Multiple Resistance at no cost: Rifampicin and Streptomycin a dangerous liaison in the spread of antibiotic resistance.

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Evidence is mounting that epistasis is widespread among mutations. The cost of carrying two deleterious mutations, or the advantage of acquiring two beneficial alleles, is typically lower that the sum of their individual effects. Much less is known on epistasis between beneficial and deleterious mutations, even though this is key to the amount of genetic hitchhiking that may occur during evolution. This is particularly important in the context of antibiotic resistance: most resistances are deleterious, but some can be beneficial and remarkably rifampicin resistance can emerge *de novo* in populations evolving without antibiotics. Here we show pervasive positive pairwise epistasis on *Escherichia coli* fitness between beneficial mutations, which confer resistance to rifampicin, and deleterious mutations, which confer resistance to streptomycin. We find that 65% of double resistant strains outcompete sensitive bacteria in an environment devoid of antibiotics. Weak beneficial mutations may therefore overcome strong deleterious mutations and can even render double mutants strong competitors.

Introduction

The effect of a mutation may depend on the genetic background where it occurs, a phenomenon termed epistasis. The existence of pervasive epistasis for mutations that affect fitness related traits has an important impact on the evolutionary dynamics of a population and the number of paths accessible to it (Weinreich et al. 2005; Weinreich et al. 2006; Phillips 2008; de Visser et al. 2011; de Visser and Krug 2014). Experimental evolution to novel laboratory environments has been used to determine the importance and type of epistasis underlying evolutionary trajectories (Salverda et al. 2011; Tenaillon et al. 2012; Kryazhimskiy et al. 2014). Recent studies suggest that the fitness of the genetic background is a key factor influencing the ability of microbial populations to adapt or re-adapt (Kahn et al 2011; Sousa et al. 2012; Schenk et al 2013).

Both in bacteria and yeast, low fit clonal populations were found to have a higher capacity to adapt than clonal populations with higher fitness (Kryazhimskiy et al. 2014; Perfeito et al. 2014). Empirical studies have also been performed to directly measure the strength of epistasis (ε). This was done by measuring both the effect of single mutations and pairs of mutations combined onto the same genetic background (de Visser and Krug 2014). Pairs of individually deleterious (Elena and Lenski 1997; Trindade et al. 2009) or beneficial, at the level of a single gene or between loci (Khan et al. 2011; Schenk et al. 2013), were studied. Far less is known about epistasis between beneficial and deleterious mutations, despite its importance to the amount of genetic hitchhiking on the evolution of asexual populations or genomic regions with reduced recombination (Johnson and Barton, 2000; Gillespie, 2000) and patterns of molecular evolution (Kondrashov and Kondrashov, 2015). An important fitness trait for bacteria is the level of resistance to antibiotics, which can occur in a wide range of concentrations across environments (Andersson and Hughes 2014). Bacterial populations show high levels of polymorphism for resistance alleles and epistasis between resistance alleles is thought to be important in explaining levels of resistance observed in natural populations (Borrell and Gagneux 2011; Müller et al. 2013) and in determining the evolutionary path towards increased resistance to certain antibiotics (Weinreich et al. 2006; MacLean et al. 2010; Borrell et al. 2013; de Visser and Krug 2014). Mutations conferring resistance also exhibit strong Genotype-by-Environment (GxE) interactions. A remarkable example of these types of interactions occurs in alleles that confer rifampicin and streptomycin resistance. Strong epistatic interactions of either positive (alleviating) or negative (increasing) type for the fitness effects of two resistance alleles, were found in different species and in different environments (Trindade et al. 2009; Ward et al. 2009; MacLean et al. 2010; Trindade et al. 2012). Some resistance alleles can even be beneficial in certain environments. For example Miskinyte and Gordo (2013) found that streptomycin (Str^R) and also rifampicin (Rif^R) resistance mutations can benefit *E. coli* survival inside macrophages. In this same species several Rif^R mutations have been also been found to confer a fitness advantage in minimal glucose medium (Trindade et al. 2012). The spontaneous emergence of Rif^R alleles has even been reported in *E. coli* evolving in poor medium under high temperature (Rodríguez-Verdugo et al. 2013). Interestingly, in that study the ancestral strain in which rifampicin emerged was streptomycin resistant.

