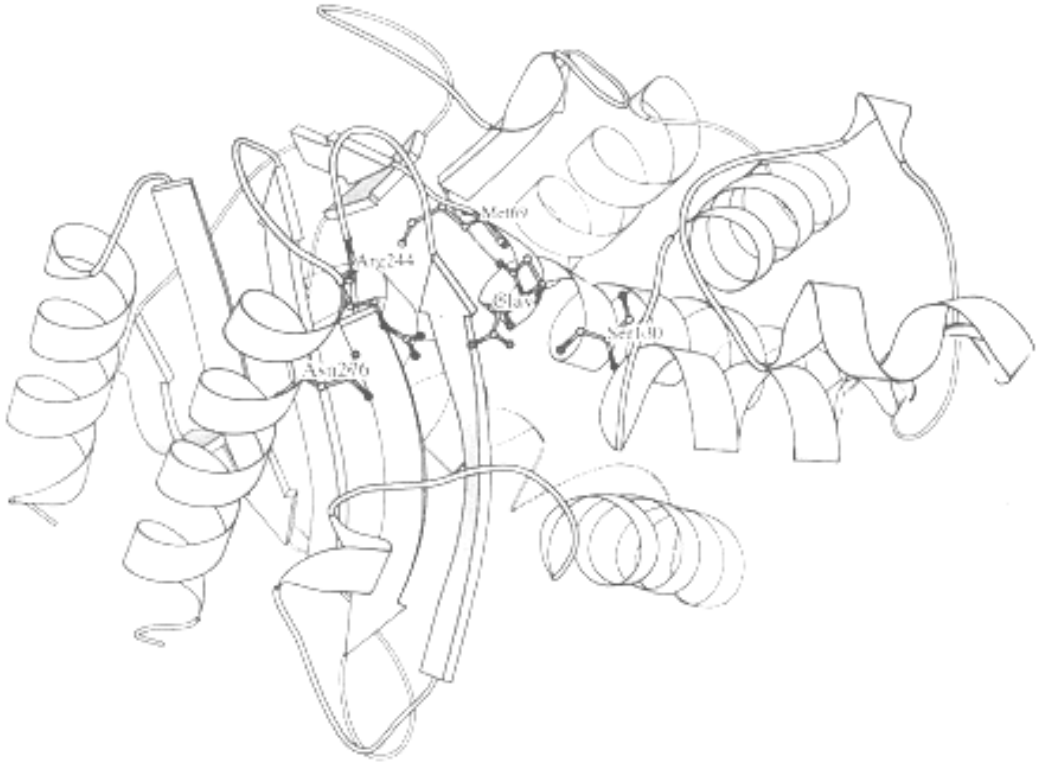
IN VITRO EVOLUTION OF TEM UNDER THE SELECTIVE PRESSURE OF MULTIPLE BETA-LACTAM ANTIBIOTICS



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Abstract

Resistant pathogens are an increasing problem worldwide and development of new antibiotics is lagging behind. The TEM encoded β-lactamase is one of the important enzymes involved in bacterial resistance against the frequently used β-lactam antibiotics. A few point mutations in the naturally occurring TEM-1 gene can increase resistance against modern β-lactams from practically nothing to high levels of resistance. In nature the enzyme is likely to evolve under the pressure of multiple antibiotics. In this study TEM-1 was evolved *in vitro* and selection for increased resistance against cefotaxime and ceftazidime was performed in single, combined and alternating antibiotic treatments. After each round of evolution the genotype as well as the resistant phenotype was established. Results show that different adaptive peaks are available for the two single antibiotics. Upon a combined pressure the same adaptive pathway is chosen as when selecting for ceftazidime alone and by this cefotaxime adaptation is hampered. Furthermore the results indicate that a combined antibiotic pressure as used in this experiment is a better alternative to alternating treatments that were previously used in selection experiments with multiple antibiotics. It gives rise to more natural combinations of mutations and the enzyme has less constrains for adaptation.

Introduction

Antibiotic resistance

The first antibiotic, named Penicillin, was discovered by Alexander Fleming (Fleming 1929) and was very effective against all sorts of infections and therefore even called 'the wonderdrug'. Almost immediately after people started to use the drug at high frequencies in the 1940's, resistant organisms were discovered (Treffers 1946). This drastically influenced medicine and medical research, because doctors had to revise their prescription strategies and scientists started a rat race to keep up with resistance by introducing new antibiotics (Barlow & Hall 2003 and Paterson & Bonomo 2005). Resistant bacteria are an increasing problem. The number of strains that are resistant to multiple antibiotics is increasing to the point where the possibility of untreatable infections becomes realistic (Paterson & Bonomo 2005, Demain & Sanchez 2009 and David & Daum 2010). Talbot *et al.* (2006) review the pipeline of new antimicrobial agents and conclude that not enough research is being done on new treatments and names ESBL producing organisms one of the most problematic groups of resistant pathogens.

To date, the β-lactam antibiotics, which include penicillins, carbapenems, cephalosporins and monobactams, are still the most frequently used antibiotics (see also Barlow & Hall 2002). Resistance to β-lactams often occurs through β-lactamases. These enzymes hydrolyze the β-lactam ring and thereby inactivate the antibiotic properties of the molecule (Poole 2004). Four classes of β-lactamases are known; CTX, metallo, SHV and TEM β-lactamases. TEM is common among micro-organisms and widespread around the world (Negri *et al.* 2000, Barlow & Hall 2002, Sun *et al.* 2009). The introduction of third-generation cephalosporins in the 1980's was seen as a major breakthrough in the fight against resistant organisms. The β-lactamases that were present at that time showed no activity against these new drugs (Paterson & Bonomo 2005). In 1984 however, the first extended spectrum β-lactamase (ESBL) encoded by TEM was discovered in a patient from France. This plasmid-borne enzyme had three substitutions (Philippon *et al.* 1989). For each existing β-lactam antibiotic there is at least one TEM variant that gives resistance among the 170 different variants that have been discovered ever since (Salverda *et al.* 2010). These variants carry one to five substitutions that confer structural differences in the enzyme that enable effective inactivation of the antibiotics (Paterson & Bonomo 2005, Weinreich *et al.* 2006 and Salverda *et al.* 2010).

Selection for increased resistance

To help the development of new antibiotics that are less susceptible to resistance one can mimic the natural evolution of TEM in the laboratory in order to predict the evolution of resistance (Baquero *et al.* 1998). Previous experiments have shown that, in principle, this is possible by running multiple rounds of *in vitro* evolution (see *e.g.* Barlow & Hall 2002 and Salverda et al. 2011). The majority of substitutions found in these selection experiments have also been encountered in clinical TEM isolates (Salverda *et al.* 2010). In most experiments TEM-1 is used as a starting point for evolution. This allele is highly resistant to penicillins and early cephalosporins. When selecting for increased resistance against different β-lactams, different mutations are selected in the gene. For example, when Blazquez *et al.* (2000) selected for increased Ceftazidime resistance they found that most selected lines obtained a histamine or a serine instead of an arginine at position 164 in the enzyme, but when Salverda *et al.* (2011) selected for increased Cefotaxime resistance they found that the glycine at position 238 was substituted by a serine in the majority of the lines.

