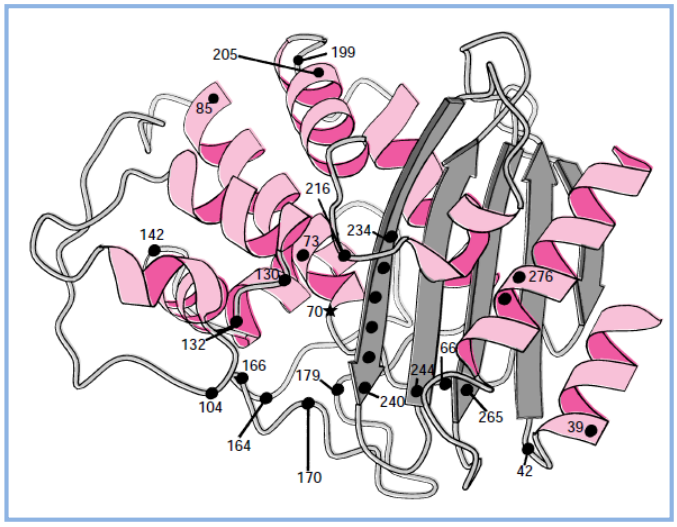
Minor Thesis Report GEN 80424

The Effect of Environmental Conditions on Epistatic Interactions between Mutations in TEM-1 β-Lactamase



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ABSTRACT

TEM-1 β-lactamase is a bacterial antibiotic resistance gene that can efficiently break down penicillins, but confers low resistance to "newer" β-lactam antibiotics, such as cefotaxime. Several mutations in TEM-1 β-lactamase increase cefotaxime resistance individually and thus are beneficial relative to the ancestral allele. However, combining these mutations are not always beneficial and may result in a decrease of the cefotaxime resistance. This suggests that there are strong negative epistatic interactions between the mutations in TEM-1 β-lactamase. Environmental conditions may influence the epistatic interactions. Therefore, we tested the effects of three environmental variables (temperature, salinity, antibiotic compound) on the epistatic interactions between four mutations in TEM-1 β-lactamase. These interactions were tested both in the absence or presence of two additional mutations that increase protein stability. To this end, sixteen new TEM-1 β-lactamase mutants were constructed, and then the minimum inhibitory concentration of the full set of 64 mutants was measured under seven different conditions. The analysis of the ruggedness measurements, fitness landscape topographies and epistasis demonstrates that the stabilizing mutations M182T and T265M reduce the negative interactions between the mutations in TEM-1 β-lactamase. In line with the impact of the stabilizing mutations, temperature also influences the interactions. Epistasis between mutations in TEM-1 β-lactamase increases at higher temperatures. But the changes are relatively subtle and the overall topography of the landscape (global maximum, location of fitness valleys) remains similar. Salinity has limited or no impact on epistasis. In contrast, the topography of the fitness landscape is quite different for Ceftazidime resistance compared to Cefotaxime resistance. The Ceftazidime fitness landscape is also less rugged. In conclusion, environmental conditions such as temperature and salinity have limited impact on the interaction between beneficial mutations in TEM-1 β-lactamase, whereas the use of a different selective agent has a strong impact.

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1. INTRODUCTION

1.1. Antibiotic resistance

Antibiotics elicit bactericidal effects by inhibiting cell wall synthesis, translation, or amino acid synthesis, as well as disrupting metabolic pathways. The most widely used antibiotics, β-lactam compounds, act by inhibiting the synthesis of peptidoglycan layer of the bacterial cell wall [2]. Since the discovery of the penicillin by Alexander Fleming in 1928, bacteria have thrived resistance to β-lactam antibiotics by three mechanisms: 1) by alerting the target site e.g. penicillin binding protein (PBP); 2) by modifying membrane permeability; and 3) by producing β-lactamases [3]. Production of β-lactamases is considered as the most common strategy to escape from β-lactam antibiotics.

The increasing bacterial resistance to β-lactam antibiotics is one of the main problems in public health, in association with the difficulty to treat infections as well as a necessity to develop new antibiotics. Therefore, studies on antibiotic resistance have gained high priority. In this context, the laboratory evolution of TEM β-Lactamase is an important tool to predict the development of antibiotic resistance.

1.2. TEM-1 β-lactamase and its important mutations

TEM-1 β-lactamase is the most common determinant of antibiotic resistance in bacteria [3]. The crystal structure of TEM-1 β-lactamase is shown in Figure 1. Since the first allele of TEM-1 β-lactamase discovered in 1963, more than 170 TEM-1 β-lactamase variants have been documented in clinical isolates [4] . Unlike TEM-1 β-lactamase, these variants confer resistance to a wider range of antibiotics, including late generation cephalosporins, monobactams, and β-

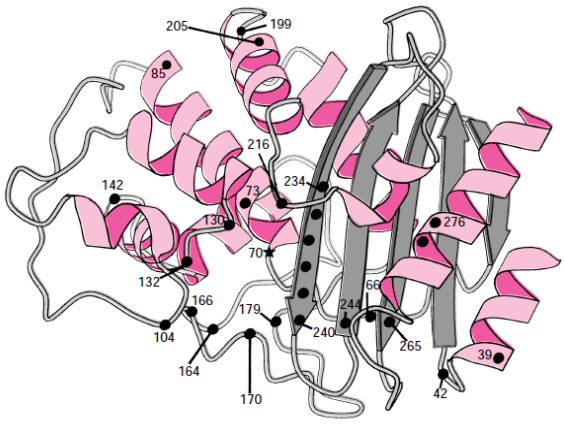

Figure 1. The crystal structure of TEM-1 β-lactamase with the α-carbon of the active-site serine marked by a star [1].

lactamase inhibitors [4]. Enhancement of the resistance phenotype of TEM-1 β-lactamase mutants results either from specific interactions with the substrate or from enlargement of the catalytic cavity [5, 6] .

Amino acid substitutions at positions 104, 164, 238, and 241 have an important role in conferring the new activity profile of the enzyme [1]. Mutation of glutamate 104 by lysine (E104K) influences the substrate's binding and catalytic activity[7]. E104K replacement alone only confers a minor alteration in resistance phenotype; however, it dramatically increases resistance when combined with mutations at position 164 or 238 [5, 7]. Moreover, a substitution of arginine 164 by serine (R164S) increases resistance to cefotaxime, ceftazidime, and aztreonam [8]. Additionally, the individual mutation G238S increases the minimum inhibitory concentration eight-fold, but concomitantly destabilizes the enzyme [9, 10]. Furthermore, amino acid 241, if residing in the loop, is involved in the stabilization of the rearranged active site form [10].

In addition, some mutations that do not have a significant effect on enzyme activity by themselves, such as M182T and M265T, are frequently found in clinical isolates and laboratory experiments. Substitution of M182T leads to new hydrogen formation of the Thr hydroxyl group with the backbone carbonyl of Glu at positions 63 and 64, ultimately decreasing the flexibility of the hinge region [10]. Mutation M182T reverses the destabilizing effect of antibiotic- or inhibitor-resistant mutations [10]. In concert with M182T, T265M shows no influence on resistance when present alone, but it expresses adaptive effects in laboratory experiments. Both M182T and T265M are commonly found in the background of other mutations and are also called compensatory mutations [4] .

In general, there is a dynamic interaction between enzyme stability and catalytic activity [11]. Clinically isolated mutants of TEM-1 β-lactamase mutants have increased activity against cephalosporin, but they are less thermodynamic stabile [12]. Substitutions affecting the size of the active site cavity may enhance activity and concomitantly diminish thermodynamic stability.

1.3. Fitness landscape and epistatic interaction

The phenotypic suppression of one allele for another allele at a different locus was originally introduced by William Bateson in 1909 as epistatic interaction [13]. This concept was later broadened to include the effects of one mutation on another mutation and may include interactions between mutations in different genes or in the same gene. The epistatic interactions between mutations play a major role in adaptation [14]. Our null hypothesis is that the interactions between beneficial mutations are dominated by negative epistasis, implying that the combined effect is less than what is expected [14].

The interactions between mutants fall into three categories: 1) magnitude epistasis, 2) sign epistasis, and 3) reciprocal sign epistasis. When magnitude epistasis occurs, the sign of the effect (beneficial or deleterious) of a mutation is fixed, while the genotypic background influences the size of their effect. Magnitude epistasis influences the curvature of a landscape by selectively choosing the likelihood mutational pathways [15, 16]. In contrast, when sign epistasis occurs, mutations are beneficial with respect to some genetic backgrounds and deleterious with respect to others. Sign epistasis affects the accessibility of mutational trajectories and results in landscape ruggedness [15, 16]. In addition, reciprocal sign epistasis represents cases in which individually beneficial mutations are deleterious in combination [17]. Reciprocal sign epistasis leads to the occurrence of multiple fitness peaks [15, 18]. Figure 2 depicts these three types of epistatic interactions.

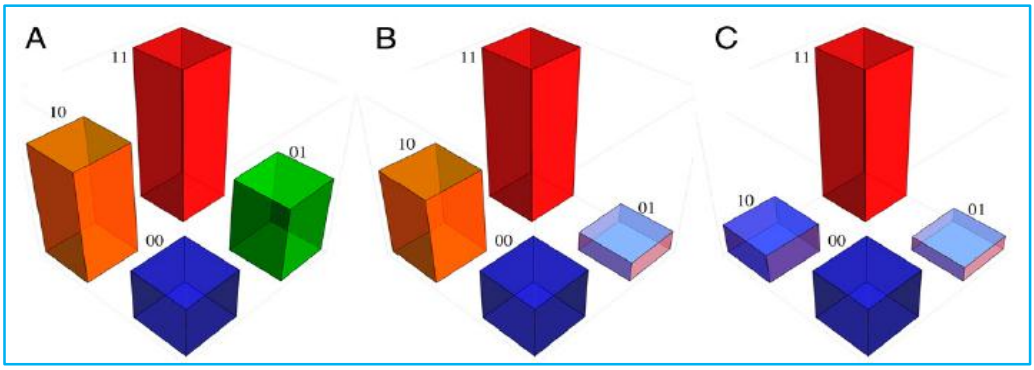

Figure 2. Illustration of three types of epistatic interactions in a two-locus fitness landscape. (A) Magnitude epistasis: one fitness peak with two accessible paths from 00 to 11. (B) Sign epistasis: one fitness peak with only one accessible path from 00 to 11. (C) Reciprocal sign epistasis: two fitness peaks with no accessible paths from 00 to 11. Paths are considered accessible when fitness increases monotonically [17].

The epistatic interactions between mutations define the structure of fitness landscape in which the relationship between genotype and fitness are depicted [16, 19]. The fitness landscapes can be smooth or rugged. A smooth landscape has only one fitness peak [17], while a rugged fitness landscape may display multiple peaks separated by valleys of lower fitness [15]. The ruggedness influences the predictability of adaptation.

1.4. Environmental effect on epistatic interactions

The conjecture that the environment affects the epistatic interactions between mutations either in the same gene or in different genes has been studied primarily in the background of deleterious mutations [20-22]. Only a single work found presents environmental effects of beneficial mutations; in this work, epistatic interactions change qualitatively as environments change [23]. Therefore, this study focused on gaining evidence regarding exposure to environmental change for beneficial mutations in TEM-1 β-lactamase.

Furthermore, we deliberately chose environmental variables exhibiting cellular adaptations to study the variability of interactions. Change in temperature and salinity, as well as antibiotics, were chosen as stressors in this study. An extreme temperature is a powerful denaturant for enzyme folding and stability [19], and ultimately, it influences the efficiency of synthesis, maintenance, and degradation of proteins [20]. Moreover, extreme salinity affects cell wall robustness, the cytoskeleton and the vacuole system [24], as well as influence the effectiveness of antibiotic. These stressors are ubiquitous in nature; thus, this study could predict the epistatic interactions between mutations in TEM-1 β-lactamase that are driven by environmental changes in nature.

