TESTING THE BENEFITS OF RECOMBINATION WITH AN ANTIBIOTIC RESISTANCE ENZYME

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ABSTRACT

The evolution of sex can be distinguished into the origin and the maintenance of sex. Sex involves recombination between closely related sequences, which is a random process. This process can speed up adaptation by bringing together beneficial allelic combinations but also break them apart. One of the ways to study recombination is by exploring the fitness landscape. The effect of recombination is shown to be dependent on epistatic interactions. Epistatic interactions affect the topology of the fitness landscape making it more rugged. Furthermore, the conditions in which recombination would be beneficial in long term are not yet well understood. This presents the necessity to explore the evolutionary effect of recombination in nature. Previous experiments have already shown recombination benefit on a model enzyme TEM-1 β-lactamase gene. Hence, this research was directed towards using laboratory evolution with and without recombination to study the adaptation of a well-adapted genotype of TEM-1 gene, called TEM-15 β-lactamase. Our results indicated that recombination does not show benefits in a well-adapted genotype at population level. However, upon analysing lines individually, some lines do indicate recombination benefit. This approach enlightened us about the evolutionary role of sex and recombination, and their impact on the complex fitness landscapes.

INTRODUCTION

The question why sex is the preferred choice for reproduction by many organisms remains an unresolved problem in biology. Sex involves random recombination of homologous DNA and RNA sequences. This process can either create beneficial allelic combinations or may also break the existing beneficial allelic bonds (Pesce, Lehman, & de Visser, 2016).

Mutations and recombination are key players in creating genetic diversity. To study the genetic diversity, it is beneficial to explore the fitness landscape. Epistatic interactions greatly affect the topology of the fitness landscape. Depending on the topology, recombination exhibits short term (A-early in adaptation) and long-term effects (B-later in adaptation) as seen in figure 1 (Pesce et al., 2016) (Salverda et al., 2011).

Sex and recombination remain an evolutionary paradox, because there have been several theories proposed with contradictory results in exploring the benefits of recombination in evolution (Salverda et al., 2011). Hence, this research was focused towards exploring the long-term benefits of a well-adapted genotype (TEM-15 β-lactamase) in the fitness landscape. To do so, we performed directed evolution of TEM-15 with and without recombination.

Paradox of sex and recombination

Sex presents an evolutionary paradox, considering it is costly and risky in evolutionary terms but at the same time is ubiquitous among eukaryotes (Otto & Lenormand, 2002). There are several hypotheses proposed to describe why sex is advantageous. Furthermore, asexual reproduction is occasionally combined with sexual reproduction (Sharp & Otto, 2016). This diversifies the plethora of hypotheses on the paradox of sex. Testing these hypotheses individually or in combinations can be challenging, making the paradox of sex not only an academically relevant question but also a topic of interest to many research fields such as plant sciences.

Recombination is a random process which eventually generates either allelic combinations which are beneficial together (Fisher-Muller effect) or may also break the existing beneficial allelic associations (Recombination load) (Pesce et al., 2016). Mutations and recombination together contribute to genetic diversity. One way to study genetic diversity is by exploring the fitness landscape (figure 1). To explore the fitness landscape, it is imperative to understand the effect of recombination, which in turn depends on the epistatic interaction. This is because epistatic interaction affects the topology of the fitness landscape resulting in different effects of short term and long-term effect (Kouyos, Silander, & Bonhoeffer, 2007).

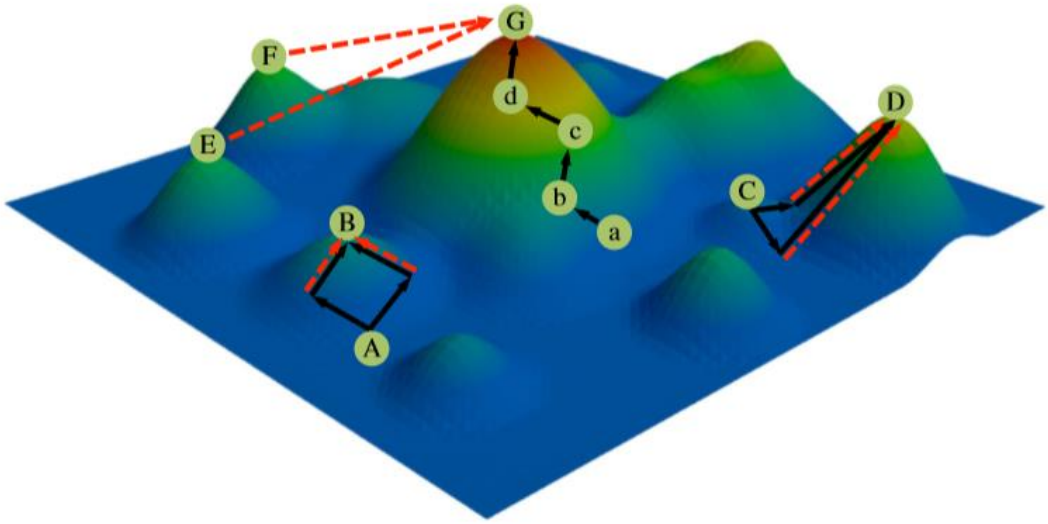


Figure 1: Schematic representation of the fitness landscape with the fitness representing the y axis and the genotype space representing the x axis. Black solid arrows indicate asexual reproduction, while red arrows indicate sexual reproduction. a,b,c and d indicate the transitions in genotype through mutations to reach point G (asexual mode). In comparison, recombination between peak genotypes E and F might lead to a faster way to reach point G. Two additional contrasting scenarios reflecting short-terms and long-term effects can be seen through points A, B, C and D. Low fitness genotypes point A and C indicate the short-term effects (occurring early in adaptation). Point A and C adapt through a series of mutations to adapt to higher fitness genotypes point B and D respectively, which indicate the long-term effects (occurring later in adaptation) (Pesce et al., 2016).

The short term effects of recombination occur within the population in an early stage of adaptation while the long term effects occur during the later stage (Pesce et al., 2016).

Epistatic interactions cause a population to prefer one pathway (Salverda et al., 2011). A mutational pathway can be defined as a collection of subsequent mutations that bridge the wild type with the highest fitness genotype as seen in figure 1 (point a,b,c,d and G). If there are two mutations involved, the pathway for these mutations will show two possible connections, either mutation A is fixed first preceded by mutation B or vice versa (Franke, Klözer, de Visser, & Krug, 2011).

Furthermore, the different types of epistasis complicate the fitness landscape. The different types of epistasis can be observed in figure 2. This implicates that the real fitness landscapes are probably rugged and contain multiple peaks and valleys (Poelwijk, Kiviet, Weinreich, & Tans, 2007). Of the various types, a stronger interaction i.e. sign epistasis is becoming more pervasive. Sign epistasis refers to stronger interactions wherein the sign of an allele's contribution to fitness changes with the genetic background making it either beneficial or deleterious (Weinreich, Watson, & Chao, 2005).

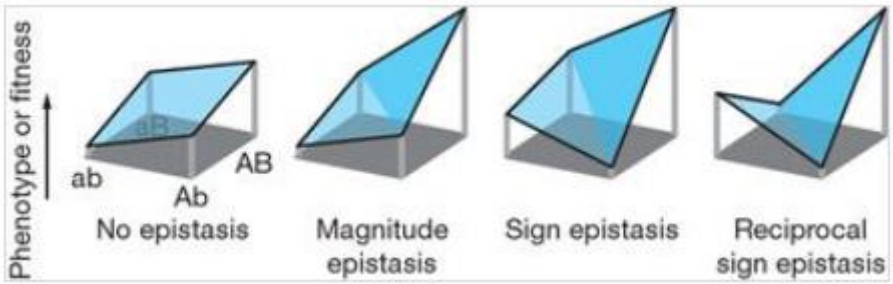


Figure 2: Different manifestations of epistasis, no epistasis- Individually both mutations are beneficial with the combination of the mutations providing benefit as high as the product of the effects of the two single mutations; magnitude epistasis- the combination of the mutations provides either higher or lower effect than the sum of the individual mutations; sign epistasis- an individual mutation is beneficial in one genetic background while it is deleterious in the other; reciprocal sign epistasis- Individually both mutations are beneficial but deleterious in each other's background (Poelwijk, Kiviet, Weinreich, & Tans, 2007).