When bacteria encounter multiple antibiotics in their environment the situation may be quite different. Adaptations that are normally selected for the single antibiotics might be less beneficial or even deleterious in the presence of an additional antibiotic. In addition, once the enzyme has specialized to some degree to a particular antibiotic, this may hamper adaptation to another. So far, most laboratory experiments are done under the selective pressure of a single antibiotic (see Salverda *et al.* 2010). In nature it is, however, more likely that bacteria are exposed to a variety of antibiotic (Novais *et al.* 2010). This is especially true in clinical settings such as hospitals where antibiotic use is high (Baquero *et al.* 1998, Weinreich *et al.* 2006, Novais *et al.* 2008). Experiments that did use multiple antibiotics conclude that selection with multiple antibiotics gives rise to more natural TEM variants and thus that this better mimics the natural environment in which the enzyme evolves (Blazquez 2000 and Barlow & Hall 2002). However, in these experiments no additional genetic variation was introduced between the exposure to the different antibiotics. Furthermore, the TEM gene was sequenced only after the final selection step and not in-between exposure to the different antibiotics, making it impossible to relate the occurrence of mutations to a specific antibiotic. Upon the exposure to multiple antibiotics - either in alternating fashion or at the same time - two evolutionary interactions play a key role, namely epistasis and pleiotropy.

Fitness peaks, mutational pathways and epistasis

Development of resistance for β-lactams occurs through the acquisition of mutations in the TEM gene followed by selection. Resistant bacteria often have multiple mutations in their TEM gene. Because of the relatively low error rate of bacterial DNA-polymerases it is unlikely that two beneficial mutations arise in the same gene at once (Gillespie 1984), so each mutation goes to fixation before the next beneficial mutation occurs. A mutational pathway is a collection of subsequent mutations that connect the wild type to a high fitness phenotype. For a pathway of two mutations there are in principle two possible connections, either mutation A is fixed first followed by mutation B or the other way around. Whether all pathways are accessible is influenced by epistasis. When the fitness effect of a mutation depends on the genetic background, this is called epistasis, different types of epistasis exist (Figure 1) each having a different effect on accessibility of pathways and selection.

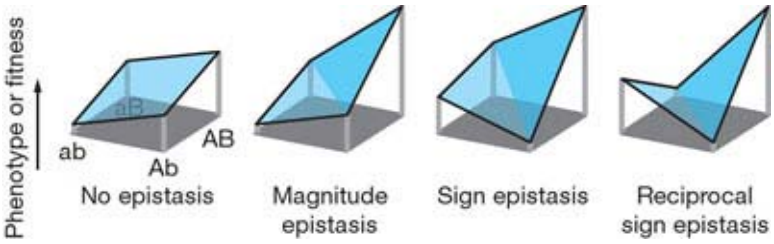


Figure 1 **Types of epistatic interactions**

No epistasis: both mutations are beneficial on their own; the combination of two mutations gives a benefit as high as the product of the effects of the two single mutations. Magnitude epistasis: the combination of two mutations gives a higher or lower effect than the sum of the product single mutations. Sign epistasis: a mutation is beneficial in one background, but deleterious in another. Reciprocal sign epistasis: Two mutations are beneficial on their own, but deleterious in each other's background. (Figure taken from: Poelwijk *et al.* 2007)

Magnitude epistasis does not limit accessibility. In the case of magnitude epistasis, both mutation A and B are beneficial in the original background. The combination of the two mutations however, has a higher or lower fitness then the product of the two single mutations together. This form of epistasis affects the selection pressure upon certain combinations of mutations and therefore the likelihood of fixation. Accessibility is influenced by sign epistasis. Sign epistasis occurs when mutations are beneficial in some genetic backgrounds, but deleterious in others (Weinreich *et al.* 2005, Weinreich *et al.* 2006). For example, consider a mutation A, which does not increase resistance in the wild type background, while mutation B does. Mutation B can therefore be selected. When mutation A is beneficial in the background of B it can be selected in the next adaptive step. In this case only one mutational pathway is accessible. Reciprocal sign epistasis is a special case of sign epistasis in which two mutations are deleterious in each other's background. Reciprocal sign epistasis is a prerequisite for having multiple peaks within a fitness landscape (Poelwijk *et al.* 2011), as is the case with TEM (Salverda *et al.* 2011). Because of these interactions a system can get stuck in a fitness peak that is lower than the global optimum because the acquired mutations make the pathway towards the highest fitness peak inaccessible. Epistasis will likely play the biggest role when the selective pressure is altered during the experiment. When there has been selection for one agent the gene has taken mutational steps towards a certain peak, when the selective agent is then changed the peaks for this agent might not be accessible due to the previous acquired mutations in the background.

Pleiotropy

Pleiotropy occurs when one genetic property has an effect on multiple phenotypic traits. When TEM evolves in an environment with multiple β-lactam antibiotics there are likely to be such pleiotropic effects. The TEM gene is then involved in the resistant phenotype against both antibiotics. In an experiment involving multiple antibiotics the level of resistance can be lower than in experiments where there is only one antibiotic present. This difference will be a measure for pleiotropic constrains. When the different antibiotics are introduced in an alternating fashion and not at the same time it is a matter of opinion if one can speak of pleiotropic effects. When one considers the alternating antibiotic pressures part of the same environment pleiotropy does play a role in this evolution. But considering the fact that the subsequent steps in the mutational trajectories are taken under different conditions I would say that only epistatic interactions play a role in this case.

Pleiotropic effects can also play a role within the enzymes mutations. When one mutation has an effect on two traits of the enzyme, for example; enhancing binding of the molecule to the binding pocket but at the same time decreases the stability of the enzyme this can be considered pleiotropy (Camps *et al.* 2007). Subsequent compensatory mutations can restore the negative effects of the previous adaptive mutation by increase stability again.

To what extent TEM is able to adapt to different β-lactams at the same time depends strongly on the structure of the antibiotic. Penicillin (PEN) is an antibiotic that can be hydrolysed by the naturally occurring β-lactamase TEM-1 and serves as a reference molecule. In this study, I used Cefotaxime (CTX) and Ceftazidime (CAZ), while a pilot study was performed with Aztreonam (AZT). These are all β-lactam antibiotics that are frequently used in a clinical setting (see also Barlow & Hall 2002). These antibiotics all share the β-lactam ring, but they belong to different subclasses based on the core ring structure (Figure 2). CAZ and CTX are third-generation cephalosporins and AZT is a monobactam. CAZ, CTX and AZT have a more complex structure than PEN. All three share side group A, but CAZ and AZT have an extra group C attached. CAZ and CTX share side group B but have different side groups D.