The degree of the effect of environmental conditions on the evolution of epistasis depends on their complexity and fluctuation. Finally, environmental stressors can result in prolonged impacts on epistasis, and consequently, they can influence genetic robustness and evolvability [25].

1.5. Research aims

This research aimed to seek the effects of the environmental factors on the epistatic interactions between mutations in TEM-1 β-lactamase. More specifically, the research attempts to address the following questions using a four locus fitness landscape of the mutations E104K, R164S, G238S and R241P:

1. Does the introduction of the stabilizing mutations M182T and T265M alter the epistatic interactions between mutations in TEM-1 β-lactamase?
2. Since temperature affects protein stability, we expect that lower temperatures ameliorate the negative interactions between the mutations and higher temperatures enhance them if stability is involved, so is this true?
3. Salinity alters both the effect of the cefotaxime and the osmotic stress of the medium. Does salinity also influence the interactions between the mutations?
4. The same four mutations also confer resistance to another β-lactam antibiotic (Ceftazidime), but at different levels. When the epistatic interactions result only from stability affects one would that combinations of mutations that are deleterious for one antibiotic are also deleterious for the other. Is this true and is the structure of the fitness landscape the same for Ceftazidime?

2. METHODS

The experiments consist of two steps: 1) construction of 16 mutants of TEM-1 β-lactamase using site-directed mutagenesis (SDM); 2) determination of the minimum inhibitory concentration (MIC) of 64 TEM-1 β-lactamase mutants under varying environmental conditions.

2.1. Construction of TEM-1 β-lactamase mutants

TEM-1 β-lactamase was used as the wild type allele. Prior to this study, three sets of 16 TEM mutants have been constructed. The first set contains all combinations of the four mutations E104K, R164S, G238S and R241P [26]. In the second and third set, the mutations M182T and T265M were introduced into all these combinations, respectively. In this study, combination M182T and T265M were introduced into all combinations of the first set. The mutants were constructed by introducing M182T into the plasmids carrying the T265M mutation (Table 1).

Table 1. Plasmid templates used for side directed mutagenesis

No	Template Plasmids	No	Template Plasmids
1	pACTEM1 (wild type)	9	R164S G238S T265M
2	E104K T265M	10	R164S R241P T265M
3	R164S T265M	11	G238S R241P T265M
4	G238S T265M	12	E104K R164S G238S T265M
5	R241P T265M	13	E104K R164S R241P T265M
6	E104K R164S T265M	14	E104K G238S R241P T265M
7	E104K G238S T265M	15	R164S G238S R241P T265M
8	E104K R241P T265M	16	E104K R164S G238S R241P T265M

Escherichia coli strain DH5αE was used as a host for all plasmids. Plasmid pACSE3 was originally utilized to construct the wild type plasmid (pACTEM1) [26, 27]. The plasmid contains an origin of replication (1-10 copies/cell), a tetracycline resistance gene, a Lac Z operon, a pTac promoter, and a multiple cloning site.

Mutation M182T was introduced using the forward and reverse primers, 3'GAGCGTGA CACCACGACGCCTGCAGCAATGG5' and 3'CCATTGCTGCAGGCGTCGTGGTGTCACGCTC5', respectively. The mutants were amplified with *pfu* polymerase using the following cycling

program: 18 cycles of denaturation at 95^{0}C for 2 min, annealing at 95^{0}C for 30 sec, extension at 55^{0}C for 60 sec, followed by 1 cycle of 68^{0}C for 6 min. PCR products were purified with the GenElute^{TM} PCR Clean-Up Kit (SIGMA-ALDRICH). The template was digested using the restriction endonuclease *DpnI*.

Plasmids were transformed into *E. coli* by electroporation and plated on LB agar (10 g trypticase peptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per litre) containing 0.1% (v/v) tetracycline, 0.1% (v/v) ampicillin, and 0.05% (v/v) isopropyl-β-D-thiogalactopyranoside (IPTG). The IPTG was used to induce the expression of TEM-1 β-lactamase. A single clone from each plate was grown (37^{0}C, 225 rpm) overnight in LB containing 0.1% (v/v). Plasmids were purified by using the GenElute Plasmid Miniprep Kit (SIGMA ALDRICH).

The constructed TEM-1 β-lactamase mutants were sequenced to verify the identity of the constructed plasmids by amplification using P3 (TCATCCGGCTCGTATAATGTGTGGA) and P4 (ACTCTCTTCCGGGCGCTATCAT) primers. The amplicons were then sequenced using primer P3. Sequences were analyzed in MEGA software by comparing them to the sequence of TEM-1 β-lactamase.

2.2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC assay for 64 alleles of TEM-1 β-lactamase mutants were conducted in standard conditions: Cefotaxime (CTX), temperature 37^{0}C and salinity 5g/L. Then the tests were further extended in two temperatures, 25^{0}C and 40^{0}C; and two salinities, 1 g/L and 25 g/L. MIC assays on other antibiotics: Ceftazidime (CAZ) and Meropenem were also determined at temperature 37^{0}C and salinity 5g/L. However, the result of the assay on Meropenem was excluded from further analysis because the range of its MIC value was narrow.

In this MIC assay, medium LB containing 0.1% (v/v) tetracycline was used. Stock solution of antibiotics in concentration of 256 mg/ml were prepared into phosphate buffer, pH 7, and stored at -80^{0}C in single use aliquots. For the cultures of TEM-1 β-lactamase mutants, their stocks were prepared by transforming 64 plasmids of TEM-1 β-lactamase into host *E. coli* and plated on LB agar. Five individual colonies were isolated and cultured in 200 µl

LB. The isolations were prepared in triple. Stock cultures (170 µl culture + 30 µl 87% glycerol) were stored at -80^{0}C for further tests.

For plate preparation, a 2-fold dilution series of antibiotics from 2048 mg/ml to 0.015625 mg/ml were prepared in phosphate buffer. Each serial dilution of antibiotics was added into medium LB with 0.05% (v/v) IPTG. Micro-titer plates of 96 wells were filled by 297 µl of the each dilution of antibiotics. Prior to the MIC assay, stock cultures were revived in LB and grown at 37^{0}C overnight.

MICs of TEM-1 β-lactamase mutants were determined from revival culture at a titer of 10^5 cells/ml. 3 µl of the revival cultures were added into each two-fold serial dilutions of antibiotics prepared in the plates. Triple MIC assays were performed towards each treatment. The tested cultures were grown overnight at 37^{0}C or at the designed temperatures. Absorbance at OD_{600} of the cultures was then measured by using a micro-titer plate reader (Victor). The lowest concentration of antibiotic without any visible growth in the culture was defined as the MIC value.

2.3. Analysis

The triple sets of MIC values from each treatment were adjusted by taking their median. The resistance levels of each treatment were determined based on their MICs and analyzed by Analysis of Variance (ANOVA). The MIC values were also used to construct the fitness landscapes (Appendix 2) and to determine the ruggedness measurements of these landscape (Appendix 1 and 3).

In addition, the relation between landscape ruggedness and epistasis interaction between mutants were quantitatively measured into four parameters: 1) the roughness-to-slope ratio, r/s, which depicts the quality of landscape in a linear model of; 2) F_{sum}, representing the amount of variations explained by two-way, three-way and four-way interactions; 3) f_s+f_r, displaying the fragment of pairwise interactions exhibiting the strongest form of epistasis, i.e. sign and reciprocal sign epistasis; and 4) N_{cp}, measuring the numbers of the accessible trajectories to the peak. The four ruggedness measures were analyzed based on the MICs of all treatments by resampling the data 10,000 times at $P<0.01$ [26].

3. RESULTS

Four mutations that individually increase CTX resistance in the TEM-1 β-lactamase background were used as a starting point. We constructed 16 (2^4) combinations of these mutations in 4 backgrounds: 1) no stabilization mutations, 2) stabilization with M182T, 3) stabilization with T265M, and 4) stabilization with a combination of M182T+T265M. As a proxy for fitness, the resistance phenotypes of these combinations to CTX and CAZ were estimated by their minimum inhibitory concentrations (Appendix 4).

To measure the effects of stressful environments on epistatic interactions, we first needed to determine the presence of epistatic interactions in all combinations of the mutations in the standard conditions (CTX, 37^0C, and 5 g/L) by examining their fitness landscape topography (Figure 3). The landscape displays 24 trajectories from ancestral genotype (TEM-1 β-lactamase) to the four-fold mutations based on their resistance levels. The acquisition of a mutation in each trajectory from the top (wild type) to the bottom (four-fold mutants) creates upward and downward pointed arrows across the landscape. Black arrows imply an increase in activity (uphill step), while red arrows represent a decrease in activity (downhill step). The blue arrows denote a similar resistance value while the purple ones indicate an accessible way to the peaks instead of to the four-fold mutations. There is a tendency for the combinations of two-fold, three-fold, and four-fold mutations to decline in their resistance phenotype. Therefore, it is suggested that negative interactions between mutants exist.

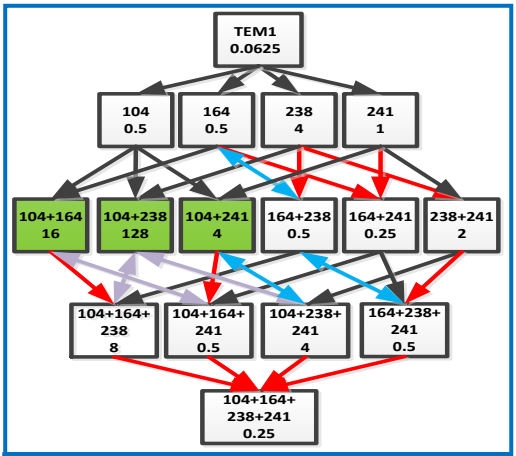

Figure 3. The fitness landscape topography of 16 combination mutations in the TEM-1 background in the standard condition.

The landscape highlights the existence of sign epistasis, which is signified by the significant uphill steps that are separated by the neutrals and the downhill steps over the 24 trajectories. Moreover, the multiple fitness peaks signify the presence of sign epistasis, including reciprocal sign epistasis.