Here we used directed evolution with or without recombination to study the adaptation of TEM-15 gene to CTX. Previously conducted experiments on the adaptation of TEM-1 gene, showed recombination benefit and therefore a well-adapted variant of TEM-1 was used as a parent gene in this experiment and is emphasised later in the report in order to test the effect of recombination later on during adaptation.

In vitro recombination in laboratory directed evolution.

As mentioned earlier, we used laboratory evolution with and without in vitro recombination to study its effect during the adaptation of TEM-15 gene in controlled conditions.

Directed evolution is an approach used in protein engineering, capable of mimicking the natural evolution of the gene of interest by adjusting its mutational spectrum towards a user defined goal (Salverda, de Visser, & Barlow, 2010). It does so by repeated cycles of mutation, selection and amplification. This approach creates novel functions for proteins. However, it has not yet been used extensively to analyse the effect of recombination. Previous experiments involved the use of organisms such as fungi which do not allow the user to control desired parameters. On the other hand, directed evolution allows the user to control parameters such as mutation rate, population size and strength of selection. Hence, we used in vitro recombination to mimic the natural evolution of TEM-15 gene and select for its increased resistance to cefotaxime (CTX)(Pesce et al., 2016) (Barlow & Hall, 2002).

In vitro mutation and recombination protocols can be combined in directed evolution experiments wherein the conditions and parameters such as mutation rate and selection pressure, can be controlled by the researcher. For instance, by varying the mutation rate, small benefits of recombination can be explored which are otherwise visible only during long evolutionary periods. Figure 3 indicates a general approach wherein; a gene library is generated creating variants using in vitro mutagenesis methods such as error-prone PCR or gene shuffling. The library is then screened/ selected for the desired function (or phenotype), followed by gene isolation. The selected genes encoding the desired improved enzymes are used as parents for the next round of evolution.

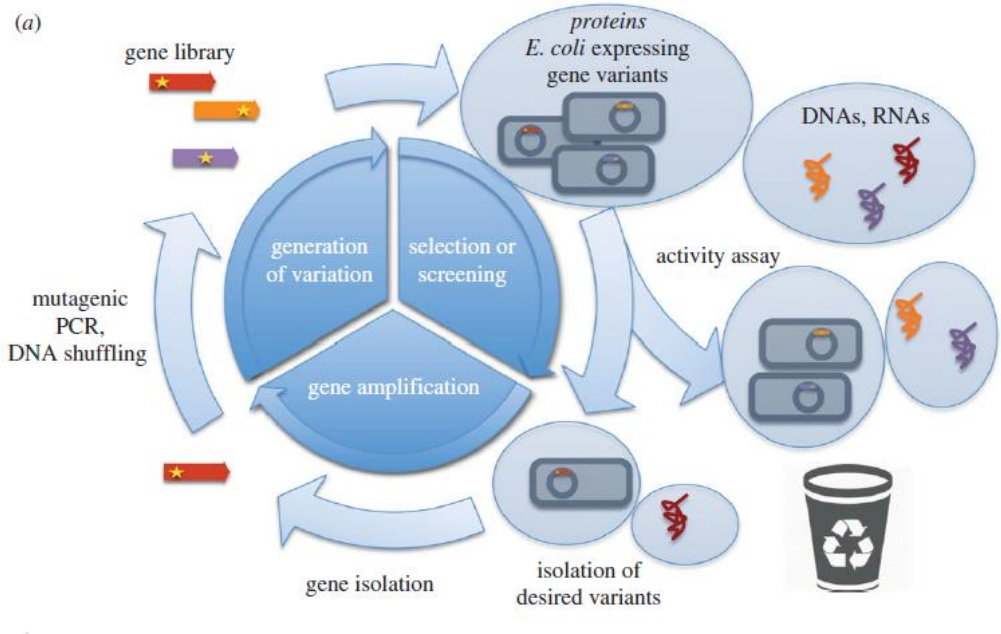


Figure 3: Schematic representation of the laboratory directed evolution approach (Pesce, Lehman, & de Visser, 2016).

Model enzyme: TEM-1 β-lactamase

TEM-1 β-lactamase is one of the most common antibiotic resistance determinants. Its mode of action involves the hydrolysis of the β lactam ring of penicillin, cephalosporins and related β lactam antibiotics. It is imperative to study this antibiotic resistance genes which is constantly evolving and acquiring novel abilities such as substrate specificities to new β lactams due to changes in its genotype which it contributes to the failure of β lactam treatment in patients (Salverda et al., 2010).TEM-1 is a model system used in protein engineering due to the availability of elaborate information on gene sequence. This information is beneficial for random mutagenesis and easy selection for increased antibiotic resistance, since it links the phenotype to the genotype. (Barlow & Hall, 2002). Furthermore, TEM-1 is naturally active against to Ampicillin but not to CTX. This makes it a good model system to study evolution in the lab since its adaptation to CTX via multiple mutational pathways can be analysed. Figure 4 showcases the chemical structures of Ampicillin and Cefotaxime with their β-lactam rings.

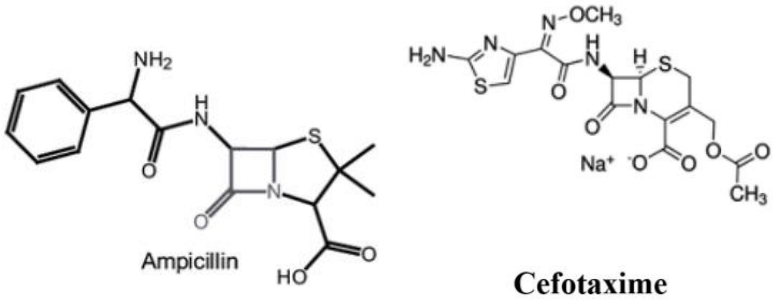


Figure 4: Chemical structure of β *lactams antibiotics. All structures contain a* β *lactam ring* (Baeza-Fonte et al., 2018).

There are several known clinical isolates of TEM-1 gene that confer resistance to CTX. One such genotype is TEM-15, which has a double mutant E104K – G238S and was used as the starting genotype in this experiment. As described earlier from figure 1, TEM-1 gene falls at point A in the fitness landscape, as a low fitness genotype. While TEM-15 gene is indicated as point B as a well-adapted genotype.

PROBLEM STATEMENT

The origin of sex and recombination is an evolutionary paradox, considering it is costly and risky in evolutionary terms but at the same time is ubiquitous among eukaryotes. Recombination generates molecular diversity. However, the process is random and hence it can either bring beneficial mutations together or break them apart. The complexity increases with the role of sign epistasis making the fitness landscape rugged with multiple peaks and valleys, leading to the asexual population to limit the production of new phenotypic variants. Previous research has been more focused on exploring effects of recombination in organisms like fungi which does not allow the user to control certain parameters. Hence, a model system (such as TEM-1) is ideal to explore the evolutionary effects of recombination. Previous experiments conducted in our lab using TEM-1 showed recombination benefits (unpublished data). However, the conditions in which recombination would be beneficial in the long-term effects is not well understood. Would recombination still be beneficial when beneficial mutations are rarer? Therefore, the long-term effects of a well-adapted genotype can be tested to implicate if recombination is beneficial or not.