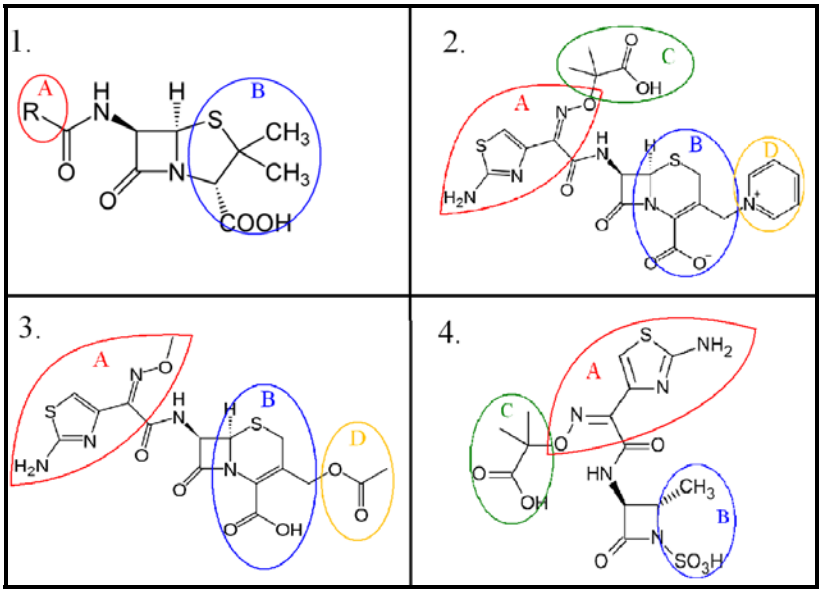


Figure 2 **Molecular structure of β-lactam antibiotics**

1. Penicillin (PEN); 2. Ceftazidime (CAZ); 3. Cefotaxime (CTX); 4. Aztreonam (AZT). Side groups are marked with coloured circles. Side group A is identical in the three modern antibiotics, but CAZ and AZT have an extra side group C attached to it. Side group B is identical in CAZ and CTX, but they differ with regard to side group D.

Research aim

The aim is to study to what extent epistasis and pleiotropy constrain evolution and what evolutionary pathways can be taken under the selective pressure of two β-lactam antibiotics. To achieve this I compared five treatments, selection was either performed with a single antibiotic (CTX or CAZ), a combination of these antibiotics or alternation between these antibiotics, using either CTX or CAZ as the first selective agent. I established the evolutionary pathways by sequencing after each round of selection. I also determined the phenotype by measuring the resistance levels against CTX and CAZ for all variants. This gives a complete overview of the adaptive process and indicates which fitness peaks can be reached under the different conditions. This study shows that different adaptive peaks are available for the two antibiotics and when the antibiotics are combined CTX adaptation is hampered because the same selective pathway is chosen as when selecting for CAZ alone. The outcomes furthermore indicate that when comparing alternating and combined selective pressure the alternating treatment gives results that are less likely to occur in nature and that adaptation is hampered to a greater extent. This leads to the conclusion that selection experiments with multiple antibiotics should be performed with a combination treatment rather than the alternating treatment that was used in all previous studies.

Material and Methods

The *in vitro* evolution of plasmid-borne TEM upon exposure to the β-lactam antibiotics CAZ and CTX was studied using *E. coli* as a host. Mutations were randomly introduced by error-prone PCR. Mutated libraries were created and genotypes with an increased resistance were selected in a selection series with increasing antibiotic concentrations. The plasmids of bacteria that grew at the highest concentration were isolated and the TEM gene was sequenced. Resistance levels towards both antibiotics were determined in a MIC assay for each selected genotype. Three rounds of selection were performed for each treatment. The experiments are based on the protocol which was first used by Barlow & Hall (2002), and which was adjusted by Salverda *et al.* (2011). A few minor adjustments were made to this protocol as indicated below. The complete protocol is enclosed in Appendix I.

Treatments

Five treatments were used to select for increased resistance. The selection series with 2-fold increasing antibiotic concentrations were prepared with the following antibiotics: 1. CTX, 2. CAZ, 3. combination CTX+CAZ (ratio 1:8), 4. alternating starting with CTX, 5. alternating starting with CAZ. For the alternating experiments 4 rounds of selection would have been ideal since there would be an equal number of rounds for each antibiotic, because of time constrains only 3 rounds were performed. In the first two treatments bacteria were exposed to a single antibiotic. Bacteria were exposed to two antibiotics at the same time in the third treatment. The initial MIC of TEM-1 for CAZ was 8 times higher than the MIC of CTX (0.0625 mg/ml for CTX and 0.5 mg/ml for CAZ). To have a similar selection pressure in the combination experiment for both antibiotics a ratio CTX:CAZ of 1:8 was used in the bottles of the selection series. The fourth and fifth treatment involved a fluctuating antibiotic pressure, alternating between the two different antibiotics in the rounds of selection. Each treatment was used in an experiment with six independent replicate lines that were started with six independent libraries. Prior to the main experiment, I ran a pilot study (data not shown) using one round of selection with AZT to determine which antibiotic (AZT or CAZ) would direct evolution in a pathway most different to that of CTX. Upon this pilot I chose CAZ as the antibiotic that will cause the most constrains for evolution in combination with CTX.

Media

LB medium was used for culturing and contained per litre 10 g trypticase peptone, 5 g yeast extract, and 5 g NaCl. LB agar was used for plating and contained LB medium with 15 g of agar per litre. LB-tet medium contained 10 mg tetracycline per litre LB medium. Mueller-Hinton I (MH) broth was used in the selection series. Mueller-Hinton II (MH II) broth was used in the MIC assays. Both MH and MH II were prepared according to manufacturer's instructions.

Bacterial strain and plasmids

Escherichia coli strain DH5αE was used as a host for all plasmids. This strain lacks a chromosomal β-lactamase gene. pACSE3 was used as vector for the expression of the different TEM variants. TEM was cloned into pACSE3 and was expressed using the pTac promoter, which is regulated by the lac repressor. This repressor is encoded by the *lacI* gene on the same plasmid. Expression of TEM was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) because this molecule binds the repressor and inactivates it. The plasmid also carries a tetracycline resistance gene, allowing for selection of plasmid-carrying bacteria by adding tetracycline to media.

Mutagenesis

Error-prone PCR using primers P3 (TCATCCGGCTCGTATAATGTGTGGA) and P4 (ATGATAGCGCCCGGAAGAGAGT) and a high error rate polymerase called Mutazyme II (Stratagene) was used to introduce mutations in TEM. PCR products were purified using a PCR clean-up kit (Sigma) according to the manufacturer's instructions. The number of mutations that were introduced per amplicon (mutation rate) was determined by sequencing 24 randomly picked colonies of a mutant library before selection (created with TEM-1 as starting point) and by counting the number of introduced transitions and transversions. In total 18 transitions and 17 transversions were found (data not shown). On average 1.46 mutations were introduced per amplicon. The TEM genes of experiments CTX, CAZ, COM and ALT starting with CTX had on average 2.42 mutations per gene in each round (including non-synonymous and synonymous mutations). Data on the synonymous mutations are shown in Appendix II, data on synonymous mutations were not available for the alternating experiment starting with CAZ.

Ligation

PCR products were digested with BspHI, DpnI and SacI for an hour at 37ºC. The digestion mixture was purified with a PCR clean-up kit (Sigma) according to the manufacturer's instructions. The DNA concentration was determined using gels or nanodrop. The equivalent of 154 ng of product was ligated into 288 ng of vector using T4 ligase. pACSE3 was used as vector and was made by digesting pACSE3 plasmid with SacI and BspHI, then phosphorylating the ends with the enzyme CIP. This made the ends unsuitable for ligation, ligation of the vector without an insert is thereby prohibited. The resulting plasmid was purified by precipitation.

Mutant libraries

The plasmids harbouring the different TEM variants were transformed into *E. coli* cells using electroporation, thus creating libraries of cells with different TEM variants. After electroporation, cells recovered in 2 ml of SOC medium for 90 min at 37ºC. Cells were then added to 500 ml flasks of LB-tet. To calculate library sizes, 50 µl was immediately taken out of the flask and plated in duplicate on LB-tet agar plates. Flasks and plates were incubated overnight (O/N) at 37ºC. Colonies counts were used to determine library size. The average library size over all experiments was 8.3×10^5 cells.