3.1. The effect of the M182T and T265M mutations on epistasis

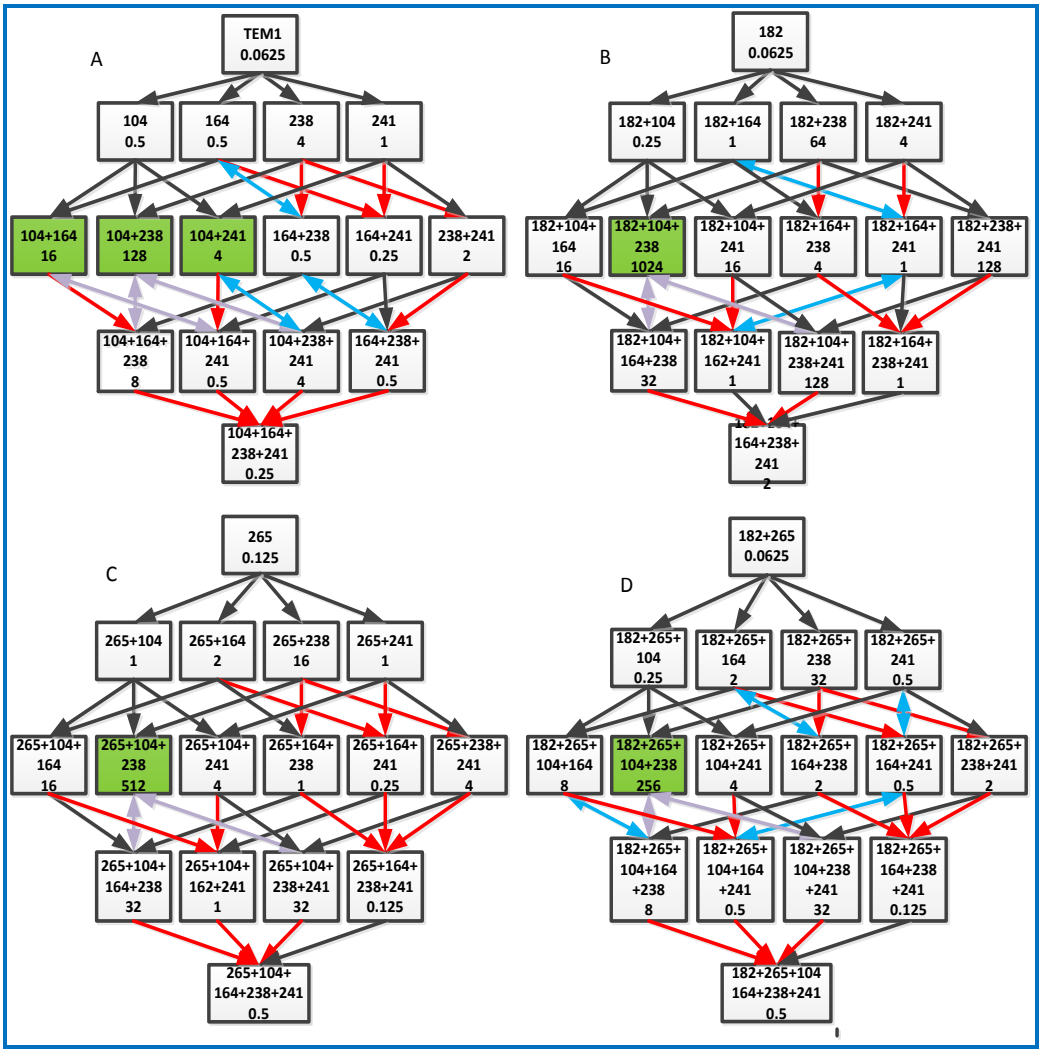

Figure 4. Fitness landscapes of the 16 combinations of the 4 beneficial mutations in the 4 backgrounds: (A) TEM-1, (B) M182T, (C) T265M, and (D) combination M182T+T265M at standard conditions (CTX, 37^{0}C, and 5 g/L).

The effects of the stabilization mutations M182T and T265M on epistasis interactions between the four mutations that increase in CTX resistance are inferred from the topographies of the fitness landscapes and the four ruggedness measurements. The fitness landscape that is built from the 16 combinations of the 4 individually beneficial mutations in the wild type background is more rugged than that from the backgrounds of M182T, T265M, and their combinations (M182T+T265M) (Figure 4). The landscape without the stabilization mutations has two or three peaks (a rugged landscape), implying abundant sign epistasis. However, the introductions of the stabilization mutations M182T

and T265M into the 16 combination mutants led to smooth landscapes with one peak, indicating low sign epistasis.

In concert with the fitness topographies, the ruggedness measures also show the same pattern, although the differences are significant only for r/s and F_{sum} (see Appendix 1). The trend of the decrease in the amount of sign epistasis after the introduction of stabilization mutations suggests the involvement of stabilization mutations in the interactions.

In addition, all fitness landscapes from four distinct backgrounds share a mutation E104K+G238S as a global maximum. Nevertheless, the same global peaks exhibit significantly varied resistance levels. The MIC value of a mutation E104K+G238S in the background of the wild type is 128, but the MIC values in the background of mutations M182T, T265M, and combination M182T+T265M are 1024, 512, and 256, respectively. In total, the resistance levels of the 16 combination mutations in the background of mutations M182T, T265M, and combination M182T+T265M increase approximately eight-fold, four-fold, and two-fold to that in the background of TEM-1, respectively. In addition, no additional effect results from the combination of mutations M182T and T265M. Therefore, the resistance phenotype increases with the introduction of mutations M182T and T265M. The introduction of mutation M182T results in the highest resistance level, and

Overall, this study denotes that the introduction mutations M182T and T265M as well as their combination reduce the negative interactions between the mutations in TEM-1 β-lactamase. On the other hand, they enhance the resistance level.

3.2. The effect of temperature on epistasis

The resistance levels change in different temperatures. The ANOVA analysis demonstrates that the resistance levels at temperatures 37^{0}C, 40^{0}C and 25^{0}C are 1.7, 0.9 and 0.27, respectively (P<0.01). To assess the effect of temperature on epistasis, we analyze the fitness landscapes topographies and ruggedness measurements. In general, the topographies of fitness landscapes among the three temperatures, 25^{0}C, 37^{0}C, and 40^{0}C, demonstrate that the temperature of 25^{0}C confers the smoothest landscape with one peak while 37^{0}C presents the most rugged landscape with two or three peaks (Figure 5). This evidence highlights greater epistasis at a high temperature than at a low temperature. Nevertheless, the topographies of the fitness landscape among the three temperatures do not significantly change, because the the positions of global maximum and valleys almost remain the same.

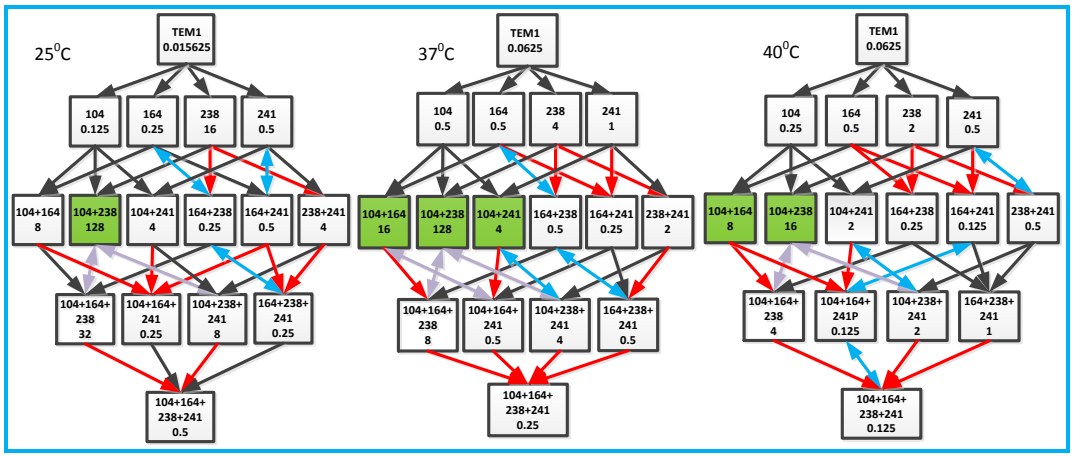

Figure 5. Fitness landscapes of the 16 combinations of the 4 beneficial mutations at temperatures of 25^{0}C, 37^{0}C, and 40^{0}C.

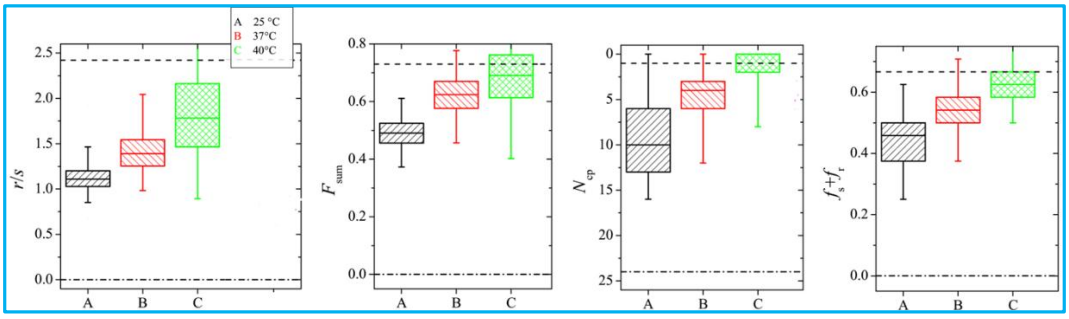

Figure 6. The ruggedness measurements of r/s, F_{sum}, N_{cp}, and F_s+F_r at 25^{0}C (A), 37^{0}C (B), and 40 ^{0}C (C).

In addition, the ruggedness measurements of the fitness landscapes demonstrate temperature dependency (Figure 6). The four ruggedness measures show the increasing ruggedness with the increasing temperature. The least rugged landscape presents at low temperature signifies the alleviation of epistasis at low temperatures.

Moreover, corresponding to the f_s+f_r parameter, we summarize that all possible combinations of two set mutations present the most abundant sign epistasis at 37^0C followed by 40^0C and 25^0C, with the number of combinations that display sign epistasis at 53, 50, and 49 (out of 96), respectively (Appendix 3). In this analysis, neutral steps in which resistance does not decrease or increase were classified as sign epistasis. This data slightly differs from average of f_s+f_r, in which the temperature of 40^0C confers the highest value.

In sum, temperature conditions affect the epistasis interactions between the mutations in TEM-1 β-lactamase but the changes in the topographies of fitness landscapes are relatively minor. Low temperature reduces the epistasis and high temperature enhance them.

3.3. The effect of salinity on epistasis

Salinity has a strong impact on the resistance level of the different TEM-1 β-lactamase mutants. The average resistance of the 64 mutants was reduced by a factor 3.3 at 25 g/L compared to the resistance at 5 g/L. At 1 g/L, average resistance was slightly higher. In contrast, the impact of resistance on epistasis on the overall topography of the TEM-1 β-lactamase fitness landscape is small (Figure 7). In all cases, the fitness peaks are located at genotypes which harbor two mutations and the global optimum is always located at the E104K+G238S mutant. One potential difference is the presence of two suboptimal peaks at E104K+R164S and E104K+R241P. The presence of this latter peak depends on whether the pathway towards E104K+G238S+R241P is accessible, since both genotypes have a MIC of 4 µg/L. A previous study which uses a more precise measurement of the CTX resistance has shown that this pathway is indeed accessible [26] and that the TEM-1 β-lactamase landscape has only two peaks under the standard conditions (5 g NaCl/L) as well. In line with these findings, changing of the salinity has little or no impact on the

ruggedness measures of the TEM-1 β-lactamase fitness landscape (Figure 8 and Appendix 3). These findings are also confirmed by examining the correlation between the resistance measurements at the different salt concentrations. These correlations are high for both 5 g/L versus 25 g/L (r = 0.97, n = 64, P<0.001) and for 5 g/L versus 1 g/L (r = 0.93, n = 64, P<0.001). Therefore, it can be concluded that salinity does not influence epistasis between the beneficial mutations in TEM-1 β-lactamase.

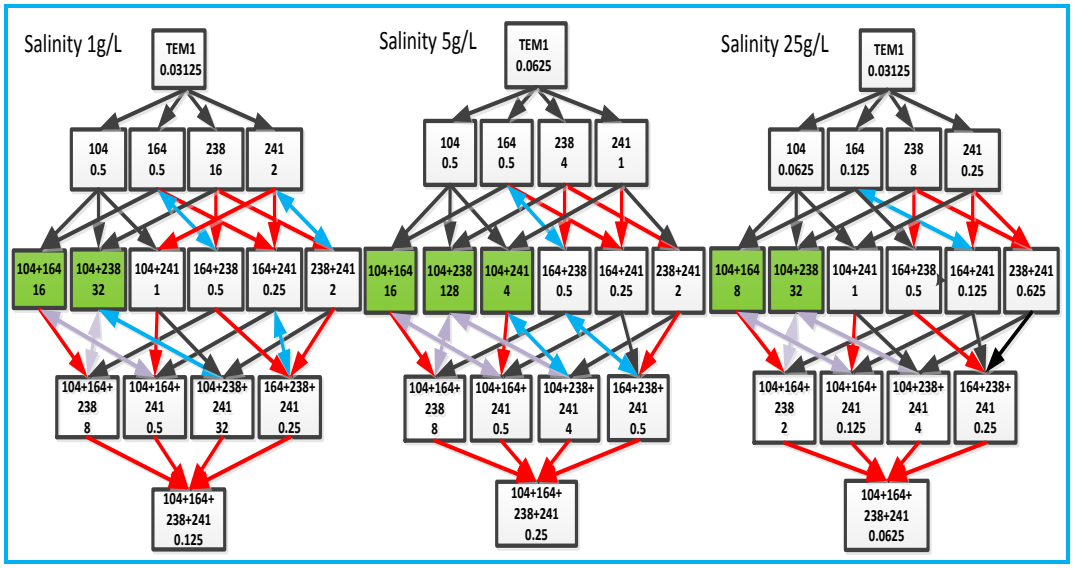

Figure 7. Fitness landscapes of the 16 combinations of the 4 beneficial mutations at salinities of 1 g/L, 5 g/L, and 25 g/L.

