MSc Biology Thesis Report

By Ruben Jorritsma

851112413060

# Epistasis and parallel evolution in a long term evolution experiment

June 14th 2014

Wageningen University

Department: Genetics

Supervisors: Martijn Schenk, Arjan de Visser

# Epistasis and parallel evolution in a long-term evolution experiment

Ruben Jorritsma

## Abstract

For many years *E. coli* has been used as a model organism to investigate various mechanisms, complications and phenomena that play a role in evolution by mutation and selection. Epistasis and parallel evolution are two of these phenomena. Here we report on a long-term evolution experiment in which 96 *E. coli* populations, carrying plasmids with the TEM-1 resistance gene, have been grown in the presence of the antibiotic cefotaxime for 500 generations. The aim of this research is to determine whether epistasis between plasmidic and genomic mutations could be observed, and what the extent, direction and cause of this epistasis is. Epistasis was indeed observed in most populations, but there is no evidence that epistasis is either predominantly positive or negative. Upon exchanging plasmids between different populations, it was found that epistasis becomes progressively negative as mutations become more beneficial. Our evidence suggests that negative epistasis may be mitigated by compensatory mutations. Another goal of this work is to assess whether large populations exhibit a higher degree of parallel evolution than small populations. It is concluded that large populations do indeed behave more deterministically with respect to their adaptation to cefotaxime.

## Introduction

### Studying adaptation in a laboratory setting using TEM-1 ß-lactamase in *E. coli*

Due to its high reproduction rate and easy, inexpensive handling, *Escherichia coli* has become a mainstay of experimental evolutionary research. Because they can quickly reach huge population sizes, *E. coli* populations can explore all possible mutations for adaptation within a relatively short time-span.

One branch of research in this area focuses on resistance to β-lactam antibiotics. β-lactam antibiotics inhibit the synthesis of the bacterial cell wall. Bacteria can acquire resistance to this class of antibiotics by producing β-lactamases, enzymes which attack the four-atom ring contained in every β-lactam antibiotic. TEM-1 is a 286 amino acid long β-lactamase that is responsible for the bulk of ampicillin resistance in gram-negative bacteria. Besides rendering bacteria resistant to ampicillin, TEM-1 also provides limited protection against related antibiotics such as cefotaxime (CTX). Several mutations in TEM-1 are known to increase resistance to CTX. Previous research has already uncovered many of the mutations that increase resistance to CTX (Barlow and Hall, 2002).

TEM-1 adaptation to CTX has been used to study various aspects of the nature of adaptation, such as the evolvability of promiscuous protein functions (Aharoni et al., 2005), multi-step walks through fitness landscapes (Novais et al., 2010), epistatic effects (Weinreich, Watson and Chao, 2005; Schenk et al., 2013), robustness (Bershtein et al., 2006), gene duplication (Bershtein and Tawfik, 2008), and the trade-off between protein activity and stability (Goldsmith and Tawfik, 2009).

This paper reports on a long-term *in vivo* evolution experiment, spanning 500 generations. In this experiment 96 lines of *E. coli* cells, containing plasmids with the TEM-1 gene, have been exposed to incrementally increasing concentrations of CTX. The resulting populations – as well as samples of the populations that have been frozen every 100 generations during the experiment – have been studied to shed light on two evolutionary phenomena: epistasis and parallel evolution.

### Fitness landscapes, adaptive walks and epistasis

The adaptive evolution of populations has been likened to a walk up a fitness slope on a multidimensional landscape consisting of combinations of properties and corresponding fitnesses (Wright, 1932). The metaphor of a fitness landscape started out as a theoretical concept, but has since come to be applied to real-world situations, as researchers started assigning fitnesses to certain combinations of traits. Advances in molecular biology have made it possible to measure the fitnesses of precisely determined genotypes, so that paths across fitness landscapes can be mapped by measuring the fitnesses of adjacent genotypes, i.e. which are separated by a single mutation. An adaptive path consists of a series of mutations, each of which increases the fitness of the organism. Often the effects of mutations are not simply additive, but influence each other's magnitude or direction, a phenomenon called epistasis.