Selection

The libraries were exposed to antibiotics in a selection series of glass bottles containing MH medium with 2-fold increasing antibiotic concentration and 50 µM of IPTG. The equivalent of 10x the library size was added to each bottle to ensure that each TEM variant was present in each bottle at least once. Bacteria from the bottle with the highest antibiotic concentration where growth was still observed were plated on LB-tet agar and grown O/N. One colony was picked and grown O/N in LB tet. Plasmids were subsequently extracted using a miniprep kit (Sigma) according to manufacturer's instructions. DNA concentration was determined and the equivalent of 1 µg plasmid was dried using speedvac and send away for sequencing with Illumina HiSeq 2000 technology (for more information visit www.eurofinsdna.com). MEGA4 software was used to align the evolved sequences with the wild type TEM-1 sequence and to identify the incorporated mutations. Plasmids were stored at -20°C. This plasmid was used as template for the error-prone PCR in the next round of evolution to introduce additional mutations. Three rounds of selection were performed for all treatments except CAZ, not all

replicates were completed for each round due to contamination or failed sequencing of the product.

Resistance measurements

To characterize the genotypes phenotypically Minimal Inhibitory Concentration (MIC) assays were performed. Prior to these assays all plasmids were transformed into a fresh isogenic background to eliminate potential chromosomal mutations in the selected isolates. MIC assays were run in triplicate in 96-well plates containing MHII medium with a 2-fold increase of antibiotic concentration ranging from 0 to 4096 µg/ml. 50µM of IPTG was added and bacteria were grown O/N at 37°C. Growth was scored by eye. The MIC indicates the lowest antibiotic concentration that gave no visible growth. I determined the resistance levels for both CTX and CAZ.

Results

Incorporated substitutions

Table 1 shows which non-synonymous substitutions were incorporated in the TEM genes after each subsequent round of selection for increased resistance. Numbering of amino acids residues corresponds to the numbering as described by Sutcliffe (1978). During the three rounds of evolution, the number of incorporated mutations decreased from 2.3 substitutions in the first round to 1.7 and 1.2 in the second and third round, respectively. A table of the synonymous mutations that were incorporated is included in Appendix II. Some lines failed to yield results, because either the sequencing failed or because the selection lines became contaminated with other resistant variants. The synonymous mutations are especially helpful in identifying such contaminations. Which results are missing is also shown in Table 1.

Table 2 shows how often the selected amino acid replacements were found in other experimental evolution studies and in clinical isolates. In the 28 lines of my five experiments 60 different substitutions were found of which 16 have been found in both clinical and laboratory isolates, 16 in laboratories, 1 has been described in a clinical isolate only and 27 have not been described (Salverda *et al.* 2010). The substitutions found most frequently in these experiments have all been found in both clinical and lab experiments.

Table 1 **Incorporated substitutions**

The substitutions incorporated into TEM after the first round (green), the second round (blue) and the third round (red). The line numbers indicated with * do not have results for the third round, line numbers indicated with ** do not have results for the second and third round as is shown at the bottom of the table when sequence is indicated with 'Failed' when the line failed to produce a sequence or with 'Cont.' when the line was found to be contaminated. For the Ceftazidime experiment only 2 rounds of selection were performed. Amino acid numbering as in Sutcliffe (1978).

Treatment	Cefotaxime						Ceftazidime						Combination						Alternating CTX-CAZ-CTX				Alternating CAZ-CTX-CAZ					
Line	1	2	3	4	5*	6	1*	2*	3*	4**	5*	6*	1	2	3*	4	5	6*	1	2	3*	4	1	2	3*	4**	5**	6
Amino acid position 10-20																												
20-30				F19Y					I11F			P27L																
30-40																	D35N											
40-50	A42G	L49M																	A42G			I47V		L49M				
50-60						F60Y					L51I				F60Y		E58G											
60-70					P62A																							
70-80																												
80-90																												
90-100		N100K	H96Q			H96Y					Y97F												G92D					
100-110	E104K	E104K	E104K	E104K	E104K	E104K	E104K		E104K		E104K	E104K	E104K	E104K			E104K			E104K	E104K	E104K						
													K104R			E110K												
110-120																		R120S							L113H			
120-130																												
130-140															T140I									T140I				
140-150																								I140V				
150-160				H153R																								
160-170							R164H		R164H	R164S	R164H	R164H		R164S	R164C	R164S	R164H	R164S		R164S	R164S	R164S	R164H	R164H	D163G	R164S		R164S
																P167T	P167L					S164R						
170-180								N175D									A172D			A172D								
								D179G							I173V		I173V							P174S			D179G	I173V
180-190	M182T							M182T				M182T				M182T	M182T				M182T				M182T			
								T189M																				
190-200			L198V				R191H									T200A												
200-210																A200T						L201V						
210-220																V216I												
220-230		A224V	A227V																									
230-240	G238S	G238S	G238S	G238S	G238S	G238S													G238S	G238S	G238S	G238S						
240-250										E240K					E240K	E240V		E240V	E240K				E240K	E240K			E240A	E240K
													R241P	R241G										K240R				
250-260																												
260-270		T265M		T265M	T265M	T265M					S266G			G267R		S268N							G267E					
270-280			R275V			T271S							R275L													T271S	R275L	
280-290								I282M																				
Sequence:																												
Round 1	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
Round 2	OK	OK	OK	OK	OK	OK	OK	OK	OK	failed	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	cont.	cont.	cont.	OK
Round 3	OK	OK	OK	OK	failed	OK							OK	OK	failed	OK	OK	cont.	OK	OK	failed	OK	OK	OK	cont.	cont.	cont.	OK

Table 2 **List of substitutions**

The identified substitutions and the number of times they were encountered in this experiment, in laboratory isolates and in clinical isolates as described in Salverda *et al.* (2010). Substitutions at the same position are grouped with a lined box.

Substitution	Number of times found		
	These experimens	Lab isolates	Clinical isolates
I11F	1		
F19Y	1		
P27L	1	1	
D35N	1	1	
A42G	2	6	
I47V	1	5	
L49M	2	3	1
L51I	1	1	
E58G	1	3	
F60Y	2		
P62A	1		
G92D	1	4	2
H96Q	1		
H96Y	1	2	
Y97F	1		
N100K	1	2	
E104R	1		
E104K	16	46	46
E110K	1		
L113H	1		
R120S	1	3	
T140I	2		
T140V	1		
H153R	1	10	3
D163G	1	1	1
R164H	7	17	19
R164S	9	21	30
R164C	1	3	4
P167T	1		
P167L	1		

Substitution	Number of times found		
	These experiments	Lab isolates	Clinical isolates
A172D	2		
I173V	3	13	1
P174S	1		
N175D	1	5	
D179G	2	4	
M182T	7	41	24
T189M	1		
R191H	1	4	
L198V	1	1	
T200A	1		
L201V	1		
V216I	1	1	
A224V	1	11	3
A227V	1		
G238S	10	60	34
E240K	6	17	27
E240R	1		1
E240V	2	1	1
E240A	1		
R241P	1		
R241G	1		
T265M	4	13	23
S266G	1		
G267E	1		
G267R	1	2	
S268N	1	1	
T271S	2		
R275L	2	3	3
R275V	1		
I282M	1		

Figure 3 shows the resistance levels as acquired from the MIC assays. Not all assays gave results due to contamination or poor quality plasmid. The adaptation as obtained in the different treatments is depicted in Figure 4, the average number of two-fold increments in resistance relative to that of TEM-1 is shown for both antibiotics.