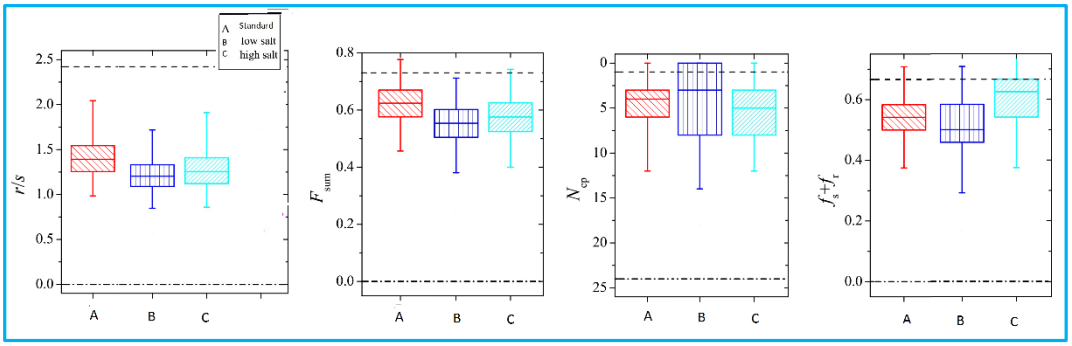

Figure 8. The ruggedness measurements of r/s, F_{sum}, N_{cp}, and F_s+F_r at salinities of 5 g/L (A), 1 g/L (B), and 25 g/L (C).

3.4. The epistatic interactions on different antibiotics

In the CTX treatment to the four individual mutations (E104K, R164S, R241P, G238S), the highest resistance phenotype was elicited by mutation G238S, followed by R241P, R164S, and E104K. Nevertheless, the CAZ treatment to the same mutations shows that the mutation R164S presents the highest resistance level, followed by E104K, R241P, and G238S. When these mutations present in combinations of two-fold, three-fold, and four-fold mutations, their resistances mostly alter without additive effect, which indicates the existence of epistatic interactions. It seems that the interactions between mutations in TEM-1 β-lactamase on CTX and CAZ are dissimilar. The fitness landscapes for CAZ displays a global peak at a two-fold mutation carrying E104K+R164S, while the fitness landscape for CTX presents a mutation carrying E104K+G238S as the global peak.

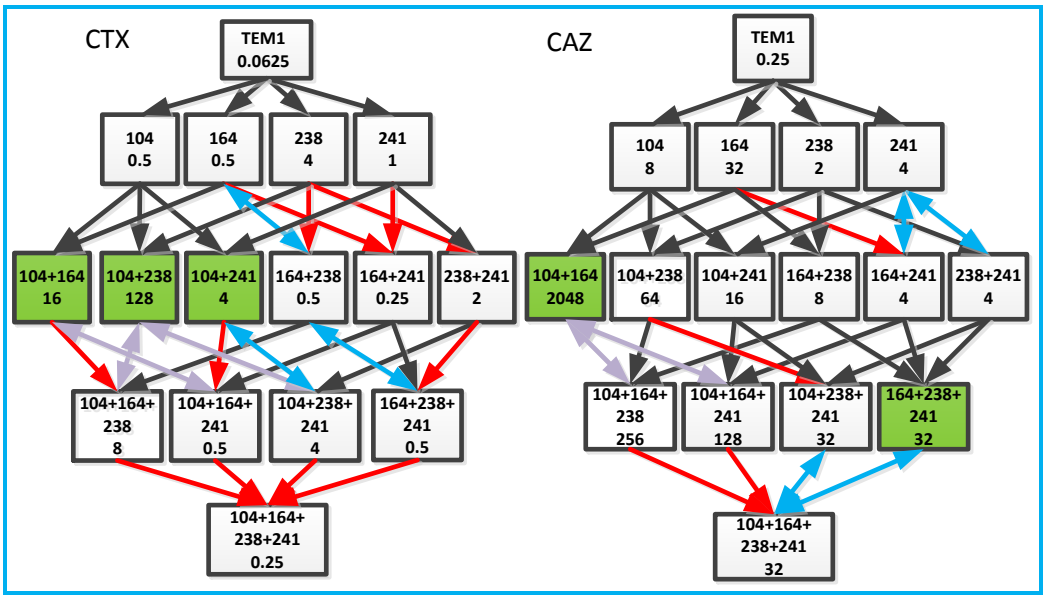

Figure 9. Fitness landscapes of the 16 combinations of the 4 beneficial mutations in the CTX and CAZ treatments

The fitness landscape topographies in CTX is more rugged landscape than CAZ (Figure 9). CTX landscape exhibits two or three peaks, in which the third peak at the 104+241 mutant is uncertain because it depends on the MICs of the 104+241 and 104+238+241 mutant. CAZ landscape seems to have one or two peaks, depend the MIC values on the seemingly neutral steps at the bottom right (32). Suppose the 164+238+241 is in reality slightly higher than the 104+164+238+241, than it would be two peaks.

In concert with the landscape topographies, the ruggedness measures of the 16 combination mutations on CAZ resistance is slightly lower than that of CTX treatment (Figure 10). These indicate that the amount of sign epistasis in CTX is slightly higher than that in CAZ. Moreover, the ruggedness parameters seem to imply no substantial effect of the introduction of mutations M182T, T265M, and the combination of M182T+T265M on the interactions between the mutations for CAZ treatment.

To conclude, change in antibiotic exhibits a strong impact on epistasis. The topographies of the fitness landscapes substantially change across different antibiotics. The fitness landscape in CAZ is slightly less rugged than that of CTX. Furthermore, the stabilization mutations confer dissimilar effects in diverse antibiotics.

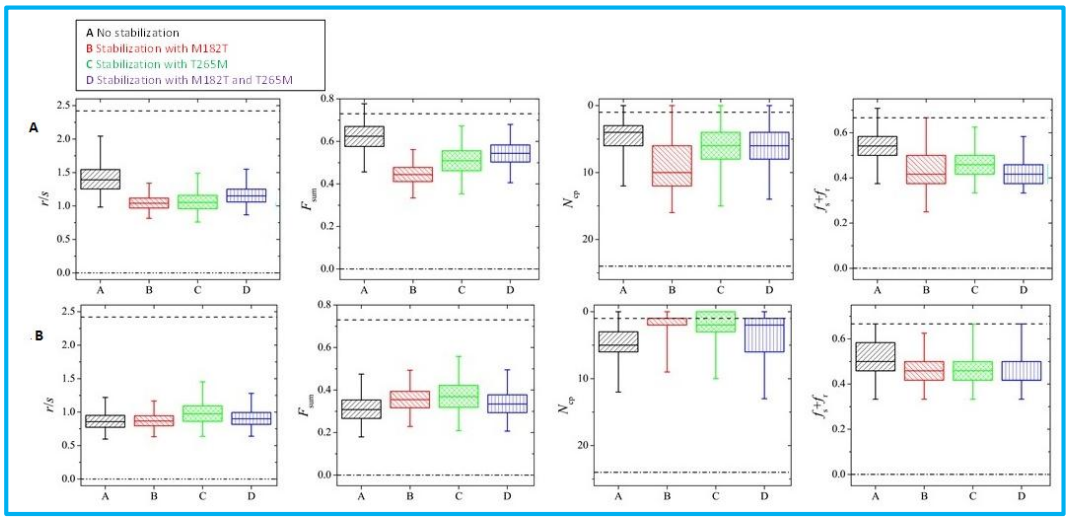

Figure 10. The four ruggedness measurements of r/s, F_{sum}, N_{cp}, and F_s+F_r in CTX (A) and CAZ (B).

4. DISCUSSIONS

Epistasis is recognized as the interactions between genes or mutations which deviate from additive effect on the phenotype [25]. Epistasis depends on their genetic background [26]. Environmental conditions are also suggested to have effect on epistasis [25]. Some studies regarding the epistatic interactions between deleterious mutations [20-22] suggest inconsistent impacts of the environmental stressors [25]. Several stressors appear to enhance epistasis, while others decrease it [22]. On the other hand, little evidence has been provided regarding exposure to environmental changes for beneficial mutations.

This study focuses on the effects of environmental conditions on epistasis between beneficial mutations in TEM-1 β-lactamase. Does epistasis change when the environmental conditions change or is epistasis - and thus the fitness landscape - constant across different environments? Four mutations in TEM-1 β-lactamase (E104K, R164S, G238S, and R241P), which raise the resistance to CTX substantially [6] were used as a starting point. We constructed 16 combinations of these mutations in four backgrounds: 1) wild type TEM-1 β-lactamase, 2) TEM-1 β-lactamase with mutation M182T, 3) TEM-1 β-lactamase with mutation T265M, and 4) TEM-1 β-lactamase with both the mutations M182T and T265M. M182T and T265M are known to increase the stability of the enzyme [4]. We measured epistasis under standard conditions (CTX, 37^0C, and salinity 5 g/L) and compared this to epistasis at temperatures that are extreme for *E. coli* (25^0C and 40^0C), at low and high salt conditions (1 g/L and 25 g/L), and while using another antibiotic (CAZ). We examined the pattern of epistasis in these stressful environments by examining the fitness landscape topography based on their resistance level and by analyzing four ruggedness measures.

In the standard conditions, we found abundant sign epistasis among the 16 mutants that were derived from the wild type TEM-1 β-lactamase. This observation confirms previous findings on the same combinations [26]. This study suggests that the observed epistasis could result from negative effects on protein stability of the involved mutations. The results of this study clearly indicate that the mutations M182T and T265M, as well as their combinations, alleviate epistatic interactions between beneficial mutations in TEM-1 β-lactamase. The fitness landscape without a stabilization mutation displays three fitness

peaks. However, after the introduction of the stabilization mutations, the landscapes become smoother and have only one peak. The mutation M182T provides the strongest effect on the interactions. There is no additional effect that results from the combination of mutations M182T and T265M. This could be because of a negative interaction between mutations M182T and T265M.

Since the stabilization mutations influence the interactions between the beneficial mutations, it is likely that any factors that affect enzyme stability also exhibit a strong impact on the interactions. Hence, temperature should affect the interactions. Therefore, the 16 combination mutations derived from the 4 beneficial mutations in TEM-1 β-lactamase were subjected to a very low temperature (25^0C) and a very high temperature (40^0C). *E. coli* grows very slowly beyond these temperatures and does not grow at all when the temperature is raised well above 40^0C. We compared resistance at these temperatures with the standard temperature (37^0C) and confirmed the expectations. The observation in the landscape with the background of wild type TEM-1 β-lactamase shows that a lowered temperature results in a smoother landscape, thus sign epistasis is more abundant at a high temperature than at a low temperature. A possible reason for this is that the enzyme molecule is more rigid at low temperatures and has a higher tolerance for (multiple) destabilizing mutations. Overall, changes in temperature conditions influence epistasis, but the fitness landscape topographies do not significantly alter.