Epistatic interactions in which an otherwise beneficial mutation is even more advantageous or less advantageous in the presence of another mutation are called magnitude epistasis. Epistatic interactions may even cause beneficial mutations to become deleterious in the presence of other beneficial mutations, a phenomenon named sign epistasis (Poelwijk, Tanase-Nicola, Kiviet and Tans, 2010). Epistatic interactions can influence the likelihood that particular mutational pathways are followed, limit the number of potential adaptive walks over fitness landscapes, and may prevent evolving populations from eventually reaching the highest possible fitness (i.e. the fittest combination of mutations) because there are few, if any, routes towards the highest peak in which all steps represent an increase in fitness, and many adaptive walks may end on local fitness peaks. Epistasis is an important factor in evolution, that needs to be studied if we are to understand the evolutionary process.

In our *in vivo* evolution experiment, cells carried a plasmid containing the TEM-1 gene, which confers limited resistance to the CTX that was present in the medium. Mutations in TEM-1 can drastically increase resistance to CTX, but there likely are several other mechanisms by which bacteria can attain antibiotics resistance. For example, Zhang et al. (2012) have listed 11 genomic genes of the bacterion *Enterococcus faecium* that are involved in antibiotic resistance. Candidate resistance genes include genes coding for membrane transport proteins (that might pump antibiotics into or out of the cell) and for proteins that bind to and immobilize antibiotics. Since resistance-increasing (and hence, adaptive) mutations could occur both on the plasmid and in the genome, this opens the door for epistasis to occur between the plasmidic and genomic mutations that occurred during the experiment.

One of the goals of this reserch was to determine the presence, magnitude and direction of epistasis in 24 of the populations of this experiment. If no epistasis has occurred between the mutations in TEM-1 and genomic mutations, the separate fitness effects of the changes in TEM-1 and genomic changes should be additive. This was tested by interchanging the plasmids between ancestral cells and the evolved lines, to measure the contributions to CTX-resistance of mutations in TEM-1 and mutations in the genome in isolation from each other. These values were then normalized to the wildtype resistance, and compared to the normalized resistance of the co-evolved combination of genome and plasmid. If the co-evolved combination displays the same level of resistance as the additive effects of the genome and plasmid, then no epistasis is observed. If it is lower, then this signifies negative epistasis. If it is more resistant than would be expected based on the individual contributions of the genome and the plasmids, then positive epistasis has occurred.

To further elucidate the nature of epitasis, an additional follow-up experiment was performed where plasmids of three populations were transformed into three different backgrounds, in which

they had not evolved. We choose a highly resistent, an intermediately resistent and a minimally resistent plasmid, and transformed these into a highly resistent, an intermediately resistent and a minimally resistent background. We hypothesized that the higher the expected resistance based on the individual performances of the background and plasmid, the more severely negative epistasis would impact the performance of the combination. The reasons for this will be given in the discussion.

### Parallel evolution

Another aim of this research was to test is whether large populations show a higher degree of parallel evolution than small populations. The rationale behind this is that larger populations possess more probabilistic resources, i.e. more mutational changes can be tested. In a population of $10^{10}$ cells, for instance, almost all possible mutations are bound to be present. In addition, chance processes (i.e. genetic drift) are more pronounced in small populations, while natural selection plays a more predominant role in large populations. Thus, on theoretical grounds we would expect the evolution of large populations to behave more deterministically than small populations. This has been confirmed by computer simulations and phenotypic observations (Rozen et al., 2008). Our research intends to confirm that large populations display more parallel evolution in both their phenotype and genotype than small populations.