Selection with a single antibiotic

These experiments serve as reference for what is possible when there is only one selective agent. The CTX experiment is done with only CTX as selective agent in all three rounds and the CAZ experiment is done with only CAZ selection. These results are compared to the combination and alternating experiments to determine the evolutionary constrains and mutational pathways that are taken. The third round of evolution was not performed for the CAZ experiment since the resistance level for CAZ after the second round already approached the maximum concentration that can be created in a selection series.

The resistance measurements indicate that resistance levels increase for both CTX and CAZ after each successive round of selection in both experiments (Figure 3). However, in the CTX experiment the adaptation for CTX is higher than that for CAZ and the reverse was seen in the CAZ experiment, where CAZ adaptation is higher (figure 4).

The genotypes that arise after selection with CTX or CAZ differ. The substitutions G238S, E104K and T265M were the most frequently encountered after selection with CTX (Table 1). G238S and E104K were actually found in all lines. In the CAZ experiment positions R164 and E104 mutated most often. Only 2 substitutions were shared among the mutants of both experiments, E104K and M182T. This suggests that de main adaptive peak differs between CTX and CAZ, which is necessary condition for the experiments to be useful. Had both antibiotics lead to the same adaptive peak, there is no conflict and one would expect to find the same adaptive peak in the combination and alternating experiments also.

Combination experiment

Selection with two antibiotics simultaneously also results in a steady increase in resistance after three successive rounds of selection (Figure 3). The resistance towards CAZ was higher in all lines compared to resistance towards CTX with the highest CAZ MIC at 2048 µg/ml and the highest MIC for CTX at 64 µg/ml. Adaptation as shown in Figure 4 increased in each round for both antibiotics. CTX adaptation remained lower than CAZ adaptation. Compared to the single

antibiotic experiment CAZ adaptation in this experiment eventually reaches the same level but CTX adaptation remains lower than in the single CTX experiment.

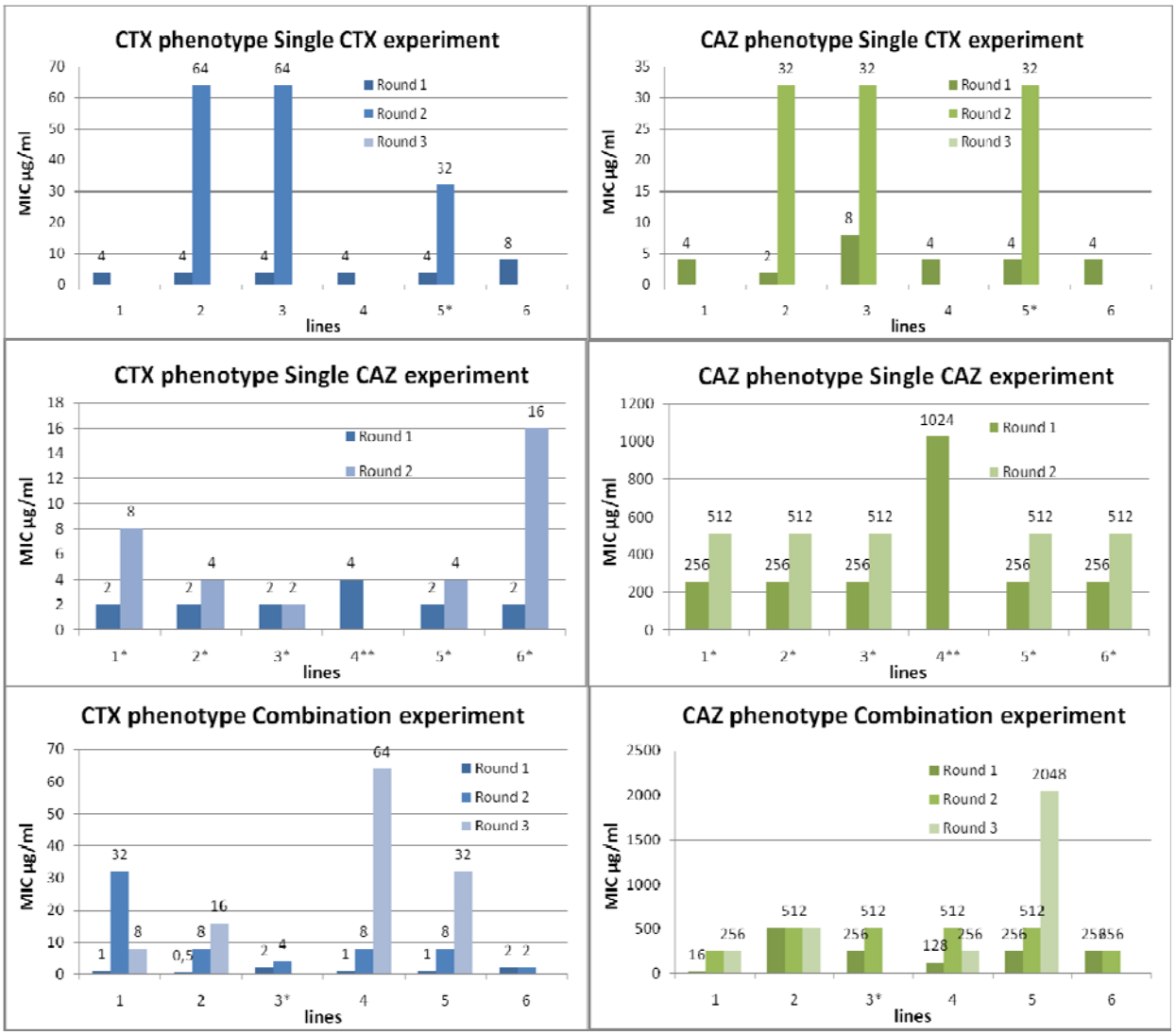
Looking at the genotypic results for the combination experiment some similarities and differences could be seen with the single antibiotic experiments. For example the combination experiment gave rise to many mutations at position R164, none at position G238, and some at position E240, these results are very similar to the single CAZ experiment. The only mutations shared with the CTX experiment were F60Y and at position R275. Not surprisingly, mutations that were found in both the CTX and CAZ experiments like E104K and M182T were also found in the combination experiment. Lines 4 and 5 of this experiment had the highest MICs and are therefore most successfully adapted to both antibiotics. These two lines share substitutions M182T and a mutation at position P167. M182T is known to have a strong effect and this experiment shows that it can be an important mutation for adaptation to both antibiotics. Mutations at position P167 have not been previously described but given the fact that it appeared twice in this experiment and that it is associated with high MICs could suggest that this mutation is a specific adaptation towards both antibiotics.

Alternating experiments

The alternating selection was either started with CTX or CAZ. MIC results for both experiments are shown in Figure 3. CAZ MICs in both experiments were higher than CTX MICs. Selection did not always result in an increased resistance for both antibiotics. For example when starting with CTX, the second round of selection was performed with CAZ and this lead on average to a decrease in CTX resistance. The same way average resistance for CAZ decreases in the third round when selecting for CTX. Interestingly, the reverse is seen in the other alternating experiment, starting with CAZ selection. In this case average CAZ resistance increases when selecting for CTX in the second round and a decrease is measured in the third round when selection shifted to CAZ.