RESEARCH AIM

The purpose of this research is to explore the long-term effects of recombination in evolutionary terms considering the role it plays in creating a complex fitness landscape. We performed directed evolution of a well-adapted variant of TEM-1, TEM-15 β-lactamase (E104K – G238S), with and without recombination in order to understand the long-term effects of recombination. By using directed evolution, we could control the evolutionary parameters such as mutation rate, population size and strength of selection.

RESEARCH METHODOLOGY

To generate the variant library of double mutant G238S-E104K (TEM-15) (figure 5), we used in vitro mutagenesis through error prone PCR (epPCR) at high mutation rate. After that the resulting library was cloned into the expression vector and transformed into *E. coli*. Followed by pre-selection of transformants on a medium containing appropriate antibiotic to get rid of deleterious mutations. Furthermore, the plasmid was extracted from the cells containing different variants of TEM-15. The selected variants were subjected to in vitro recombination through Staggered Extension Process PCR (StEP PCR). As a control the remaining were maintained for asexual reproduction. The recombinant TEM-15 alleles were cloned back into the vector and then transformed into host bacteria prior to long selection. Long selection identified the fittest clone by exposure to increasing concentrations of antibiotic. The fittest genotype was selected for

further characterization. The genetic changes of the selected alleles were characterized using Sanger sequencing the phenotypic changes were observed by quantifying the Minimal Inhibitory Concentration (MIC).

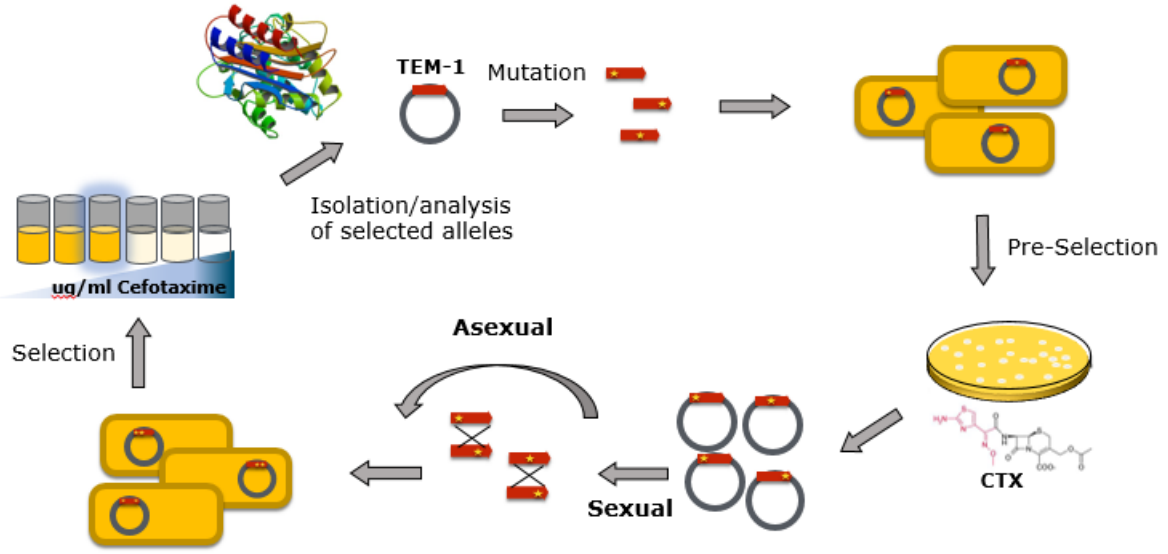


Figure 5: Overview of the experimental set up to analyse selected alleles

RESULTS

Laboratory Directed Evolution

Random mutagenesis

To estimate the number of mutations introduced by the epPCR, the mutation rate was determined by sequencing 24 randomly picked colonies of sample 3. A total of 79 transitions and 89 transversions were observed. This technique was able to achieve a mutation rate of 6 mutations per gene.

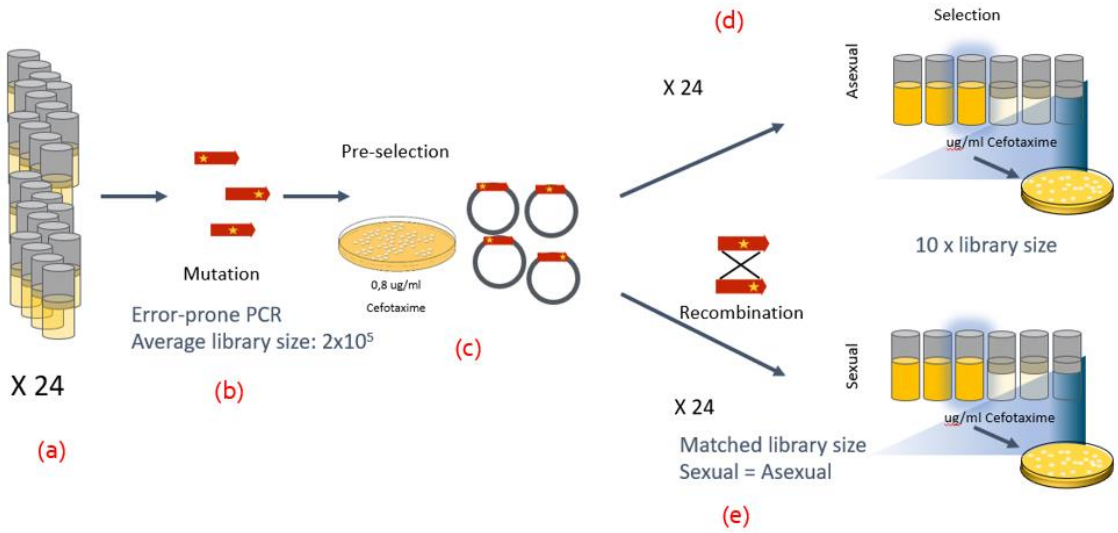


Figure 6: Selection with paired line- (a) 24 TEM15 lines are prepared. (b) To create random mutagenesis, the lines are exposed to epPCR with an average library size of 2x10^5 *and transformed. (c) The lines are preselected at 0.8 µg/ml of CTX and re-transformed. (d) 24 lines of asexual are selected on a gradient concentration of CTX and MIC determined. € 24 lines of sexual are obtained through StEP PCR, followed by selection and MIC assay.*

Pre-selection

To oust out all non-functional mutations, a pre-selection procedure was carried out. For pre-selection, the cultures were plated on LB agar with CTX concentration of 0.8 µg/mL and incubated overnight at 37°C. Followed by plasmid extraction of the O/N cultures.

Recombination

To promote cross over events, the transformants were subjected to Staggered Extension PCR (StEP PCR). As observed in figure 7, the principle of StEP PCR involved using full length homologous genes as templates for synthesising chimeric progeny genes.