Of the 96 populations in this *in vivo* experiment, 24 were 'large' populations with effective population sizes of $2.1 \times 10^8$. The other 72 populations were 'small', with effective population sizes of $2.1 \times 10^6$. These population sizes were strategically chosen to bring out the difference between large and small populations. Given a mutation rate in *E. coli* of approximately $2 \times 10^{-10}$ per genome per bp (Lee et al., 2012), the size of the TEM-1 gene (850 bp), the number of generations (500), the number of plasmids per cell (approximately 10) and the effective population size, it follows that about 178,500 mutations in TEM-1 have been explored in the large populations, but only 1785 in the small populations. This means that most mutations in TEM-1 have occurred multiple times in the large populations, but not in the small populations.

Full genome sequencing has been performed on all populations. This allows for an extensive genotypic analysis of the extent to which the large populations share similar mutations, as contrasted to the small populations. These results will be published elsewhere (M.F. Schenk et al., unpublished data).

Here we report phenotypic evidence for a higher degree of parallel evolution among large populations than among small populations. Five large and five small populations were picked and their resistance to CTX compared to the WT was determined at different stages during the *in vivo* experiment, namely after 100, 200, 300, 400 and 500 generations. It was then determined whether the small populations displayed more variability in their normalized resistance to CTX than the large populations, by comparing the standard deviations among the small populations to those among the large populations.

## Materials and methods

### Antibiotics

The antibiotics that are used for adaptation experiments and fitness measurements belong to the β-lactam class of antibiotics. These antibiotics are characterized by a β-lactam ring, which is the target of β-lactamases, a group of enzymes that can be responsible for antibiotics resistance in bacteria. The structural formulae of ampicillin and cefotaxime are depicted below.

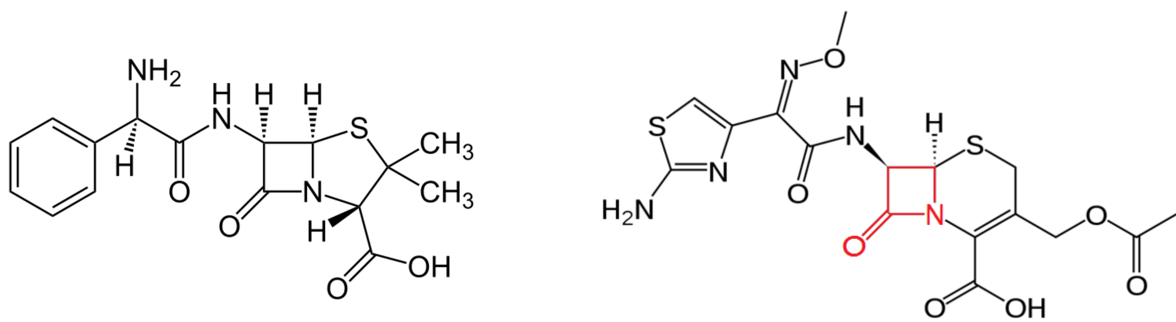


**Figure 1: β-lactam antibiotics relevant for this project. Left: ampicillin. Right: cefotaxime. The β-lactam ring of cefotaxime is shown in red.**

An additional antibiotic to be supplemented is tetracycline. This prevents the occurrence of plasmid loss at the onset of the experiment. In addition, tetracycline will serve as a measure against contamination. The presence of tetracycline in the medium or agar will inhibit the growth of cells lacking the plasmid (pACTEM), which contains a tetracycline resistance gene.

### Growth medium

Lysogeny broth (LB) is used as the primary growth medium. LB contains 10 g trypticase peptone, 5 g yeast extract and 5 g NaCl per liter.

LB agar is used in the final step of competition experiments and in plasmid curing procedure. It contains 15 g agar per liter in addition to the ingredients for LB.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) binds the repressor (encoded by the lacI gene on pACTEM) which inhibits the expression of TEM-1. In every culturing procedure in which TEM-1 needs to be expressed 50 µM IPTG is added to the medium.