In both experiments the first rounds gave rise to similar mutations as in the single experiments, G238S was selected in all 4 lines when selection started with CTX and 4 out of 6 lines gained a mutation at position R164 in the experiment started with CAZ. Interestingly, mutation T265M is not encountered in this experiment whereas half of the lines had this

mutation in the first round of the CTX experiment. When selection pressure altered in the second round 3 out of 4 lines in the first experiment gained R164S in addition to G238S. Lines from the experiment started with CAZ did not gain specific mutations for CTX like G238S in the second round, but other mutations such as at position E240 were selected. In the third round half of the lines did not gain any substitutions and line 4 of the first experiment even lost mutation R164S in this round.



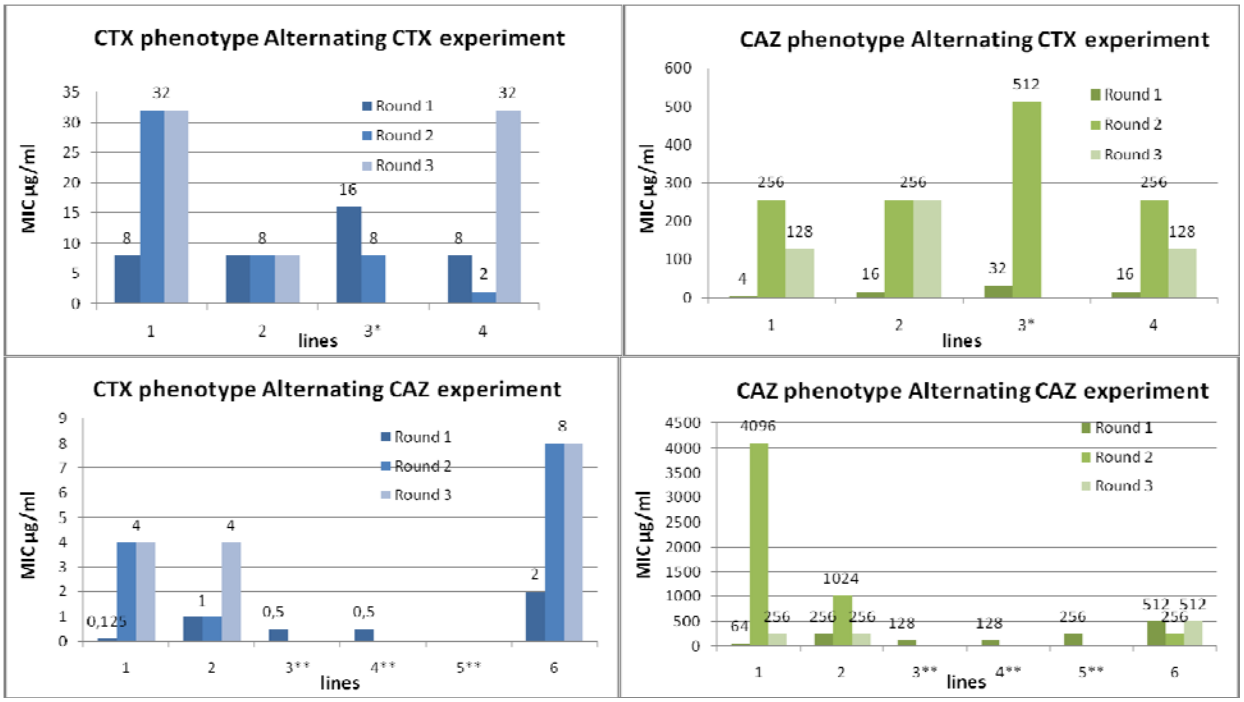


Figure 3 **Phenotipic characterization of TEM isolates**

Minimal Inhibitory Concentration (MIC) assay results are shown per experiment per antibiotic. MIC results for CTX are shown in blue, MIC results for CAZ are shown in green. No bar indicates that no MIC results are available for this line. Lines where the third round of selection failed are indicated with * and where the second round of selection failed are indicated with **.

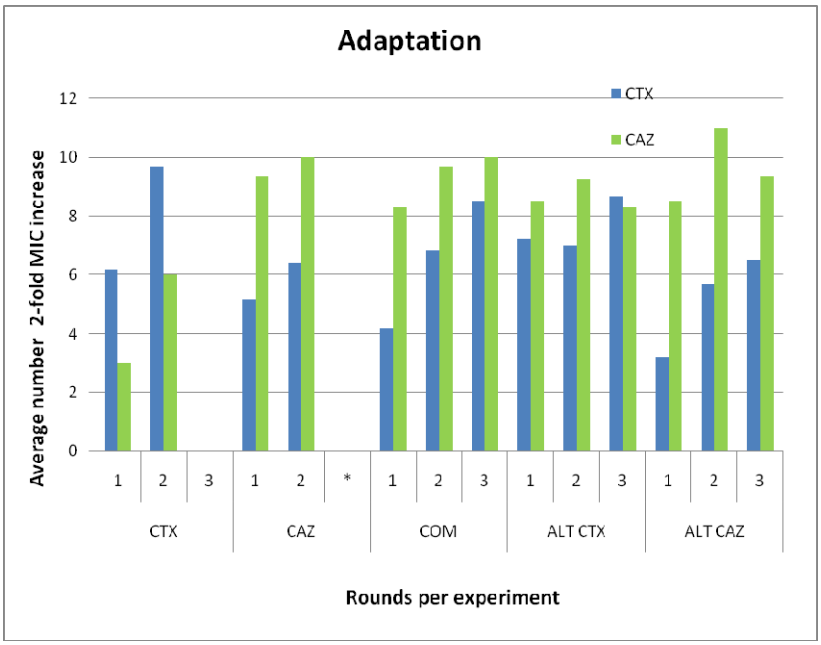


Figure 4 **Average number two fold increments (per experiment per round) relative to the TEM-1 MIC**

MIC of TEM-1 for CTX is 0.0625 µg/ml and for CEF 0.5 µg/ml. The average number of two-fold increments relative to the TEM-1 MIC was used as a measure for adaptation. Rounds of selection that were not performed are indicated with *. Rounds that lack a bar in the figure had no MIC results.

Discussion

Incorporated substitutions and mutation rate

The rate of incorporated substitutions goes down after each round, this is due to the fact that once some mutations are fixed, the number of beneficial mutations that are available goes down. The average of mutations incorporated by Mutazyme II was around 1 (1.46) in this study but, the average number of mutations in the selected genes is much higher at 2.42. The incorporation rate is higher because with an average of 1.46 the libraries can contain genes with many more mutations. Using PEDEL software (Guinivere at http://guinevere.otago.ac.nz/aef/STATS/index.html) the library composition can be calculated. Table 3 shows the results as obtained from the program. Of all variants present in the library 25% account for double mutants but only 6% of all possible combinations of mutations is present (Cx/Vx=0.06), this number goes down to 12% of triple mutants and the fraction of all the possible triple mutants that is present is close to zero. Some lines obtained four additional mutations in one round of selection, according to these calculations there are 36000 such variants in each library that can be selected. By chance these combinations of mutations can be beneficial and therefore selected.