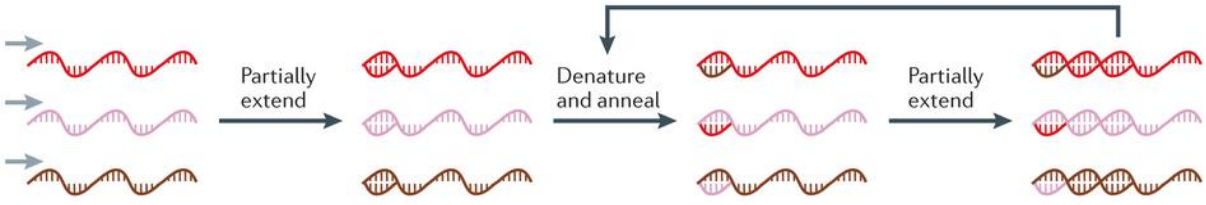


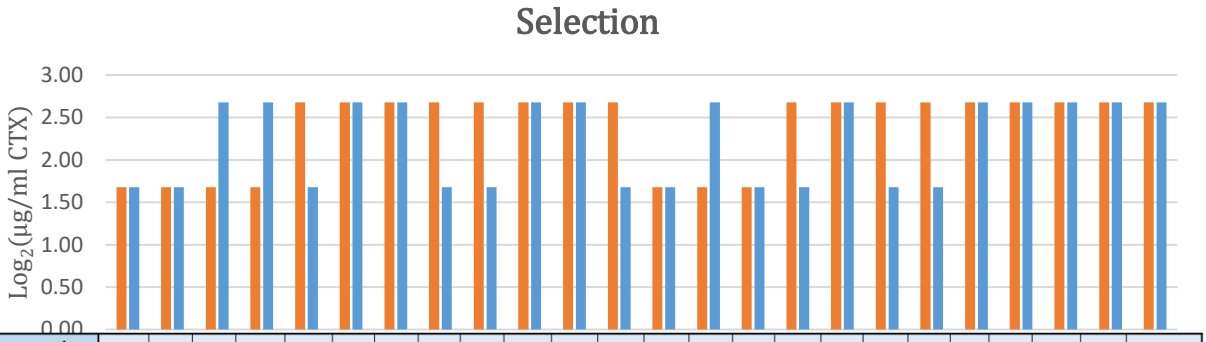
Figure 7 : Schematic representation of StEP PCR protocol (Zhao, Giver, Shao, Affholter, & Arnold, 1998)

Based on the protocol, the denatured templates were primed, followed by repeated cycles of denaturation and very short annealing and extension steps. These partially extended primers randomly annealed to different templates based on sequence homology and extend further, this gave rise to recombination events. StEP PCR was further continued until full length genes are formed (Zhao & Zha, 2006). The library size was kept constant throughout the protocol to ensure the lines can be compared to each other at every step.

For this experiment the population size of around 10x the library size was used for each step. The average library size obtained through recombination was 7.26E+05.

Long Selection

To find the different resistant genotypes present in each line, approximately 10X library size of the cultures were plated on big plates with varying concentrations of CTX (12.8µg/ml, 6.4µg/ml and 3.2 µg/ml) and incubated overnight at 37°C. As observed in figure 8, for each line the largest colony growing at the highest concentration were picked and their genotypes were determined.



	Treatment/Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
#colonies	Sex	14	52	5	87	1	13	1	4	5	57	10	3	1	127	26	3	15	24	1	12	2	7	14	4
	Asex	9	26	5	6	31	1	6	3	28	6	2	17	10	1	8	122	2	27	16	1	2	5	12	4

Figure 8: Selection of asexual and sexual lines in terms of CTX concentrations along with the number of colonies present at the highest concentration for each treatment.

Phenotypic Characterization

To determine the resistance level of each genotype, the minimum inhibitory concentration was measured (Appendix 1). The gradient concentrations used of CTX can be seen in Appendix 2.

To avoid inoculum effect, we used10^4 cells per µl. 1µl of all lines were then spot plated on each concentration. The plates were incubated for 24 hours at 37°C. The growth was determined by visual inspection, and MIC was stipulated as the lowest concentration of antibiotic that completely prevented visible growth.

The MIC of all lines were compared to wild type which was 1.6µg/ml. To analyse if recombination has beneficial effects on the MIC, the recombination benefit (ΔMIC) was calculated by subtracting the MIC of asexual lines from sexual lines as seen in figure 9. The plot indicates 12/24 lines showing recombination benefit. However, at population level the data is statistical insignificant. Therefore, the lines were analysed individually to understand the recombination benefit exhibited by 12 lines. MIC fold increase was determined as compared to the wild type shown in figure 9. Higher resistance to CTX was shown by 12/24 lines with sexual treatment. While, 8/24 lines showed higher resistance to CTX with asexual treatment. The remaining lines that is 4/24, show same resistance to CTX with both treatments.

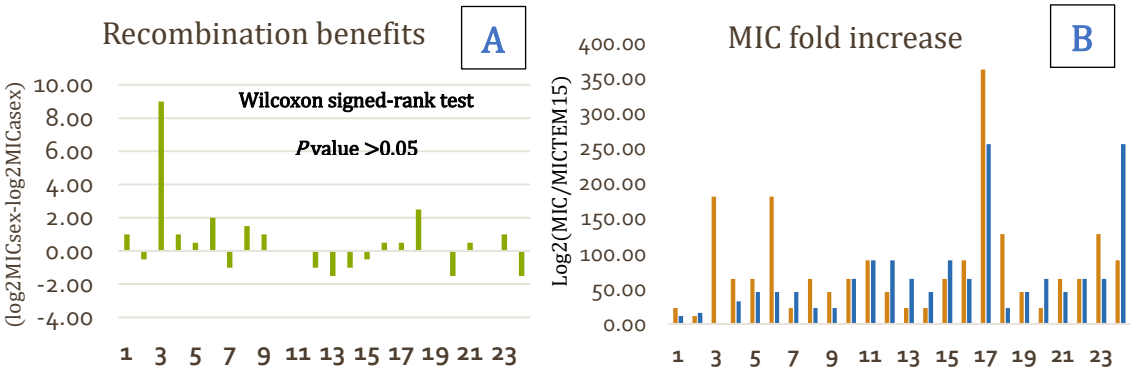


Figure 9: (A) Recombination benefit is calculated ΔMIC = MIC_{Sex}-MIC_{Asex}, along with the P value. (B) The MIC fold increase of all lines is calculated with respect to the MIC of wild type TEM15.

The lines were individually analysed. The asexual treatment of line 3 showed MIC of 0.57µg/ml which is below that of wild type. Two synonymous mutations are seen that is A11* and H153* present in the signal peptide and H alpha helix respectively (figure 12).

In line 3, sexual treatment showed 9-fold increase in resistance to CTX as compared to the asexual treatment as seen in figure 9. The probable reason is the presence of two stabilizing mutations M182T and R241H occurring together in the sexual treatment. The double mutant is not found in any other lines except the sexual treatment of line 3. Recombination brings the two beneficial mutations together and thereby increasing the resistance level to CTX.
In line 17, sexual and asexual treatments had an MIC of 579.26 and 409.6 respectively. The high resistance in both treatments can be due to the presence of mutation A42G. The genotype consisting of A42G mutations along with double mutant E104K – G238S has been previously known to confer the highest level of resistance(Palzkill, 2018). In line 24, the asexual treatment shows an MIC of 409.6 as compared to sexual which is 144.82.

Genotypic Characterization

The recombination benefit is not evident from the MIC of all lines. Hence, investigating the mutations at molecular level can help in understanding if the position and type of mutation influences the resistance. To characterize the lines in terms of genotypes, after 24 hours colonies growing at the highest concentration of CTX are picked, purified and sent for sequencing using sanger sequencing to identify selected mutations.