In addition to the temperature, the effect of salinity on epistasis was also examined. Salinity correlates strongly with osmotic stress of the medium and influences the stability of CTX. Current study demonstrates that salt concentration has a strong impact on the resistance level of the TEM-1 β-lactamase mutants. In the collection of gene deletions in *E.coli,* high salinity results in the alleviation of the negative impact of the deletions on the maximum growth [20]. In this study, low, standard and high salt concentrations (1 g/L, 5 5 g/L and 25 g/L, respectively) were applied to the 16 combination of beneficial mutations in TEM-1 β-lactamase. The comparison of the fitness landscape topographies and the ruggedness measures between the three salinities implies that little or no impact on epistasis. In general, the fitness landscape topographies among the three salinities could be inferred to share the same two peaks, because the additional peaks at salinity 5 g/L is built from the same MICs of E104K+R241P and E104K+G238S+R241P mutants, which is

indeed accessible in another assay [26]. The ruggedness measures also reveal little different values among the treatments. Moreover, the high correlation between the resistance measurements at salinity 5 g/L versus 1 g/L and 25 g/L confirms the conclusion that salinity does not influence epistasis between the beneficial mutations in TEM-1 β-lactamase.

Finally, we studied the epistatic interactions in the presence of a different antibiotic. The same four mutations also conferred resistance to Ceftazidime, but to a different degree [4]. The most beneficial mutation for CTX resistance is G238S, followed by R164S/R241P and E104K. For CAZ resistance, R164S is the most beneficial mutation, followed by E104K, R241P and G238S. Despite these differences, we expected that the mutations have a negative interaction on both antibiotics given that the negative interaction results from the destabilizing effect of the mutations. Current study finds that the epistatic interactions are different in distinct antibiotics, which are clearly depicted by their fitness landscape topographies. Their landscapes exhibit dissimilar positions of a global peak and valleys as well as the numbers of peaks. The global maximum in CAZ landscape is E104K+R164S mutant, while CTX landscape presents E104K+G238S mutant as a global peak. The fitness landscape of CTX shows two or three maxima, while that of CAZ shows one or two maxima depending on the MIC values of 164+238+241 mutants and 104+164+238+241 mutants. Furthermore, the three-fold interactions of the mutations in CTX tend to show a pattern of diminishing returns, while most of the three-fold interactions in CAZ demonstrate positive interactions. Overall, these observations signify the alteration of the fitness landscape topography across different antibiotics.

In addition, the four ruggedness measures also depict that CTX landscape is more rugged than CAZ landscape. This points to a slightly higher amount of sign epistasis for CTX compared to CAZ. The slight difference of their molecular structures (CTX and CAZ) could be a trigger for the discrepancy of the effect of the mutational interactions in the enzyme. The molecular structures of the substrates may influence the enzyme's substrate specificity, enantioselectivity, prochiral selectivity, regioselectivity, and chemoselectivity [28]. Thus, the mutational interactions would have a dissimilar effect on the enzyme properties and yield different resistance phenotypes.

Another highlight generated from the latter experiment is that the stabilization mutations M182T, T265M, and their combination, do not have a significant impact on the interactions in CAZ, as they do in CTX. There are three possible argumentations for this evidence. First, the interactions were different to begin with because the epistasis interactions between TEM-1 β-lactamase mutants in CTX and CAZ are not exactly the same, which are depicted in their fitness landscape topographies. Second, the stabilizers are not global stabilizers, but their effect depends on the involved mutations. The fitness landscape topographies seem to imply that both M182T and T265M to have the largest effect in combination with G238S. The CTX landscape is dominated by the effect of G238S and the CAZ landscape is dominated by the effect of R164S. Hence, the stabilizing mutations have a strong effect in the CTX landscape and not in the CAZ landscape. Lastly, the negative interactions are not only caused by general stability defects, but also probably due to specific structural interactions between mutations.

In this study, the mutants conferring the same MIC values present a negative issue, because we cannot infer which mutant is better, thus it leads to a huge impact on the ruggedness of the landscape. Another drawback found is that the setting of real no sign epistasis and magnitude sign epistasis as no sign epistasis. The argument for the latter is the limitation of the MIC assay, which is incapable of distinguishing between no sign epistasis that results from an additive effect and magnitude sign epistasis.

Overall, environmental conditions such as temperature and salinity have limited impacts on the interaction between beneficial mutations in TEM-1 β-lactamase and their effects on the fitness landscapes are relatively minor, whereas the use of a different selective agent (antibiotic) has a strong impact on epistasis and significantly alters the fitness landscape.

5. CONCLUSIONS

The conclusions that can be inferred from this study are:

- The stabilization mutations: M182T, T265M, and their combinations, alleviate the epistatic interactions between mutations in TEM-1 β-lactamase. However, the same combination mutations do not confer the same interactions across different antibiotics.
- Temperature influences epistasis but the changes in fitness landscapes are relatively minor. The epistatic interactions between mutations in TEM-1 β-lactamase increase with the increasing temperatures.
- Salinity confers little or no impact on the epistasis between mutations in TEM-1 β-lactamase. The fitness landscapes remain the same across different salinities.
- Epistasis and fitness landscapes significantly change in across different antibiotics. CTX landscape is more rugged than CAZ landscape.

6. REFERENCES

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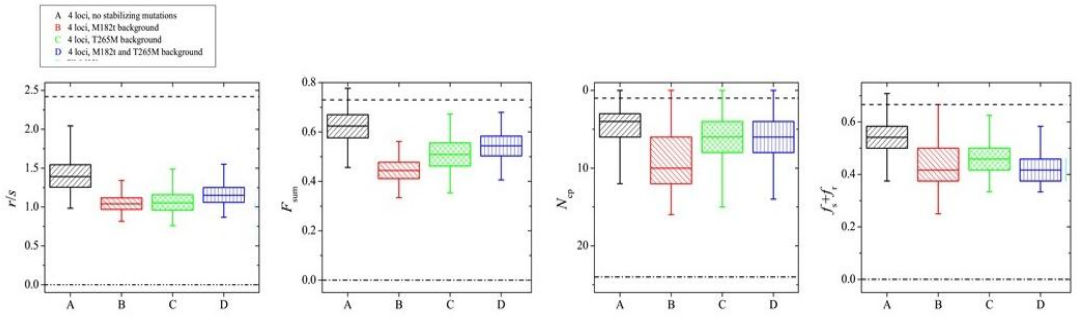
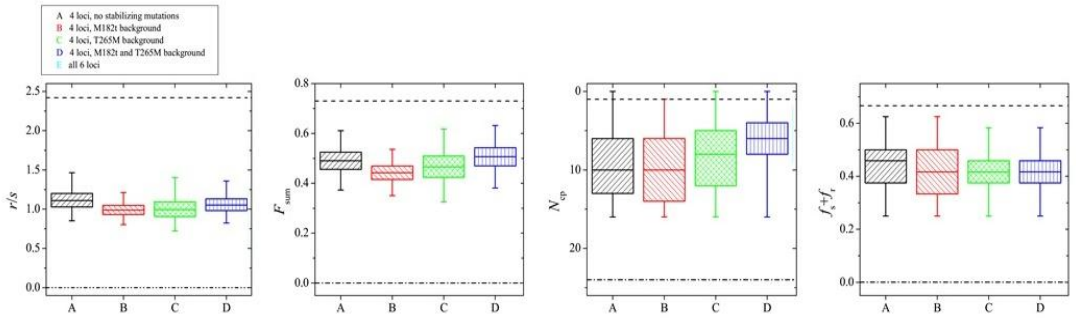
7. APPENDIXES

Appendix 1. Figures of the four ruggedness measurements

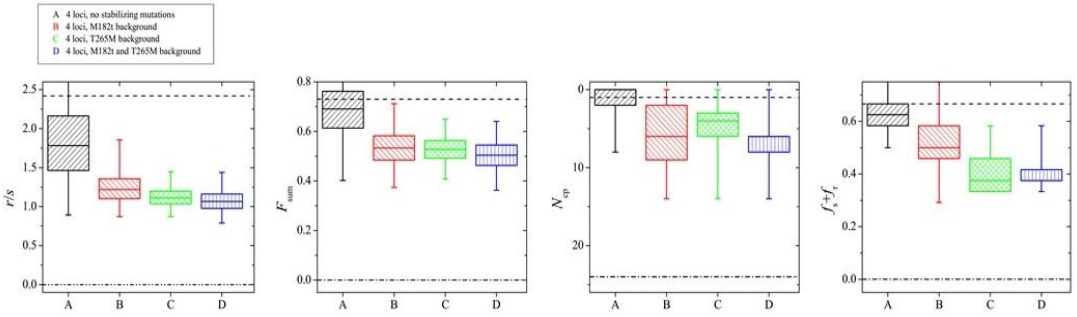
1. The ruggedness measurements at standard conditions: CTX, 37^{0}C and 5g/L



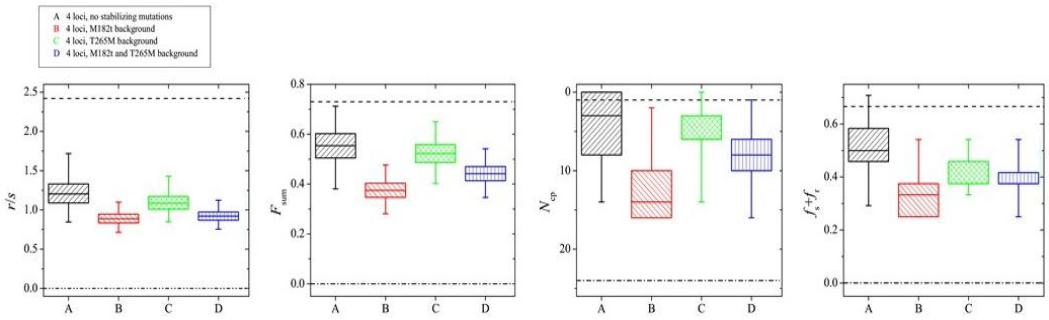
2. The ruggedness measurements at temperature 25^{0}C



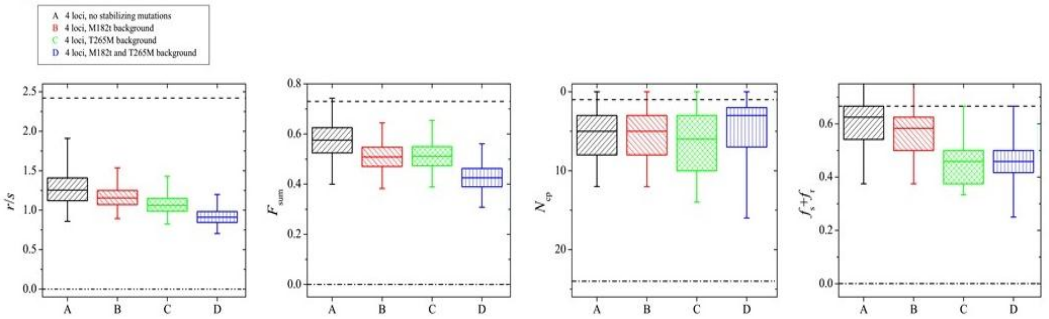
3. The ruggedness measurements at temperature 40^{0}C



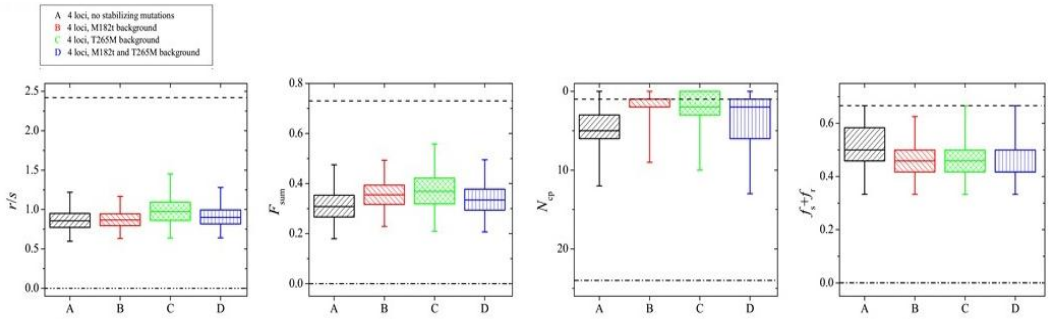
4. The ruggedness measurements at low salinity (1g/L)