Table 3 **Composition of libraries**
Calculation of the composition of the libraries as used in the experiments. *x* = exact number of mutations in the gene. *Px* = probability of *x* mutations in the gene, given mutation rate 1.46. *Lx* = expected number of sequences in library with exactly *x* mutations. *Vx* = number of possible sequences with exactly *x* mutations. *Cx* = expected number of distinct sequences with exactly *x* mutations. *Cx/Vx* = fraction of all possible sequences with x mutations that are present in the library.

x	Px	Lx	Vx	Cx	Cx/Vx
0	0.2322	1.93e+05	1	1	1
1	0.3391	2.817e+05	2583	2583	1
2	0.2475	2.057e+05	3.332e+06	2.057e+05	0.06173
3	0.1205	1.001e+05	2.862e+09	1.001e+05	3.497e-05
4	0.04397	3.653e+04	1.842e+12	3.653e+04	1.984e-08
5	0.01284	1.067e+04	9.471e+14	1.067e+04	1.126e-11

Since in nature only one substitution can be fixed at a time, the average incorporation of 2.42 is rather unnaturally high. This also makes it difficult to determine if selected substitutions are really beneficial or that these just hitch-hiked along with the other real beneficial mutations in the gene. The mutation rate I used is already lower than the

average of 2 used in other studies performed with this protocol (Barlow & Hall 2002, Salverda *et al.* 2011). But, in future studies the mutation rate could be brought even further down to at least below 1.0 so that fewer mutations are selected at the same time and the situation becomes more natural.

Single antibiotic experiments

To examine the extent to which selection with multiple antibiotics constrains evolution, it is required to select for antibiotics in which mutational pathways lead towards different global optima. Otherwise one expects evolution to follow the same pathway in all treatments. The two β-lactams that were used in these experiments (CTX and CAZ) share the same core structure, but have different side-groups attached to the β-lactam ring. This may lead to a trade-off in the activity of TEM, such as for example some mutations that increase CTX resistance, lead to a reduction in resistance towards ampicillin (Holloway *et al.* 2007). The results of the selection with a single antibiotic show that different mutations are selected for both antibiotics and that resistance levels for CAZ stay relatively low when selecting for CTX and vice versa. This indicates that the two antibiotics indeed cause a different selective pressure for TEM and is therefore a good pair to use in the combination and alternating experiments. Selection for one of the antibiotics is however always accompanied by some degree of adaptation to the other, meaning that using either CTX or CAZ in the clinical setting can lead to resistance for both antibiotics or that the beneficial mutations for these antibiotics partially overlap.

All lines that were selected for increased CTX resistance seem to following the same main pathways that includes the mutations G238S and E104K. This is very similar to the results obtained by Salverda *et al*. (2011). For example, the mutations A42G, G238S and T265M seem to be specific for CTX selection and did not occur when selecting for CAZ. Selection with CAZ revealed two alternative pathways, one involving a mutation at position R164 that was observed in five lines, whereas the sixth line had the striking combination of four mutations very close to each other in the gene e.g. N175D, D179G, M182T and T189M. The first three substitutions have been found in experimental evolution experiments before (Salverda *et al.* 2010). The MICs for both CTX and CAZ (Figure 3) show that this alternative fitness peak does not have lower resistance than the ‘normal’ R164:E104 combination.

Combination experiment

By combining CTX and CAZ during selection, we examined the availability of mutations that have an effect on both traits simultaneously. The experiments with the single antibiotics already showed that mutational pathways which simultaneously increase CTX and CAZ resistance do exist, but also showed that the preferred pathways for a single antibiotic causes the resistance of the other antibiotic to lag behind. The combination experiment showed that an intermediate solution is available whereby resistance against both antibiotics increases more than the lagging resistance in the single experiments. Some constrains remain, especially in the case of CTX adaptation (Figure 4).

Overall the substitution results from the combination experiment are most similar to the CAZ experiment, R164 is the most mutated position and characteristic mutations for CTX, like G238S and T265M are missing. But the fact that R164 is favoured over G238S makes sense since mutations at position R164 are among the mutations that confer the highest resistance against CAZ but also increases resistance against CTX considerably (Giakkoupi *et al.* 2000). This could be why CAZ adaptation for this experiment is almost the same as for the CAZ experiment (on average 0.65 lower over 2 rounds) but CTX adaptation is lower than the CTX experiment (average 1.42 lower over 2 rounds). It seems that CTX adaptation is limited by the selection of R164 mutations instead of G238S.

Alternating experiment

By alternating CTX and CAZ during selection, we examined to what extent adaptation to a particular antibiotic hampers adaptation to a second selective agent. The experiments with the single antibiotics already showed that preferred pathways differ between CTX and CAZ. The genotypes that are realized during adaptation showed divergent mutations. By alternating both antibiotics we determined whether it is possible to “jump” between the adaptive pathways and whether adaptation is constrained. The alternating experiments show that the outcomes depend strongly on the antibiotic that was used in the first round. Selecting for CTX first gives a different set of substitutions than when you select for CAZ first. Also when selection is started with CAZ CTX MICs stay lower overall 3 rounds than when selection is started with CTX.

Mutations G238S and R164S do not occur together in nature but have been found in lines of the alternating experiment starting with CTX. Data from Giakkoupi *et al.* (2000)

show that going from G238S to G238S:R164S gives a two-fold increase in resistance against CAZ, even though R164S alone would have had a resistance level 16 times higher than that of G238S:R164S. In the third round of selection, with CTX treatment, G238S:R164S is not favourable since resistance against CTX for the double mutant is 8.3 times lower than for G238S alone. Therefore, it is not surprising to find a reversed mutation as seen in line 4 resulting in loss of R164S. Half of the lines of these experiments do not gain additional mutations in the third round. This indicates that there are little beneficial mutations left after rounds of alternating antibiotic pressure and that the peaks for resistance against the other antibiotic are not available due to epistasis. The fact that unnatural combinations such as G238S:R164S are selected is an indication that experiments with alternating antibiotic pressures do not mimic the natural evolutionary conditions closely.

In the case of the CTX alternating experiment CTX adaptation peaks in the rounds where there was selection for CTX and CAZ adaptation peaks in rounds where there was selection for CAZ (Figure 4). This is what is expected since selection for one antibiotic can have a negative effect on the adaptation for the other antibiotic. In the CAZ alternating experiment however, CAZ adaptation goes up in round 2 where there was selection for CTX. This can be explained by the fact that CAZ adaptation also increases when selecting for CTX as seen in the single CTX experiment. But the drop in the third round is unexpected. These results seem very unlikely because there was selection for CAZ resistance in this round. Furthermore, CTX adaptation in the CAZ alternating experiment are the lowest of all experiments, this is not what is expected since there was selection for CTX in one of the rounds. It should be considered that many lines of the alternating experiments either failed or were contaminated, this could hamper the reliability of the conclusions. Therefore, further experiments should be done to add the missing data.

Mutations

The most frequently found mutation in these experiments was E104K. It was found in 16 out of 28 lines. Of the 12 lines that did not incorporate E104K, 9 have a mutation at position E240, either E240V, E240R, E240A or E240K. Mutations at positions E104 and E240 never appeared in combination, therefore it is likely that these mutations inhibit fixation of the other due to sign epistatic interactions. Sowek *et al.* (1991) concluded from site directed mutagenesis experiments that mutations at these positions serve similar roles

in increasing resistance against β-lactam antibiotics. Therefore it is likely that the mutations interfere with each other. F60Y and A172D both appeared twice in these experiments but have not been found in other lab experiments or in clinical isolates (Salverda *et al.* 2010). Further testing could give information about the possible beneficial effects and what structural changes are caused by these mutations. It is interesting to note that mutation E240R, found in line 2 of the CEF alternating experiment has been reported in a clinical isolate but had never been found in a lab experiment before (Table 2).