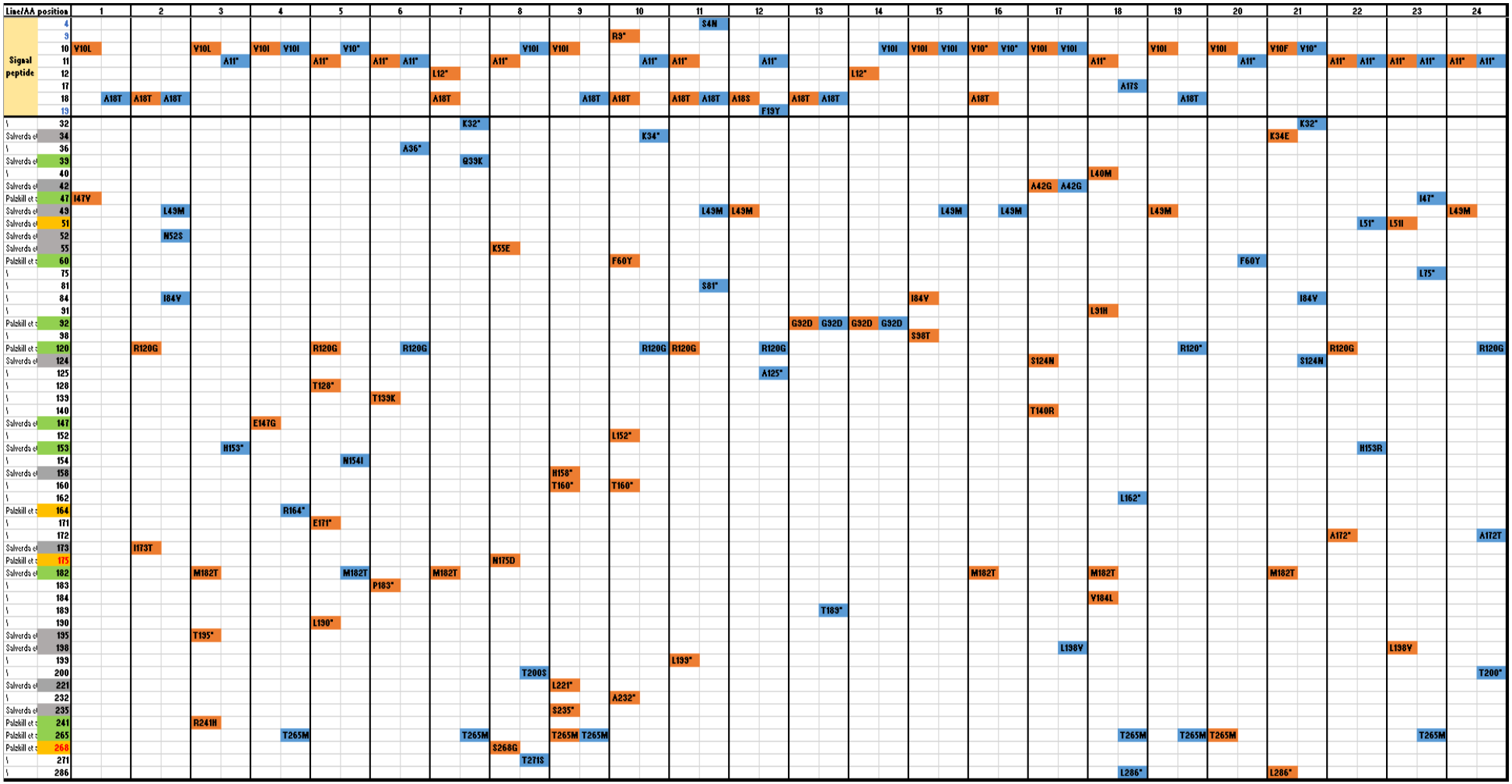


Figure 10: Genotypes of all lines determined after long selection. Orange indicating sexual treatment and blue indicating asexual treatment. Grey bars indicate mutations which have been previously identified with unknown effect. Green bars indicate mutations which have exhibited known stabilizing effect. Yellow bars indicate mutations which show activating effect. White bars indicate mutations, which have previously not been observed and hence novel.

As seen in figure 10, with long selection varied number of genotypes were selected.

By observing the number of mutations occurring at the population level and individually as seen in figure 11, 10/24 lines indicate that recombination brings a greater number of mutations as compared to the asexual treatment. While 7/24 lines show that asexual lines have more mutations. The remaining lines show that both treatments have the same number of mutations. However, upon performing Wilcoxon test the P value is >0.05 indicating the result is not statistically significant to conclude that sexual treatment has greater number of mutations. Therefore, the mean number of mutations was determined which shows that sexual treatment has slightly a greater number of mutations than asexual. The difference is due to non-synonymous mutations which are observed to be more in sexual treatment as compared to asexual treatment.

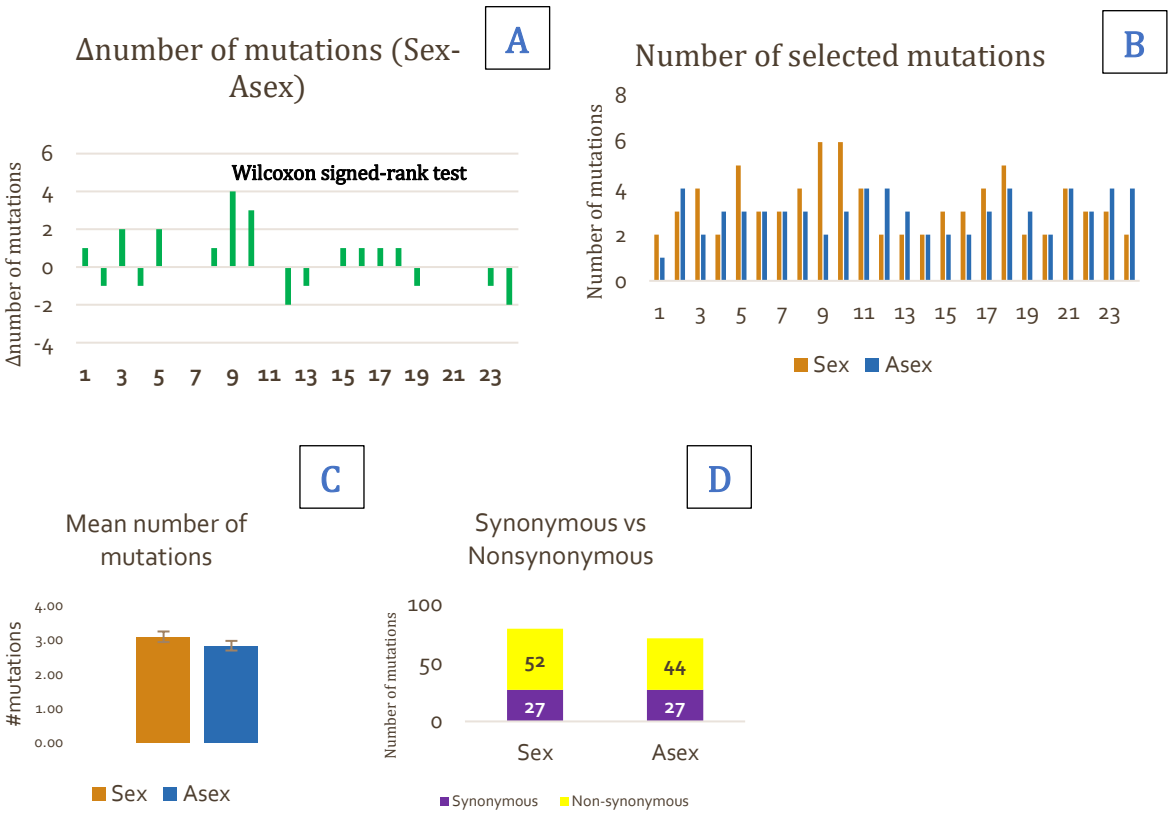


Figure 11: (A) Number of mutations at population level is estimated by subtracting asexual treatment from number of mutations in sexual treatment. (B) Number of mutations present in all lines per treatment. (C) Mean number of mutations. (D) Total number of mutations per treatment dissected into synonymous vs nonsynonymous.

On comparing the lines within treatments, asexual treatments of line 15 and 16 showed similar genotypes with difference in one synonymous mutation (V10I, V10*). This may indicate that the two lines would have had the same genotype but due to the occurrence of a point mutation, one of the lines showed synonymous mutation. None of the lines of sexual treatment showed same or similar genotypes.

All lines belonging to both treatments were compared as well. Mutations V10L and A18T are observed to occur in almost all lines. While mutation A11 exists only in synonymous form and very often. Three stabilizing mutations, R120G, M182T and T265M are seen to occur in many lines. On the other hand, stabilizing mutations like G92D occurred only in 2/24 lines.

The effect of recombination is difficult to be interpreted by observing the mutations alone and hence further analysis is required.