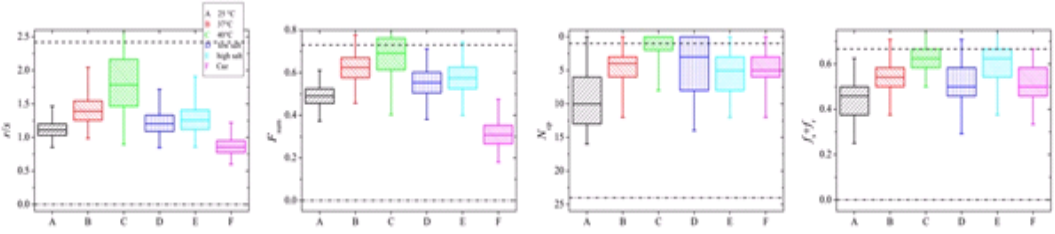
5. The ruggedness measurements at high salinity (25g/L)



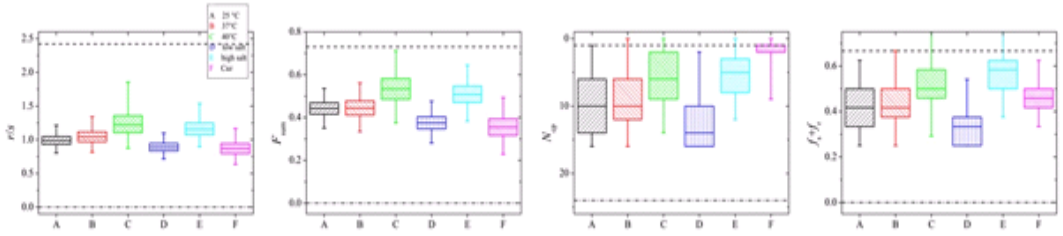
6. The ruggedness measurements on Ceftazidime



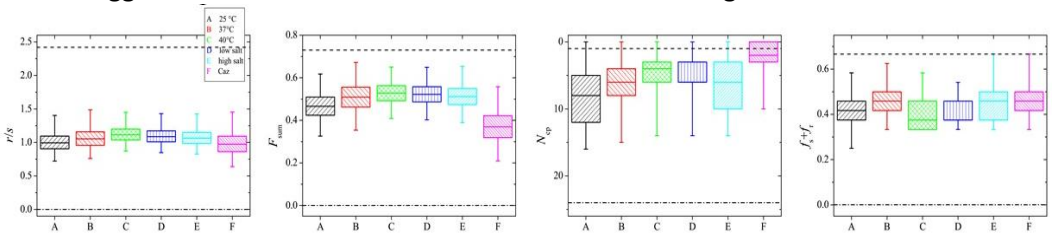
7. The ruggedness measurements on 4 loci without stabilization mutation



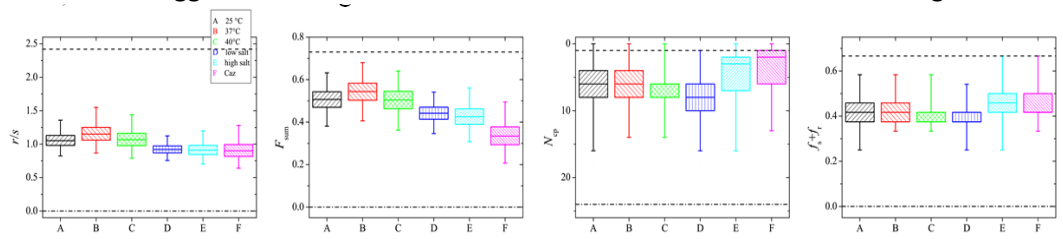
8. The ruggedness measurements on 4 loci with M182T background