### The *in vivo* experiment

During the long term evolution experiment 24 large and 72 small populations of *E. coli* cells have been exposed to gradually increasing concentrations of CTX for 50 days, amounting to 500 generations. The 96 populations were equally divided between the two strains REL606 and REL607, which differ only in one selectively neutral aspect: when grown on arabinose REL606 colonies are stained red, while REL607 colonies are white (Sniegowski, Gerrish and Lenski, 1997). This difference in coloring helps to control for contamination and allows for competition experiments. Each cell contained approximately 10 copies of pACTEM, a plasmid carrying a lacI gene, a tetracycline resistance gene, and the TEM ß-lactamase gene (TEM-1), which is an ampicillin resistance gene that also confers limited resistance to CTX (Barlow & Hall 2002). The cells were grown in LB medium with tetracycline and a starting concentration of 0.03 µg/ml CTX. The 24 large populations were grown in tubes containing 1 ml of medium and the 72 small populations in 10 µl of medium in microtiter wells.

Each day a 1/1000 dilution of the medium was transferred to a new tube or microtiter well containing the same medium. One day equals approximately 10 generations, which can result in a 1000-fold increase in population size. Ancestral populations carying the original TEM-1 gene will reach a cell density of 70% in the presence of 0.03 µg/ml CTX, while mutants with increased resistance may reach higher cell densities. Each line that had reached a cell density exceeding 80% received a 19.3% increase in CTX concentration the next day. (Four such increases constitute a doubling of the CTX concentration.) Samples of each line have been collected and stored at five day intervals, so the timing and order of adaptive changes can be tracked over the duration of the experiment.

### Analyzing the products of the *in vivo* experiment

In order to examine the occurrence of epistasis between the plasmid and the bacterial genome, we needed the resistance levels of these four combinations for each population:

1. The ancestral bacterial strain with the original plasmid
2. The evolved bacterial strain with the evolved plasmid
3. The ancestral bacterial strain with the evolved plasmid
4. The evolved bacterial strain with the original plasmid

To attain item (3), the evolved plasmids had to be isolated and transformed into the ancestral cells. To attain item (4), the evolved cells had to be 'plasmid cured' (their plasmids removed), and subsequently the ancestral plasmids had to be transformed into the evolved cells. To attain item (2), the evolved cells were cured and then were retransformed with the same plasmids. This was done to maintain identical handling of all combinations, to facilitate a fair comparison.

To isolate the evolved plasmids, the cells from generation 500 were grown overnight in 5 ml LB + Tet. The following day the plasmids were extracted using the Sigma GenElute™ Plasmid Miniprep Kit according to the manufacturer's manual.

To remove plasmids while leaving the cells intact (plasmid curing), we relied on selection against the plasmid in the absence of any antibiotic. Since the plasmid and its protein products lay a heavy burden on the cell's resources, losing the plasmid is selectively advantageous when it is not needed. To this end, the cells were cultured in LB with IPTG but without CTX and tetracycline for two days, diluted to a concentration of ~$10^6$ cells per liter, and 40 µl was plated on an LB agar plate without antibiotics. The next day, colonies on this plate were be used to infest agar plates with tetracycline. Colonies on the first plate that fail to grow on the second plate are likely to have lost their plasmids, since their lack of growth in the presence of tetracycline indicates they have lost their tetracycline resistance gene, which is located on the plasmid.

To equip cells with plasmids, the cells had to be made competent. This was done using the Sigma RapidTransit™ Kit according to the manufacturer's manual. Finally, the cells were transformed by applying an electrical pulse that increases the permeability of the cell membranes so the plasmid can enter the cell. The cells were allowed to recover and were then plated on LB agar with tetracycline, on which only cells with the plasmid can grow.