Furthermore, the position of the mutations along the length of the gene was investigated as seen in figure 12. Most of the mutations appear in the signal peptide. The signal sequence directs export of the protein to the periplasmic space of *E.coli*. This signal sequence is not a part of the mature protein since it is removed in the periplasm. However, it has earlier been investigated that the mutations in signal peptide are likely to change protein abundance and thereby affect fitness and MIC (Schenk, Szendro, Krug, & de Visser, 2012).

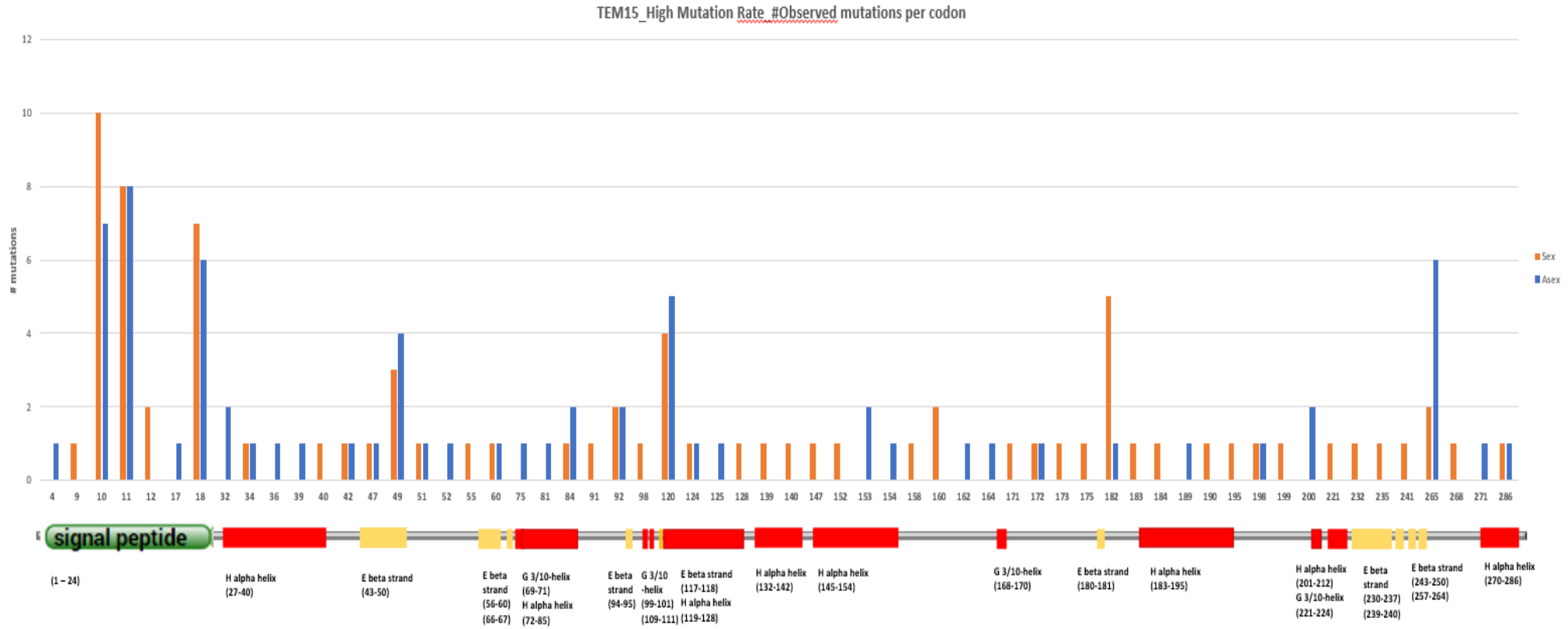


Figure 12: Mutations per codon along the length of the gene. Occurrence of substitutions in clinical and laboratory isolates of TEM-1 β-lactamase. The amino acid numbering according to Ambler et al. (1991) is on the x-axis. Structural features of the enzyme according https://www.rcsb.org/pdb/protein/P62593?addPDB=1XPB are indicated below the length of the gene.

Figure 13 shows the next most occurring mutation which is mutations with known stabilizing effects. 19 stabilizing mutations are seen in asexual treatment as compared to sexual which shows 17 stabilizing mutations. To further investigate the stabilizing mutations per treatment, the position of the known stabilizing mutations on the crystal structure of TEM-1 gene are observed. 7/10 stabilizing mutations are found to be on the exposed region of crystal structure while the remaining are buried.

As observed in figure 13, 34/150 mutations have not been identified before. These new mutations occur mostly only once in the lines and hence are non-adaptive (Appendix 3).

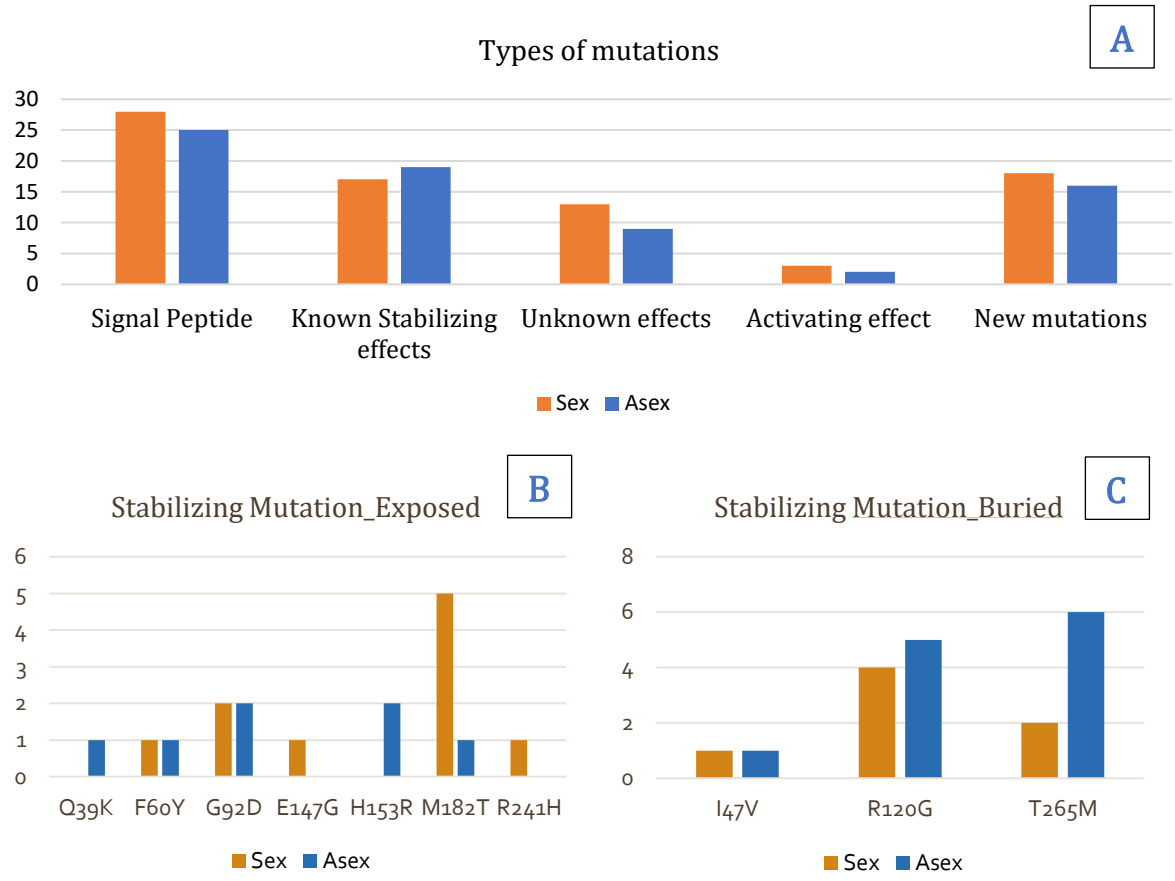


Figure 13: (A) Mutations from all lines with different effects on the resistance phenotype. (B) Stabilizing mutations which are exposed on the crystal structure TEM gene. (C) Stabilizing mutations which are buried in the crystal structure of TEM gene.