Conclusions

Selection for CTX and CAZ lead to different selective peaks, the initial incorporation of G238S for CTX and substitutions at position R164 for CAZ divides the pathways. Overall it seems that no alternative fitness peak is available for this combination of antibiotics. Instead the same pathway is chosen as when selecting for CAZ resistance alone. Pleiotropic constrains do hamper resistance increase towards both antibiotics but adaptation towards CTX resistance seems to be more affected then CAZ adaptation. This is probably because of the initial incorporation of R164S instead of G238S. Substitutions P167, A172D, R241 and G267 remain interesting because they were selected multiple times and only under the selection pressure of both antibiotics. Therefore, these can be specific adaptations to this combination of antibiotics.

Combining the two antibiotics in the same treatment seems a better way to select for increased resistance against both antibiotics than the alternating fashion which was used in previous studies. The adaptation towards both antibiotics is less constrained because of reciprocal sign epistasis in the combination experiment and this treatment did not give rise to unnatural combinations of mutations as in the alternating treatments.

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Appendix I

Protocol

Step 1 Error-prone PCR

- PCR mix Error-prone PCR:

10x Mutazyme buffer	2.5 µl
dNTP	0.5 µl
P3 (10 pmol)	0.5 µl
P4 (10 pmol)	0.5 µl
Mutazyme	0.5 µl
Template	1130 ng (contains ~100 ng TEM) /x (concentration ng/µl) = y µl
MQ	20.5 – y = z µl
TOTAL VOLUME	*25.0 µl*

- Program Mutazyme (P3/P4):

1		2 min at 95°C
2	30x	30 sec at 95°C
		30 sec at 60°C
		75 sec at 72°C
3		10 min at 72°C

- Pour 1% agarose gel (100 ml 0.1x TBE/TAE + 1 gram Agar + 10µl Ethidium bromide)
- Dilute 1 µl PCR-mix in 9 µl MQ
- Put 5, 2.5 and 1 µl of the diluted mix on gel
- Put 5, 2.5 and 1 µl of Mutazyme 1.1 kB (20 ng/µl) on gel to determine the concentration of PCR product

Step 2 Purification

- Clean-up using PCR cleanup-kit according to manufacturer's instruction
- Dilute in 50 µl of MG at final step

Step 3 Digestion

- Digest mixture

BspHI	1.0 µl
DpNI	1.0 µl
SacI	1.0 µl
BSA	0.5 µl
Buffer 4	5.9 µl
PCR product	50.0 µl
TOTAL VOLUME	*59.4 µl*

- Digest for 1 h at 37°C

Step 4 Purification

- Clean-up using PCR cleanup-kit according to manufacturer's instruction
- Dilute in 50 µl of MG at final step
- Pour 1% agarose gel (100 ml 0.1x TBE/TAE + 1 gram Agar + 10µl Ethidium Bromide)
- Dilute 5x in MQ and put 4, 2 and 1 µl of the diluted mix on gel
- Put 4, 2 and 1 µl of Mutazyme 1.1 kB (20 ng/µl) on gel to determine the concentration of DNA

Step 5 Ligation

- Ligase mixture

T4 Ligase	1.0 µl
10x ligase buffer	2.0 µl
Vector	x µl (288 ng of vector in 20 µl)[1]
Insert	y µl (154 ng of insert in 20 µl)[2]
MQ	17.0 – x – y µl
TOTAL VOLUME	*20.0 µl*

- Ligate O/N at 4°C

Step 6 Precipitation

- Incubate 5 min at 65 °C to destroy ligase
- Transfer ligase mixture to a 1.5 ml eppendorf tube
- Do all steps and spinning at 4 °C
- Add 0.6v ice-cold isoproponal and mix well (add 12 µl to 20 µl ligase mixture)
- Add 1µl of glycogen
- Spin down for 15 min at 13,000 rpm (pay attention: lids on outside!)
- Carefully remove supernatant with pipette and add 300 µl ice-cold 70% ethanol
- Spin down for 5 min at 13,000 rpm (pay attention: lids on outside!)
- Carefully remove supernatant with pipette and add 300 µl ice-cold 70% ethanol
- Spin down for 5 min at 13,000 rpm (pay attention: lids on outside!)
- Carefully remove supernatant with pipette
- Dry under vacuum for 15 min
- Dissolve in 8 µl MQ

Step 7 Electroporation

- Put cuvettes at -20 °C

[1] 1007/√4891bp = 14.40 ng/µl; 14.40 ng/µl x 20 µl = 288 ng vector in a 20 µl volume

[2] (288 ng x 871 bp x (3/1)) / 4891 bp = 154 ng insert

- Put SOC at 37 °C
- Switch on shaker bath at 37 °C
- Put 500ml LB bottles at 37 °C
- Take competent cells from -80 °C freezer (box 14.11/2.11) and keep cells on ice
- Add aliquot of 3.8 µl precipitated ligation-mix to a 40 µl batch of competent cells
- Electroporate at 1.8 kV in cuvette
- Add 1 ml of SOC and mix well
- Repeat with second aliquot
- Let cells recover at 37 °C for 90 min
- Mix 1 µl of SOC+cells with 99 µl LB and plate out 50 µl of mixture on LB+tet
- Add SOC + cells to 500 ml LB + 500 µl tet (15mg/ml)
- Plate out 50 µl of medium on LB-tet to determine lib. Size (2 times)
- Incubate O/N at 37 °C (no shaking)

Step 8 Selection

- Prepare required number of bottles of Mueller-Hinton (# lines x # antibiotic concentrations)
- Count the number of transformants on the LB+tet plates to determine library size
- Add appropriate antibiotic (stored in box 7.10 in -80 °C) concentration to bottles
- Add IPTG (25µl per 50ml)
- Determine the concentration of E. coli in incubated bottles by determining the OD 600nm of the 10x diluted mixture and using a 3.7 x 10^8 calculation factor
- Add 10x effective library size
- Make a stock of the enriched library (850 µl + 150 µl 87% glycerol)
- Incubate O/2N at 37 °C (no shaking)

Step 9 Picking a single clone

- After 48h, select the bottle with the highest [Ctx] which confers growth
- Plate out on LB + tet
- Grow O/N at 37 °C
- Pick a single colony an inoculate 5 ml of LB + tet (2 times)
- Grow O/N at 37 °C (shaking)
- Put 10µl of the culture into fresh medium
- Grow O/2N (shaking)
- Make a stock of the culture (850 µl + 150 µl 87%glycerol)
- Miniprep the remainder and dissolve in 100 MQ at the final step
- Measure the concentration on the Smartspec (100µl of 10x diluted mix) or on
- Nanodrop (1µl of undiluted mix)
- Put the equivalent of 1µg plasmid into fresh tube
- Dry for 30 min in speedvac
- Send away for sequencing

Appendix II

Incorporated synonymous mutations

Mutations that do not lead to amino acid substitutions that were incorporated into the TEM gene during the first round (shown in green), second round (shown in blue) and third round (shown in red) of selection. The line numbers marked with * lack a third round, while line numbers marked with ** lack results for the second and third round. The results of the CAZ alternating experiment are missing and therefore not included in the table.