Resistance measurements were performed by determining the minimal inhibitory concentration (MIC) of antibiotic in which the bacterial lines can no longer grow. Each MIC essay was done in triplicate in microtiter plates, in which every next well contains a higher concentration of CTX. The CTX-concentrations ranged from 0.005 to 8192 µg/ml. All resistance levels were then normalized by dividing the MIC by the MIC of the wildtype, and then taking the log2 of this figure. The resulting log2-scores show how many 'doublings' are required to go from the MIC of the WT to the MICs of the particular lines. In the remainder of this paper, the log2-values will be used as a simple measure for resistance.

## Results and discussion

### Epistasis between plasmid and genome

For 22 of the large populations, we succeeded to determine the MICs of the evolved background with the ancestral plasmid, the evolved plasmid in the ancestral background, and the co-evolved combination of plasmid and background. These were converted to log2-scores, which are given in Table 1. By adding the log2-scores of the evolved backgrounds and plasmids, we attained 'expected' values for the co-evolved combination – expected under de hypothesis that there is no epistasis. A value for the epistasis is acquired by subtracting the expected log2 from the observed log2 in the co-

evolved combination. This value can be positive, negative, or zero. It is giving in the last column of Table 1.

**Table 1: Log2-scores of the evolved backgrounds with the ancestral plasmids, the evolved plasmids with the ancestral genomic backgrounds, and the co-evolved backgrounds and plasmids together. The final column gives the epistasis between plasmid and genome that is observed for each population. These values are based on the averages of three MIC essays.**

| # | Evolved background | Evolved plasmide | Expected co-evolved whole | Observed co-evolved whole | Epistasis |
|---|---|---|---|---|---|
| 1 | 8,4 | 0,5 | 8,9 | 8,4 | -0,4 |
| 3 | 11,9 | 0,0 | 11,9 | 11,8 | -0,2 |
| 4 | 9,8 | 6,7 | 16,5 | 13,9 | -2,5 |
| 5 | 3,9 | 10,0 | 13,9 | 14,8 | 0,9 |
| 6 | 4,7 | 6,7 | 11,4 | 13,7 | 2,3 |
| 7 | 6,7 | 8,8 | 15,5 | 14,1 | -1,4 |
| 8 | 8,8 | 5,8 | 14,7 | 13,3 | -1,3 |
| 9 | 7,0 | 2,3 | 9,3 | 9,7 | 0,3 |
| 11 | 13,8 | 0,8 | 14,6 | 13,9 | -0,7 |
| 12 | 6,2 | 6,1 | 12,3 | 15,0 | 2,7 |
| 13 | 5,0 | 10,3 | 15,3 | 15,5 | 0,2 |
| 14 | 10,6 | 0,0 | 10,6 | 10,6 | 0,0 |
| 15 | 5,1 | 12,5 | 17,6 | 15,5 | -2,1 |
| 16 | 4,3 | 10,1 | 14,4 | 14,8 | 0,4 |
| 17 | 11,1 | 3,0 | 14,1 | 11,0 | -3,1 |
| 18 | 9,8 | 0,0 | 9,8 | 10,7 | 0,8 |
| 19 | 11,0 | 0,0 | 11,0 | 10,8 | -0,2 |
| 20 | 7,4 | 6,0 | 13,4 | 15,5 | 2,1 |
| 21 | 10,3 | 4,1 | 14,4 | 12,4 | -2,1 |
| 22 | 7,2 | 5,9 | 13,2 | 14,7 | 1,5 |
| 23 | 11,6 | 0,7 | 12,3 | 14,7 | 2,4 |
| 24 | 11,7 | 1,0 | 12,7 | 11,4 | -1,3 |

It must be noted that these values are approximations. The above table is based on three different MIC essays, but the individual MIC essays can differ notably from one another. Still, the comparisons between different populations are reproducible from one MIC essay to another.

The observed levels of epistasis are shown in the bar chart in Figure 2. In seven cases no evidence for epistasis has been found. Eight populations showed negative epistasis. Seven populations showed positive epistasis.