Correlation between substitutions and mutant MIC's

To measure the predictability of evolution in terms of divergence, the genetic and adaptive divergence is estimated as shown in figure 14. To estimate the relative genetic divergence, the average of pairwise Hamming distance of all genotypes is divided by the sum of both hamming distances relative to TEM-15. As observed in figure 10, high mutation rate generates vast number of genotypes. However, the average genetic divergence indicates that genetically the lines are not very divergent compared to the wild type (figure 14). Similarly, the relative phenotypic divergence is estimated by average pairwise difference in MIC-step improvement relative to TEM-

15, divided by the sum of MIC-step improvements of both lines. The average adaptive divergence indicates that the lines show the same level of phenotypical divergence. The same can be seen in figure 9, where the lines share MIC.

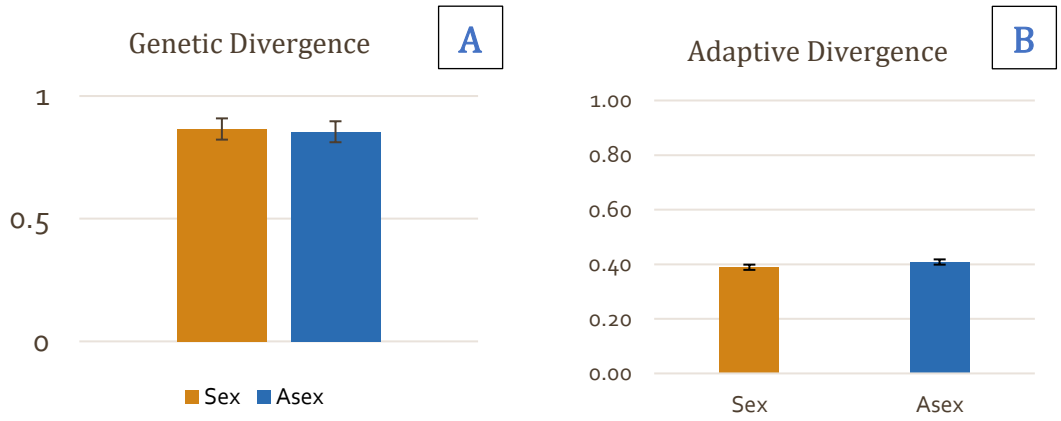


Figure 14: (A) Average of pairwise Hamming distance of all genotypes divided by the sum of both Hamming distances relative to TEM-15. (B) Average pairwise difference in MIC-step improvement relative to TEM-15, divided by the sum of MIC-step improvements of both lines.

To investigate the predictability of evolution in terms of divergence within population, genetic and adaptive divergence were estimated by measuring the hamming distances. As observed in figure 15, by correlating the genotypic and phenotypic aspects, three evolutionary principles can be observed in all the lines. The most common mechanism amongst the lines (11/24) was Fisher-Mullers effect impliying that two mutations which are beneficial together are brought together faster by recombination.This will allow the simultaneous substitution of both mutations. 4/24 lines show background selection. Background selection occurs when a bad mutation is observed to hitchhike with a good mutation. This in turn brings down the resistance level of the gene. Recombination is able to separate the two mutations and thereby maintaining only the good mutations. The remaining lines exhibit recombination load, which leads to two known scenarios by recombination. Firstly, two mutations which are individually good but when brought together by recombination are bad for the enzyme levels. This implicates the presence of negative epistasis. Secondly, recombination disrupts the already existing good mutations.

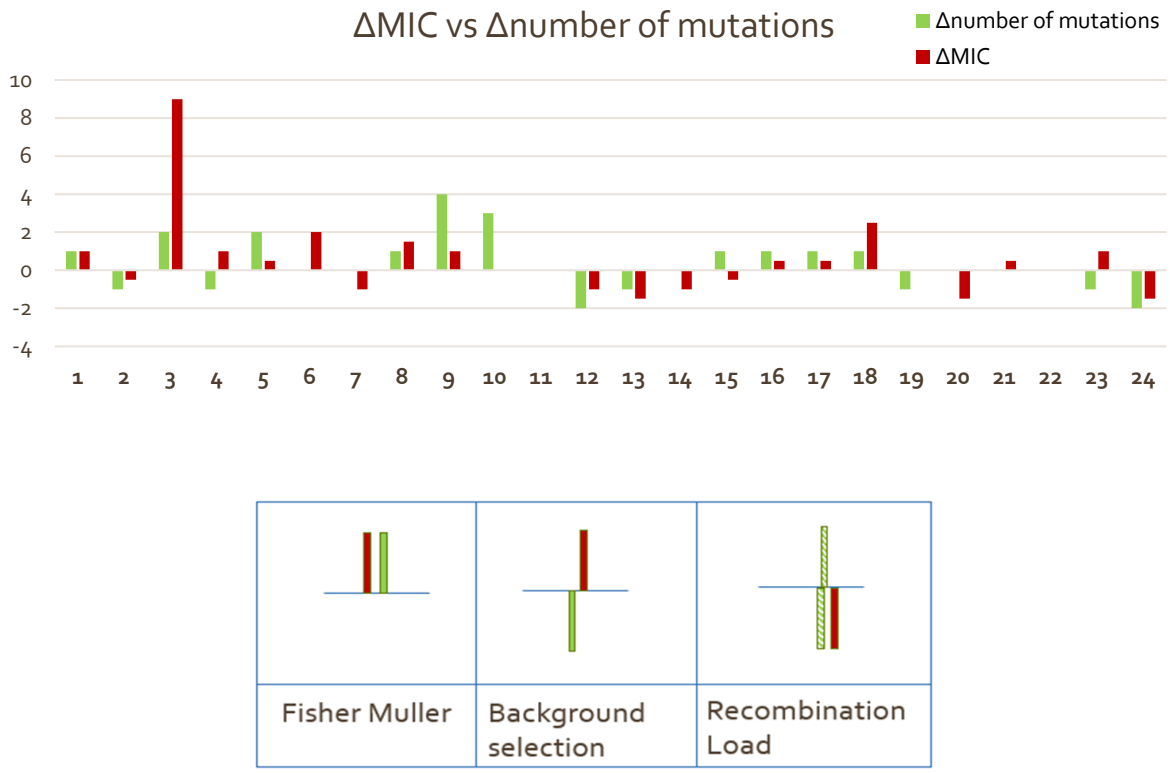


Figure 15: Correlation of genotypic and phenotypic characterization of all lines with three evolutionary principles namely Fisher Muller effect, background selection and recombination load.

DISCUSSION

This research is focused on investigating the long-term effects of recombination of well-adapted genotype TEM-15 (E104K – G238S) in the fitness landscape, considering that the conditions in which recombination is beneficial in long-term are not well known. The results obtained indicate that there is no recombination benefit for a well-adapted genotype. Probably because the mutation rate is so high that already the fittest genotypes are present in the population and hence recombination does not have a major impact at population level. However, when the lines are individually investigated, recombination does bring along beneficial mutations together and, in some cases, it breaks apart bad mutations from the good ones.

There are a few possible reasons on why recombination did not show beneficial effects in this research. Firstly, beneficial mutations in TEM-15 have known to exhibit negative epistasis due to which recombination shows no benefit. Secondly, recombination benefit exhibited by certain lines may have been missed due to the resolution of MIC. By increasing the resolution, the true MIC of certain lines can be determined which will ultimately affect the recombination benefit at the population level.

One of the major challenges encountered was the unpredictability of evolution, which lead to low repeatability of phenotypic and genomic evolution in this experiment.