9. The ruggedness measurements on 4 loci with T265M background

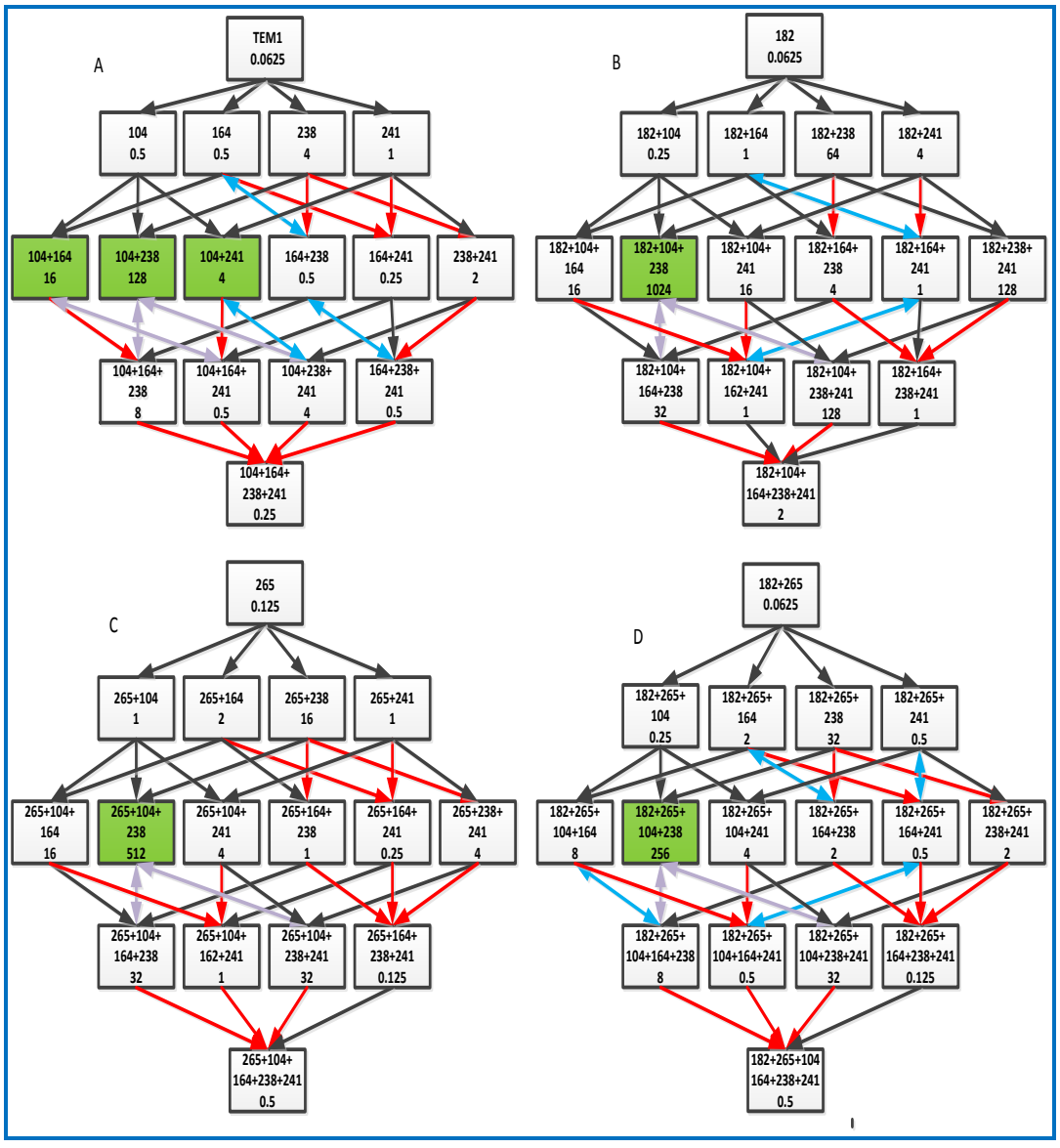


10.The four ruggedness measurements on 4 loci with M182T and T265M background

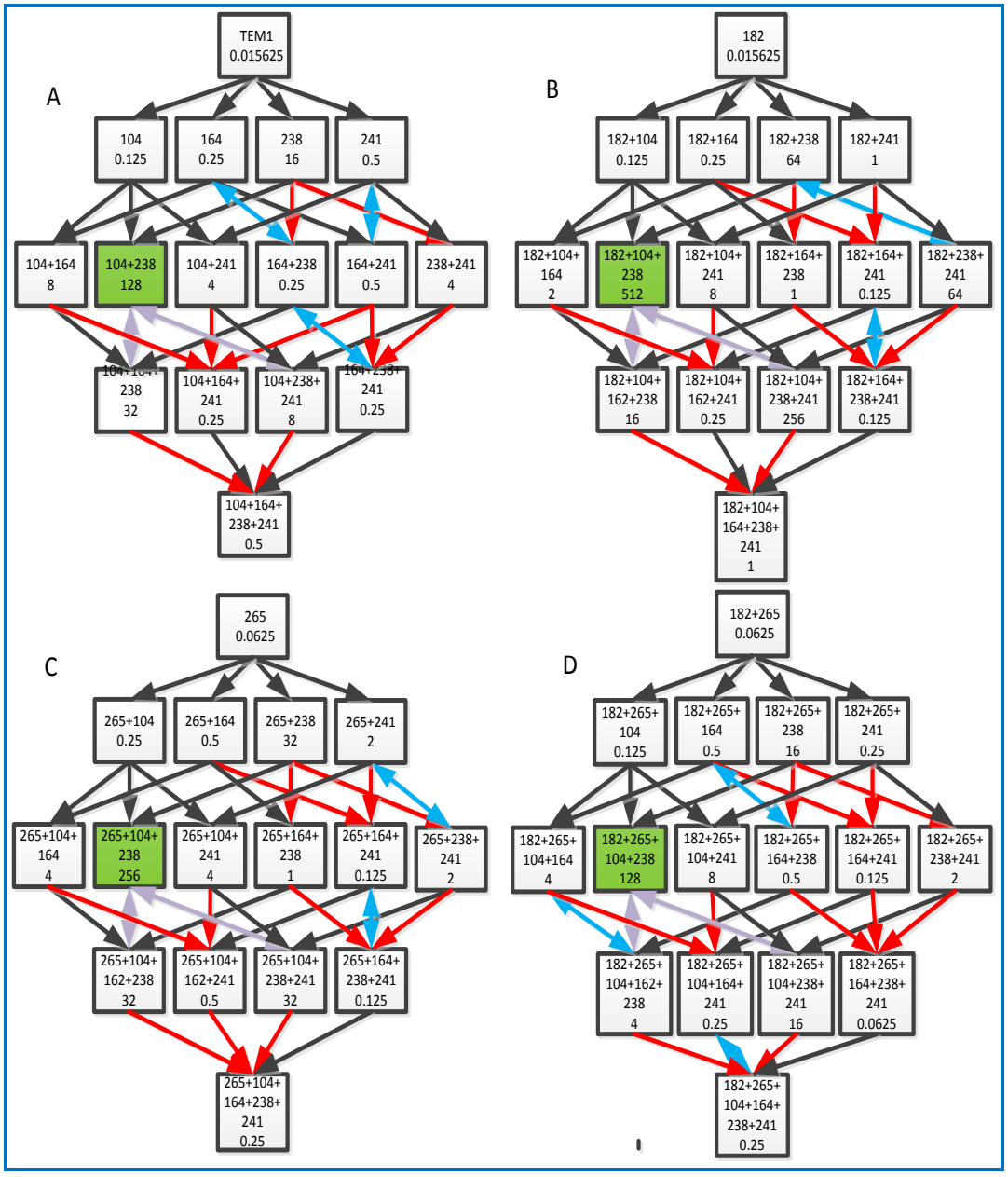


Appendix 2. Topographies of the fitness landscapes

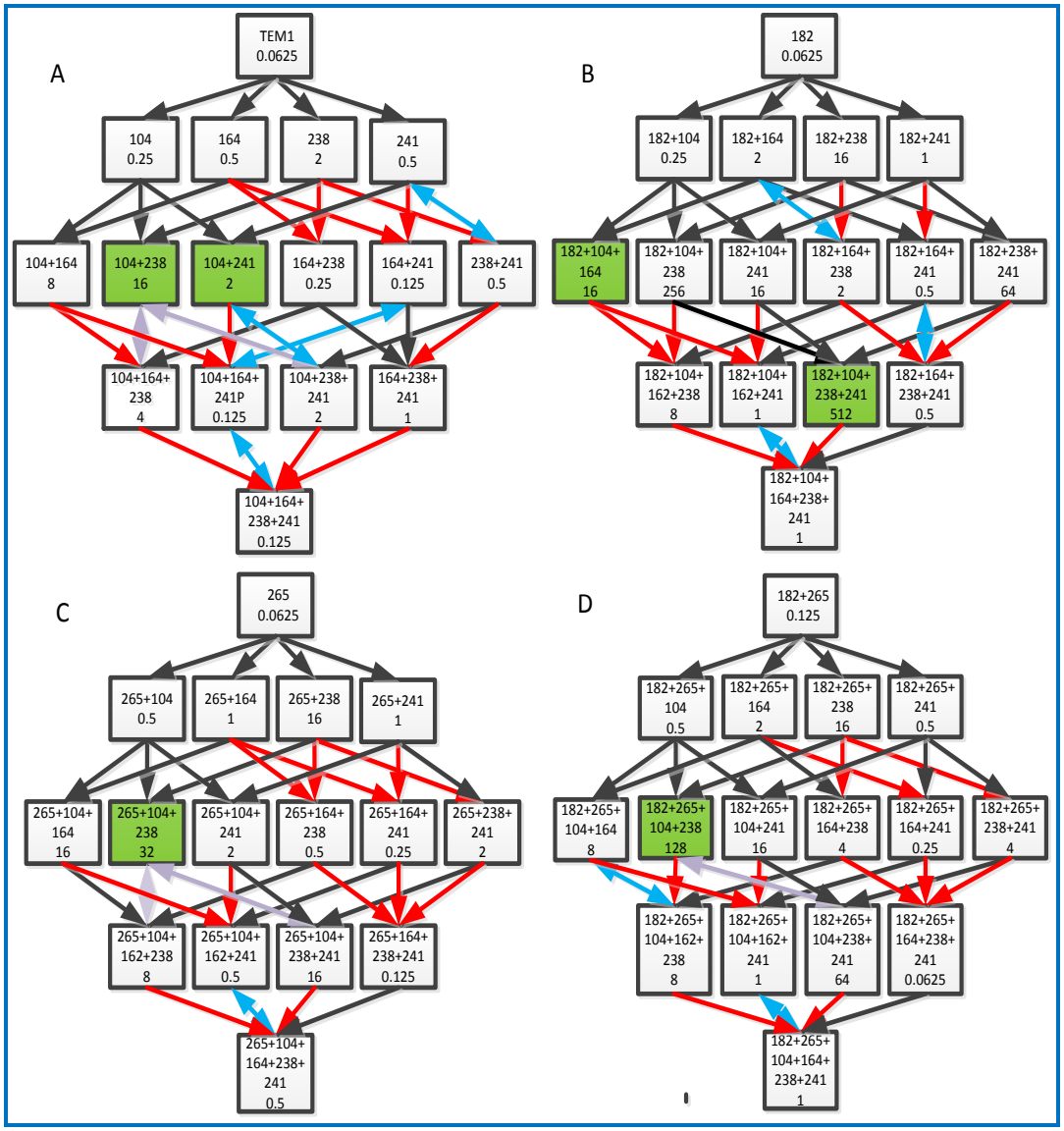
1. Fitness landscape topographies at standard conditions: CTX, temperature 37^0C and salinity 5g/L



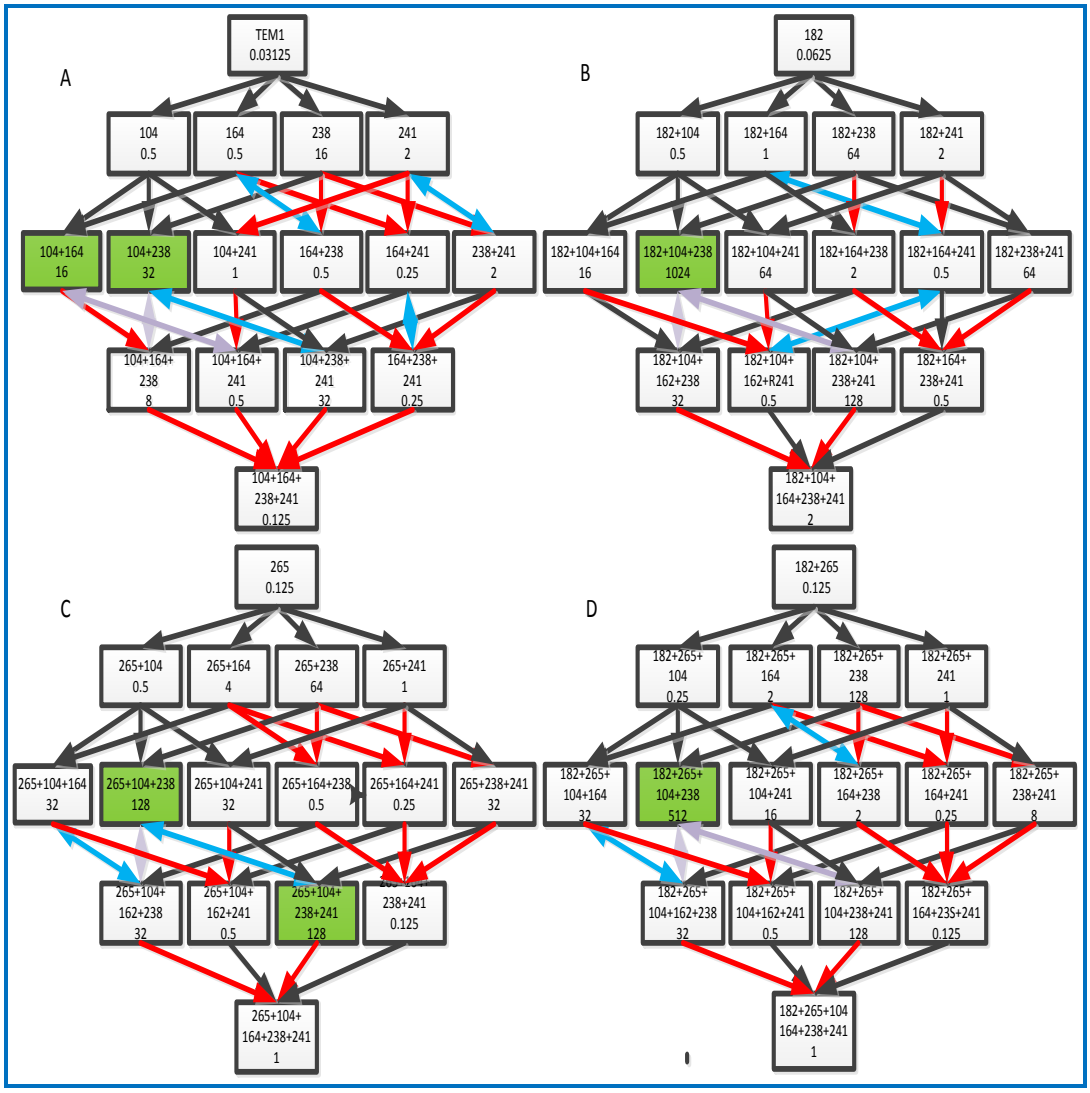
2. Fitness landscape topographies at temperature 25^0C



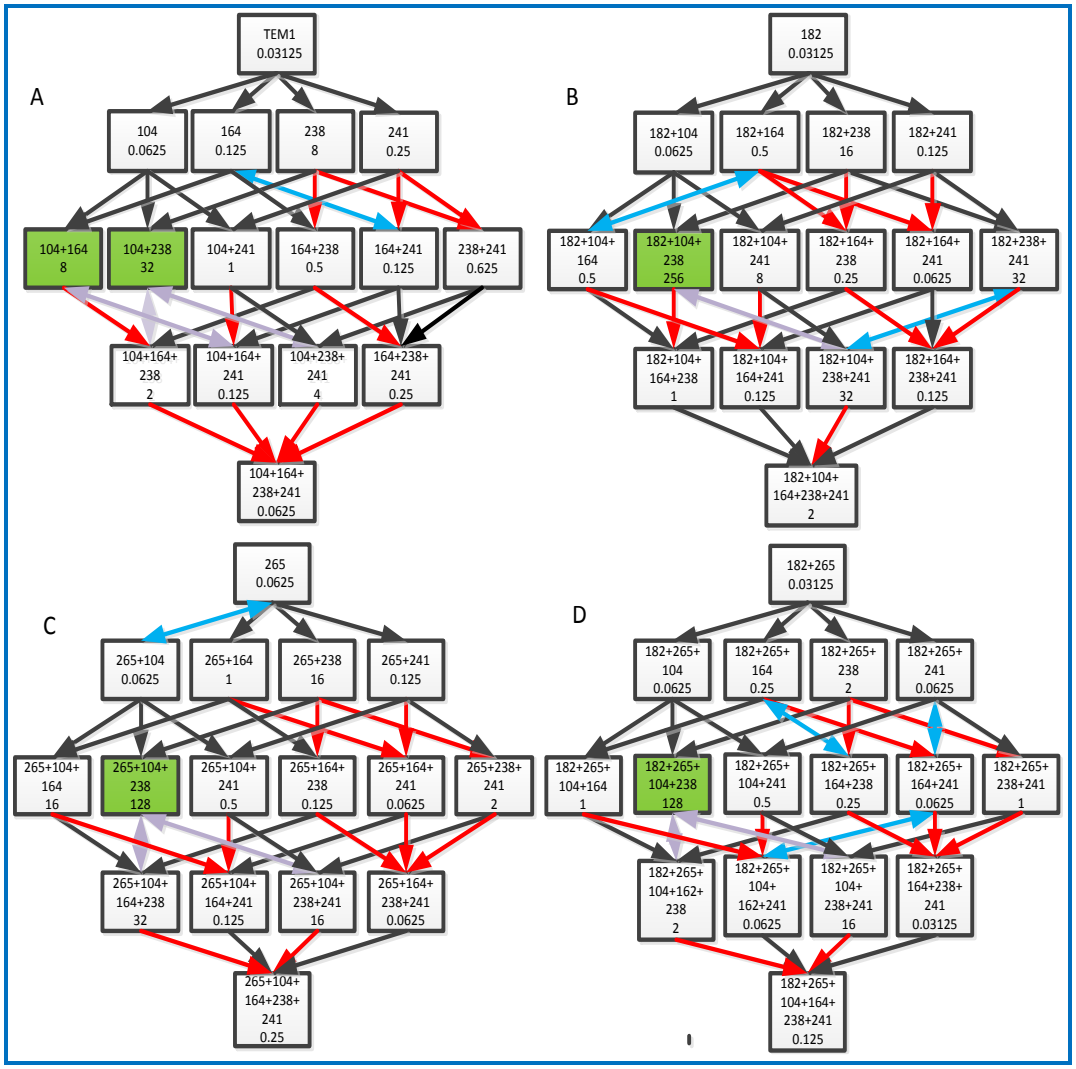
3. Fitness landscape topographies at temperature 40^0C