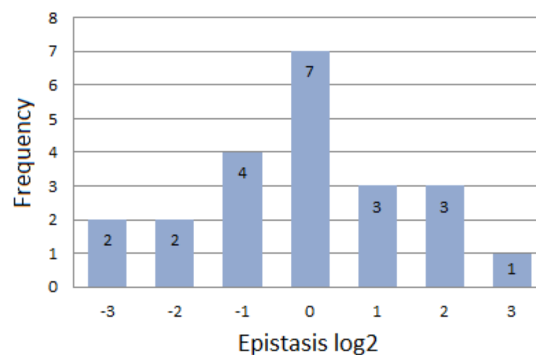


**Figure 2: The observed levels of epistasis between plasmid and genome among 22 populations.**

### Possible causes for negative epistasis

The mechanisms leading to epistasis between plasmids and genome are obscure. One possible explanation for the occurrence of negative epistasis is that as one attains progressively higher resistance to CTX, one approaches physiological limits that make it harder to progress. If this is the case, then a highly resistent plasmid combined with a highly resistent genome, should exhibit more severe negative epistasis than a less resistent plasmid combined with a less resistent genome. We have tested this by transforming plasmids 12, 13 and 15 into backgrounds 5, 1 and 11. Separate MIC essays were performed, leading to the results shown in Table 2.

**Table 2: Nine combinations of evolved backgrounds with evolved plasmids. See text for details.**

| Background # | Plasmid # | Expected log2 | Observed log2 |
|---|---|---|---|
| 5 | 12 | 9 | 9.5 |
| 5 | 13 | 11.5 | 10.5 |
| 5 | 15 | 16 | 12 |
| 1 | 12 | 13.5 | 11 |
| 1 | 13 | 16 | 11.5 |
| 1 | 15 | 20.5 | 14 |
| 11 | 12 | 19 | 15.5 |
| 11 | 13 | 21.5 | 15.5 |
| 11 | 15 | 26 | 16 |

These data do appear to support the prediction that progressively higher expected resistent levels lead to progressively more severe negative epistasis. In Figure 3 the observed log2-values are plotted against the expected log2-values.

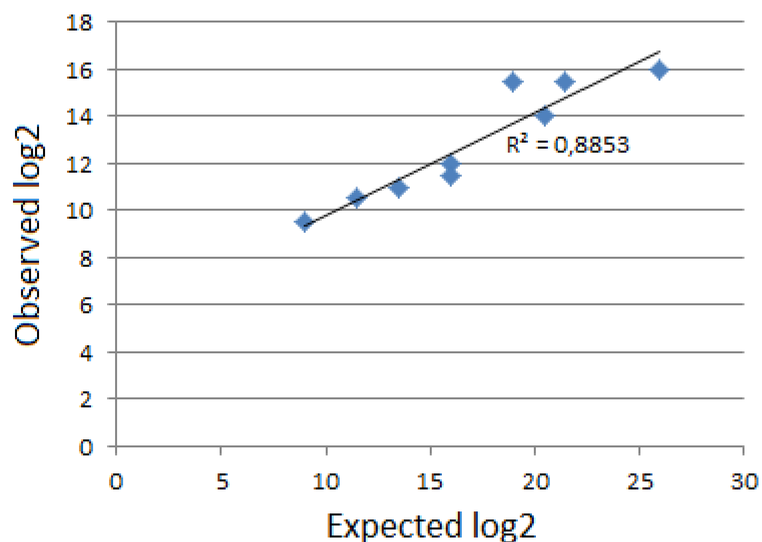


**Figure 3: The observed resistance to CTX plotted against the expected resistance. The formula for the trendline is y = 0,4336x + 5,4617 ($R^2$ = 0,8853). See text for details.**

The observed resistance steadily rises along with the resistance that is expected (in the absense of epistasis) based on the individual contributions of the plasmids and genomes. However, the linear trendline that is fitted to this data has a slope of only 0,43 ($R^2$=0,89). This shows that as the expected resistance rises, the observed resistance levels do not rise at the same pace. This must be contributed to negative epistasis that becomes more severe as resistance levels increase.