CONCLUSION

The aim of the experiment was to explore the benefits of recombination during the adaptation of well adapted genotype TEM-15 (E104K – G238S). The purpose of the experiment was not fulfilled when viewed at population level, since the benefit of sex is not observed at high mutation. However, upon assessing the lines individually recombinant lines showed some benefit over nonrecombinant lines. This indicates that the benefits are primarily dependent on the history of each line rather than the population. By correlating the genotype with the phenotype, the most common mechanism observed was Fisher-Muller effect and background selection. This implicates that recombination does bring beneficial mutations together or is involved in separating a bad mutation from a good mutation. Hence, this research contributes to exploring the long-term effects of recombination in a well-adapted genotype on the fitness landscape.

However, this research can be further improved by increasing the resolution of MIC. By doing so, the true MIC of each line can be estimated and thereby determine the phenotypic divergence of each line compared to the wild type.

MATERIALS & METHODS

Random mutagenesis

To create a library of mutant TEM15 alleles, random mutations using epPCR was done as indicated in figure 6. epPCR was carried out by using primers, FOR1: 5'-CTT AGA CGT CAG CTG GCA CTT TTC G-3' and REV2: 5'-CGT TAA GGG ATT TTG GTC ATG AG-3'(Biolegio). To achieve high mutation rate along, GeneMorph II random mutagenesis kit which includes Mutazyme II DNA polymerase (Agilent Technologies) was used. The PCR product was then purified using NucleoSpin Gel and PCR clean up kit (Macherey-Nagel) as per the manufacturer's instructions. The cycling program consisted of: denaturation at 95°C for 2 min, 30 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 60°C), and extension (75 sec at 72°C), followed by a final step at 72°C for 10 min.

Media

LB medium was prepared with 10g Bacto tryptone, 5g yeast extract and 10g NaCl in 1 litre. This medium was used to culture the cells and prepare dilutions. LB agar was prepared containing LB medium with 16g agar per litre. LB agar was used for plating. LB-CHF medium and plates were prepared containing 10mg chloramphenicol per litre LB medium. The cells were inoculated on LB medium containing chloramphenicol with desired concentration of CTX.

Bacterial Strains and plasmids

Escherichia coli strain DH5aE was used as a host for all plasmids. This strain is devoid of a chromosomal β-lactamase gene. Plasmid pUC-MCS was used as the vector for cloning and expressing TEM variants. The plasmid consists of chloramphenicol-resistance marker with a replication rate of approximately 10 replications per cell. TEM gene was under the control of native TEM-1 β-lactamase promoter, present in the plasmid hitherto. The plasmid carries a chloramphenicol (CHF) resistance gene which permits selection of plasmid-carrying bacteria when CHF was added to the media.

Ligation of epPCR samples

The PCR products are double digested with enzymes NotI, NcoI and DpNI overnight at 37°C. The double digested product is purified using NucleoSpin Gel and PCR clean up kit (Macherey-Nagel) as per the manufacturer's instructions. Prior to ligation, the double digested samples are quantified using gel electrophoresis and Nanodrop. 20 ng of insert if ligated into 40ng of vector using T4 ligase. The ligated samples are then followed by digestion with NheI for 2 hours at 37°C.

Transformation of epPCR samples

The plasmids harbouring TEM15 variants is transformed in *E. coli* cells by electroporating the cells in 950 µg of prewarmed SOC (recovery medium). After electroporation, the samples are incubated for 1.5 hours at 37°C. To calculate the library sizes, 100 µl of 10^{-3}dilutions are plated on LB-CTX agar plates and incubated overnight at 37°C. The average library size is estimated as 4.85 x 10^{5}cells. To validate the

transformation efficiency, colony PCR was done for two selected transformant lines, one with high and other with average cfu. Followed by purification using NucleoSpin Gel and PCR clean up kit (Macherey-Nagel) as per the manufacturer's instructions and quantification using nanodrop.

Characterization of selected alleles

The PCR products were sent for sequencing using LIGHTrun protocol (https://www.eurofinsgenomics.eu/). To identify the integrated mutations, Benchling (https://benchling.com/) software was used to align the evolved variants with the wild type TEM-15 sequence. The transformants were amplified on LB-CHF agar plates at 30°C for 12 hours.

Re-cloning

After carrying out long selection, plasmid was purified of the genotypes growing at the highest concentration of CTX. The respective plasmids were then transformed in competent *E.coli* cells. The transformed lines are further used for MIC assay analysis.

APPENDIX

Appendix 1: Minimum Inhibitory Concentrations (µg/ml) of all lines in triplicates

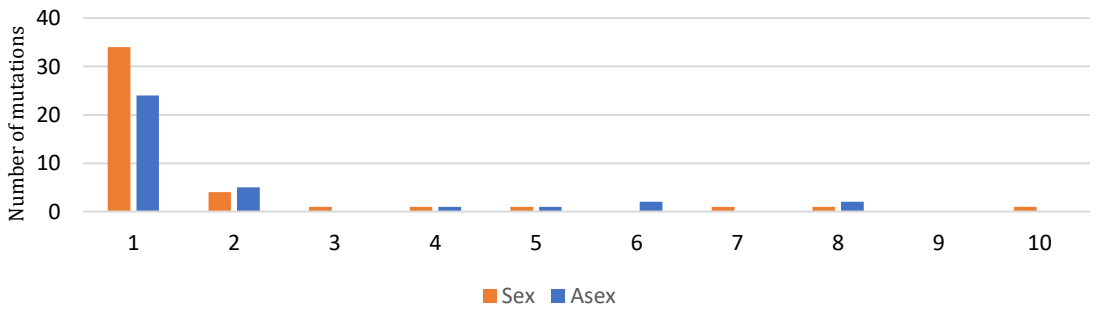
Sex	MIC in triplicates		
Line	#1	#2	#3
1	25.6	36.2	36.2
2	18.1	18.1	25.6
3	204.8	289.6	289.6
4	72.41	102.4	102.4
5	102.4	102.4	144.82
6	51.2	51.2	51.2
7	25.6	36.2	36.2
8	102.4	102.4	144.82
9	72.41	72.41	72.41
10	102.4	102.4	102.4
11	144.82	144.82	204.8
12	72.41	72.41	102.4
13	36.2	36.2	36.2
14	36.2	36.2	36.2
15	102.4	102.4	102.4
16	102.4	144.82	144.82
17	579.26	579.26	579.26
18	289.63	204.8	204.8
19	72.41	72.41	102.4
20	36.2	36.2	51.2
21	102.4	102.4	102.4
22	102.4	102.4	102.4
23	144.82	204.8	204.8
24	102.4	144.82	144.82

Asex	MIC in triplicates		
Line	#1	#2	#3
1	18.1	18.1	18.1
2	25.6	25.6	36.2
3	0.57	0.57	0.57
4	36.2	51.2	51.2
5	72.41	72.41	102.4
6	72.41	72.41	102.4
7	51.2	72.41	72.41
8	36.2	36.2	36.2
9	36.2	36.2	36.2
10	72.41	102.4	102.4
11	102.4	144.82	144.882
12	144.82	144.82	204.8
13	72.41	102.4	102.4
14	51.2	72.41	72.41
15	102.4	144.82	144.82
16	72.41	102.4	102.4
17	409.6	409.6	579.26
18	25.6	36.2	36.2
19	51.2	72.41	72.41
20	102.4	102.4	144.82
21	51.2	72.41	72.41
22	102.4	102.4	144.82
23	36.2	36.2	36.2
24	289.63	409.6	409.6

Appendix 2: Gradient concentration of Cefotaxime used to determine the Minimum Inhibitory Concentration of each line per treatment.

CTX Gradient (µl/ml)																	
0	0.57	0.8	1.13	1.6	9.05	12.8	18.1	25.6	36.2	51.2	72.41	102.4	144.82	204.8	289.63	409.6	579.26
	√2		√2		√2		√2		√2		√2		√2		√2		√2

Appendix 3: Distribution of frequency of mutations in all lines.



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