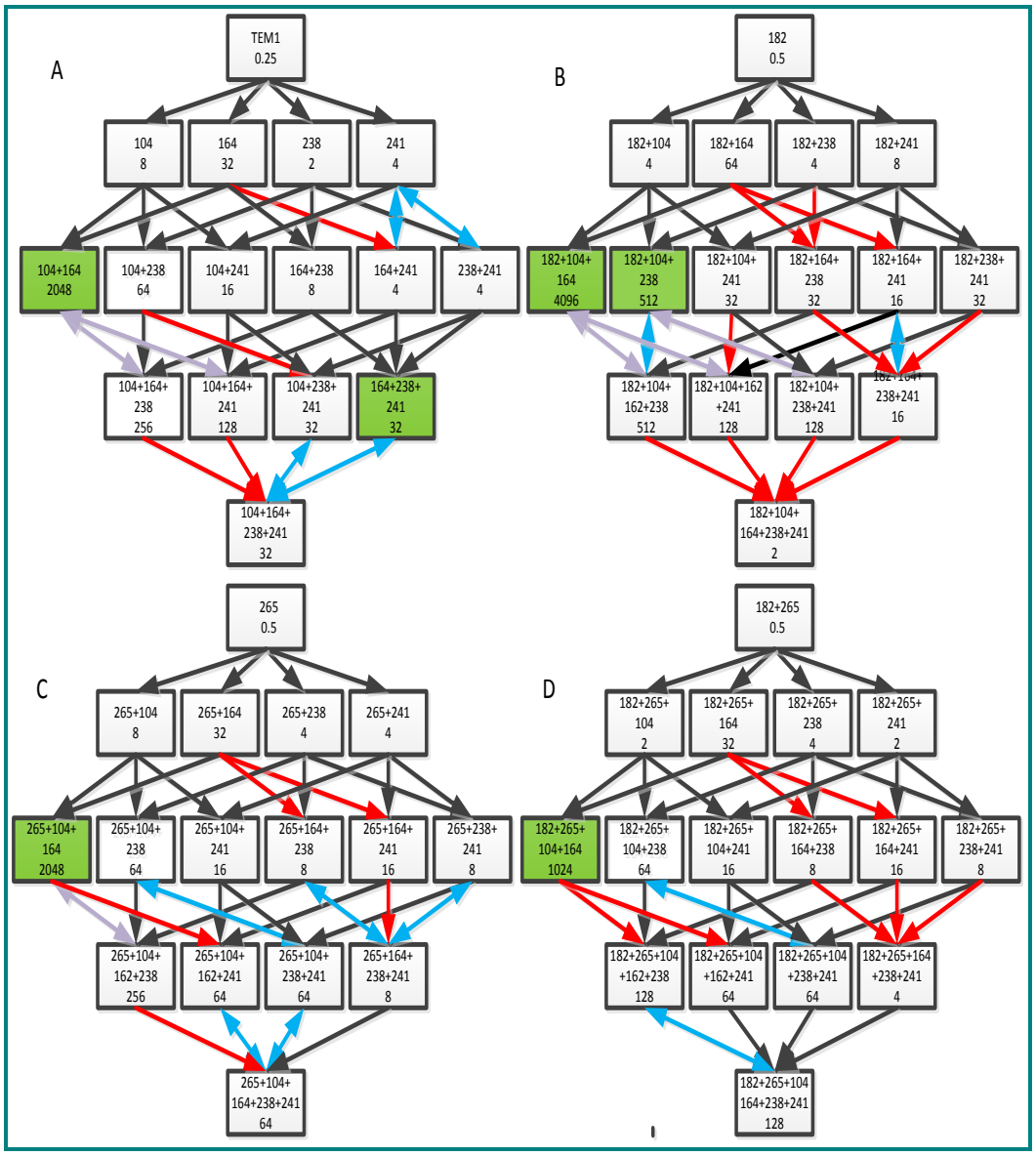
4. Fitness landscape topographies at salinity 1g/L



5. Fitness landscape topographies at salinity 25g/L



6. Fitness landscape topographies on Ceftazidime



Appendix 3. Ruggedness Measures on Pairwise Interaction

The interactions between all possible sets of two mutations were classified into sign epistasis, reciprocal epistasis or no sign epistasis (Figure 11). Here, the highest MIC value was considered to be the peak. The starting point of the mutational trajectory was pull from its antipode or the lowest MIC value. If the peak is accessible from both pathways, it is assigned as no sign epistasis. In this context, real no sign epistasis and magnitude epistasis were classified as no sign epistasis. Furthermore, both the accessible peak from one path and neutral effect were set as sign epistatic. Reciprocal epistasis is a case for the highest interaction and a sub-maximal peak which were accessed from both low values. The numbers of each type of the interactions from all treatment were summarized in Table 2.

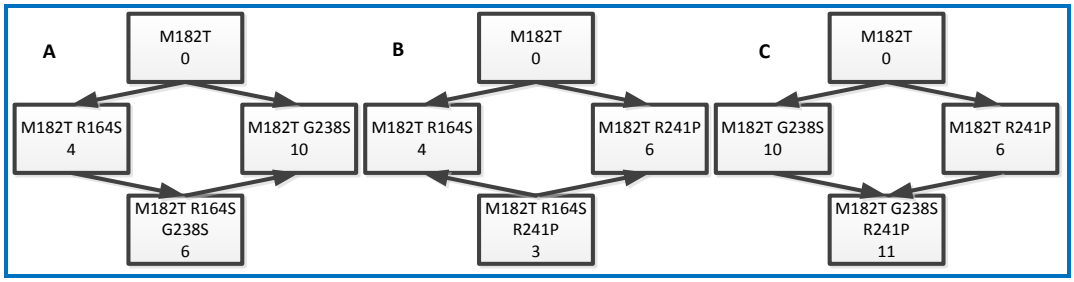


Figure 11. A. Sign epistasis, B. reciprocal sign epistasis, C. No sign epistasis

Table 2. Summary of F_s+F_r in all treatments

Treatments	Sign epistasis			Reciprocal epistasis	No sign epistasis
	Sign epistasis	Neutral	Total		
Cefotaxime					
Temperature 37°C and Salinity 5g/L	15	27	42	11	43
Temperature 25°C	14	24	38	11	47
Temperature 40°C	9	22	31	19	46
Salinity 1g/L	19	28	47	14	35
Salinity 25g/L	21	21	42	14	40
Ceftazidime					
Temperature 37°C and Salinity 5g/L	20	32	52	8	36

Appendix 4. Minimum inhibitory concentration values

Codes	Mutants	Treatments					
		25^{0}C	37^{0}C	40^{0}C	Salinity 1g/L	Salinity 25g/L	CAZ
A. Without stabilization mutation							
1	No	0.015625	0.0625	0.0625	0.03125	0.03125	0.25
2	E104K	0.125	0.5	0.25	0.5	0.0625	8
3	R164S	0.25	0.5	0.5	0.5	0.125	32
4	G238S	16	4	2	16	8	2
5	R241P	1	1	0.5	2	0.25	4
6	E104K R164S	8	16	8	16	8	2048
7	E104K G238S	128	128	16	32	32	64
8	E104K R241P	4	4	2	1	1	16
9	R164S G238S	0.5	0.5	0.25	0.5	0.5	8
10	R164S R241P	0.25	0.25	0.125	0.25	0.125	4
11	G238S R241P	4	2	0.5	2	0.0625	4
12	E104K R164S G238S	32	8	4	8	2	256
13	E104K R164S R241P	0.25	0.5	0.125	0.5	0.125	128
14	E104K G238S R241P	16	4	2	32	4	32
15	R164S G238S R241P	0.25	0.5	1	0.25	0.25	32
16	E104K R164S G238S R241P	0.5	0.25	0.125	0.125	0.0625	32

Codes	Mutants	Treatments					
		25^{0}C	**37^{0}C**	**40^{0}C**	**Salinity 1g/L**	**Salinity 25g/L**	**CAZ**
B. With stabilization mutation M182T							
17	M182T	0.03125	0.0625	0.0625	0.0625	0.03125	0.5
18	M182T E104K	0.125	0.25	0.25	0.5	0.0625	4
19	M182T R164S	0.25	1	2	1	0.5	64
20	M182T G238S	64	64	16	64	16	4
21	M182T R241P	1	4	1	2	0.125	8
22	M182T E104K R164S	8	16	16	16	0.5	4096
23	M182T E104K G238S	512	1024	256	1024	256	512
24	M182T E104K R241P	16	16	16	64	8	32
25	M182T R164S G238S	2	4	2	2	0.25	32
26	M182T R164S R241P	0.25	1	0.5	0.5	0.0625	16
27	M182T G238S R241P	32	128	64	64	32	32
28	M182T E104K R164S G238S	32	32	8	32	1	512
29	M182T E104K R164S R241P	0.25	1	1	0.5	0.125	128
30	M182T E104K G238S R241P	64	128	512	128	32	128
31	M182T R164S G238S R241P	0.25	1	0.5	0.5	0.125	16
32	M182T E104K R164S G238S R241P	1	2	1	2	2	2

Codes	Mutants	Treatments					
		25^{0}C	37^{0}C	40^{0}C	Salinity 1g/L	Salinity 25g/L	CAZ
C. With stabilization mutation T265M							
33	T265M	0.0625	0.125	0.0625	0.125	0.0625	0.5
34	T265M E104K	0.25	1	0.5	0.5	0.0625	8
35	T265M R164S	0.5	2	1	4	1	32
36	T265M G238S	32	16	16	64	16	4
37	T265M R241P	2	1	1	1	0.125	4
38	T265M E104K R164S	4	16	16	32	16	2048
39	T265M E104K G238S	1024	512	32	128	128	64
40	T265M E104K R241P	4	4	2	32	0.5	16
41	T265M R164S G238S	2	1	0.5	0.5	0.125	8
42	T265M R164S R241P	0.25	0.25	0.25	0.25	0.0625	16
43	T265M G238S R241P	4	4	2	32	2	8
44	T265M E104K R164S G238S	32	32	8	32	32	256
45	T265M E104K R164S R241P	0.5	1	0.5	0.5	0.125	64
46	T265M E104K G238S R241P	64	32	16	128	16	64
47	T265M R164S G238S R241P	0.125	0.125	0.125	0.125	0.0625	8
48	T265M E104K R164S G238S R241P	0.5	0.5	0.5	1	0.25	64

Codes	Mutants	Treatments					
		25^{0}C	37^{0}C	40^{0}C	Salinity 1g/L	Salinity 25g/L	CAZ
D. With stabilization mutation M182T and T265M							
49	M182T T265M	0.0625	0.0625	0.125	0.125	0.03125	0.5
50	M182T T265M E104K	0.125	0.25	0.5	0.5	0.0625	2
51	M182T T265M R164S	1	2	2	2	0.25	32
52	M182T T265M G238S	16	32	16	128	2	4
53	M182T T265M R241P	0.25	0.5	0.5	1	0.0625	2
54	M182T T265M E104K R164S	8	8	8	32	1	1024
55	M182T T265M E104K G238S	256	256	128	512	128	64
56	M182T T265M E104K R241P	4	4	16	16	0.5	16
57	M182T T265M R164S G238S	0.5	2	1	2	0.25	8
58	M182T T265M R164S R241P	0.125	0.5	0.25	0.25	0.0625	16
59	M182T T265M G238S R241P	2	2	4	8	1	8
60	M182T T265M E104K R164S G238S	8	8	8	32	2	128
61	M182T T265M E104K R164S R241P	0.25	0.5	1	0.5	0.0625	64
62	M182T T265M E104K G238S R241P	32	32	64	128	16	64
63	M182T T265M R164S G238S R241P	0.0625	0.125	0.0625	0.125	0.03125	4
64	M182T T265M E104K R164S G238S R241P	1	0.5	1	1	0.125	128
94	pACSE3	0.015625	0.03125	0.0625	0.0625	0.03125	0.25

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