Also, note that these data come from transforming 'alien' plasmids into backgrounds in which they had not evolved, contrary to the data shown in Table 1 and Figure 2. The co-evolved plasmids + backgrounds in Table 1 and Figure 2 did not show any systematic trend towards negative epistasis, since there was notable positive epistasis also. The data in Table 2 and Figure 3, however, show resoundingly negative epistasis. This suggests that the co-evolved genomes and plasmids may have accumulated compensatory or complimentary mutations that mitigate or even remedy the problem of negative epistasis.

### Parallel evolution more pronounced in large populations

Another aim of this research was to determine whether large populations evolve more deterministically than small populations. To this end, we measured the resistance levels of five large and five small populations after 100, 200, 300, 400 and 500 generations. The results are shown in Figure 4 and Table 3.

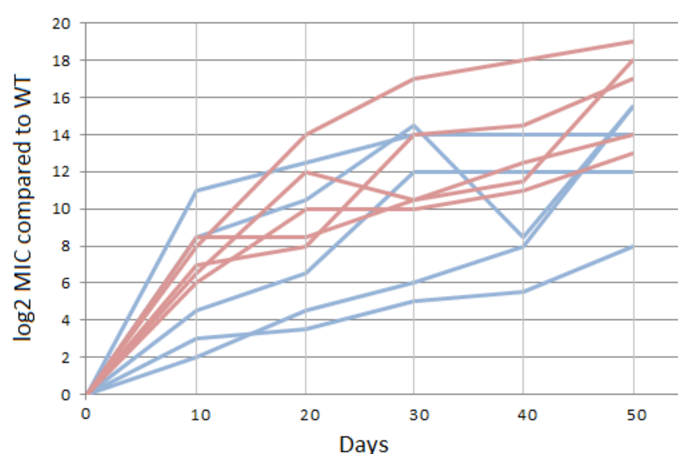


**Figure 4: The evolution of CTX-resistance of five large populations (red lines) and five small populations (blue lines) over the course of 50 days. This equals about 500 generations.**

**Table 3a: The resistance levels of five large populations over the course of the 50-day *in vivo* experiment. The bottom row shows the standard deviation for each column.**

| | 10 days | 20 days | 30 days | 40 days | 50 days |
|---|---|---|---|---|---|
| Large pop 1 | 6.5 | 12 | 10.5 | 12.5 | 14 |
| Large pop 2 | 7 | 8 | 14 | 14.5 | 17 |
| Large pop 3 | 6 | 10 | 10 | 11 | 13 |
| Large pop 4 | 8.5 | 8.5 | 10.5 | 11.5 | 18 |
| Large pop 5 | 8 | 14 | 17 | 18 | 19 |
| Std dev | 1.04 | 2.50 | 3.03 | 2.85 | 2.59 |

**Table 3b: The resistance levels of five small populations over the course of the 50-day *in vivo* experiment. The bottom row shows the standard deviation for each column.**

| | 10 days | 20 days | 30 days | 40 days | 50 days |
|---|---|---|---|---|---|
| Small pop A1 | 3 | 3.5 | 5 | 5.5 | 8 |
| Small pop A4 | 4.5 | 6.5 | 12 | 12 | 12 |
| Small pop B3 | 11 | 12.5 | 14 | 14 | 14 |
| Small pop B7 | 8.5 | 10.5 | 14.5 | 8.5 | 15.5 |
| Small pop C6 | 2 | 4.5 | 6 | 8 | 15.5 |
| Std dev | 3.82 | 4.47 | 4.49 | 3.38 | 3.14 |

In order to assess whether large populations evolve more deterministically, the standard deviations of the resistance levels for each of the five stages in the experiment were calculated for both groups of populations. If parallel evolution is more pronounced in the large populations, then their standard deviations should be smaller than those for the small populations. A Mann–Whitney U test was performed, resulting in a probability of 0,992 that there exists a systematic difference between the two groups, so the difference is significant ($P<0.01$).

## Conclusions

Epistasis between genome and plasmid has been observed in 15 out of 22 large populations. This epistasis was positive for seven populations and negative for 8 populations – no clear trend in the direction of epistasis could be perceived. When transforming plasmids into backgrounds in which they had not evolved, a clear increase in epistasis was observed with increasing resistance. This may imply that there is a physiological limit to the level of resistance that is attainable, and that negative epistasis becomes more severe as this limit is approached. It also implies that the large populations (which, on average, did not display negative epistasis) have accumulated compensatory mutations that mitigate the effects of negative epistasis.

By examining the adaptation to increasing concentrations of CTX over the course of 500 generations, we have indeed observed that large populations behave more deterministically with respect to their phenotypic evolution.

## Acknowledgments

The author thanks Martijn Schenk, Arjan de Visser and Bertha Koopmanschap for practical and theoretical support.

## References

Aharoni, A., Gaidukov, L., Khersonsky, O., Gould, S.M., Roodveldt, C. and Tawfik, D.S., 2005. The 'evolvability' of promiscuous protein functions. *Nature Genetics*, 37, pp. 73-76.

Barlow, M. and Hall, B.G., 2002. Predicting evolutionary potential: In vitro evolution accurately reproduces natural evolution of the TEM β-lactamase. *Genetics*, 160, pp. 823-832.

Bershtein, S., Segal, M., Bekerman, R., Tokuriki, N. and Tawfik, D.S., 2006. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. *Nature*, 444, pp. 929-932.

Bershtein, S. and Tawfik, D.S., 2008. Ohno's model revisited: Measuring the frequency of potentially adaptive mutations under various mutational drifts. *Molecular Biology and Evolution*, 25, pp. 2311-2318.

Goldsmith, M. and Tawfik, D.S., 2009. Potential role of phenotypic mutations in the evolution of protein expression and stability. *Proceedings of the National Academy of Sciences of the United States of America*, 106, pp. 6197-6202.

Novais, A., Comas, I., Baquero, F., Canton, R., Coque, T.M., Moya, A., Gonzalez-Candelas, F. and Galan, J.C., 2010. Evolutionary trajectories of b-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance. *Plos Pathogens*, 6(1), e1000735.

Poelwijk, F.J., Tãnase-Nicola, S., Kievit, D.J. and Tans, S.J., 2011. Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes. *Theoretical Biology*, 272, pp. 141-144.

Rozen, D.E., Habets, M.G.J.L., Handel, A. and de Visser J.A.G.M., 2008. Heterogeneous Adaptive Trajectories of Small Populations on Complex Fitness Landscapes. *PLoS ONE*, 3(3).

Schenk, M.F., Szendro, I.G., Salverda, M.L.M., Krug, J. and de Visser, J.A.G.M., 2013. Patterns of Epistasis between Beneficial Mutations in an Antibiotic Resistance Gene. *Mol. Biol. Evol.*, 30(8), pp. 1779-1787.

Sniegowski, P.D., Gerrish, P.J. and Lenski, R.E., 1997. Evolution of high mutation rates in experimental populations of E. coli. *Nature*, 387(6634), pp. 703-705.

Weinreich, D.M., Watson, R.A., Chao, L., 2005. Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. *Evolution*, 59(6), pp. 1165-1174.

Wright, S., 1932. The roles of mutations, inbreeding, crossbreeding and selection in evolution. *Genetics*, 6, pp. 356-366.

Zhang, X., Paganelli, F.L., Bierschenk, D., Kuipers, A., Bonten, M.J.M., Willems, R.J.L. and van Schaik, W., 2012. Genome-Wide Identification of Ampicillin Resistance Determinants in Enterococcus faecium. *PLoS Genetics*, 8(6), e1002804