Here we study the fitness effects of rifampicin and streptomycin resistance in poor nutritional medium devoid of antibiotics, where, according to competitive assays, rifampicin mutations are beneficial and streptomycin resistance incur a fitness cost (Trindade et al. 2012). We ask three questions: How costly is double resistance in this environment? How pervasive is epistasis between beneficial and deleterious alleles? How do the benefits of a single Rif^R allele vary with the fitness of the genetic background where it emerges?

Results and Discussion

In order to determine the effect on fitness of double resistance we performed competitive fitness assays between the double resistant and a sensitive strain in minimal media supplemented with glucose. The sensitive strain carries a genetic marker, which is neutral in this environment (Trindade et al. 2012). Figure 1a shows the results of the competitive fitness assays. From the 20 double mutants studied only 3 have a significant fitness cost. We find that 85% of the double resistant strains have no significant cost and therefore are not expected to be eliminated from the population when it grows in poor medium. Hitchhiking of deleterious mutants is observed in the double mutants - *K88E+H526N*, *K43T+H526N*, *K88R+H526N, K88R+D516V* and *K88E+D516N*. These represent cases where the deleterious effect of the Str^R mutations is not enough to impair fitness below wild type levels (that is below 1) and therefore are still expected to outcompete the sensitive strain. Most importantly, 65% of all clones are actually expected to outcompete the sensitive strain even in the absence of any antibiotic. This implies that if single resistance alleles are segregating in populations, double resistance can be a likely end result of natural selection, in environments where only glucose is present and the selective pressure of antibiotics is inexistent.

For further understanding the consequences of the resistances, we studied their 'trait effects': growth rate (r) and carrying capacity (K). Each of these traits contributes to the competitive ability of the resistant clones and have different relevance when we consider natural populations, which are likely structured (Hall et al 2014). For example in a metapopulation where extinction and recolonization occurs, the growth rate and carrying capacity will also be important determinants of the maintenance of resistance. Figure 1b indicates that only two Rif^R mutants are beneficial for r and all show a deleterious effect on their carrying capacity (Fig 1c),

despite their competitive superiority. It also shows that many doubles resistant clones have increased growth rates.

The absence of costs for competitive fitness in the majority of double mutants could be due to an epistatic interaction between the two. To answer if this was the case we estimated the level of pairwise epistasis between each pair of mutations. This is measured as the difference between the observed relative fitness of the double mutant (Fig. 2a) and the expected fitness, based on the effects of each individual mutation: $\varepsilon = W_{Rif.Str} - W_{Rif} * W_{Str}$. Figure 2b shows the estimated values of epistasis for competitive fitness. We find that positive epistasis is detected between all pairs of Rif^R and Str^R alleles, which are *per se* beneficial and deleterious, respectively, when considered individually. In only a single combination of double resistance (D516V/K43N) was the level of epistasis not significant, but still resulted in a positive mean. All other combinations showed a level of ε significantly above 0.

We also studied the pattern of epistasis for the traits r and K of the mutants *vs* the sensitive strains. Fig. 2c-f shows that an overall level of positive epistasis is also observed for both r (mean ε =0.14, 95%CI [0.06,0.21], 60% of the cases with significant positive epistasis) and K (mean ε =0.20, 95%CI [0.10,0.30], 75% of the cases with positive epistasis). Hence the costs of double resistance are also smaller than expected when these genotypes grow independently.

Next we tested for a correlation between the effect of each beneficial resistance mutation and the fitness of the genetic background where it emerges. Recently, a negative correlation was found between the amount of fitness increase and the initial fitness in bacteria and yeast (Perfeito et al. 2014, Kryazhimskiy et al. 2014, Couce and Tenaillon, 2015). Furthermore the effect of specific beneficial mutations was found to negatively correlate with the fitness of the genetic background (Kryazhimskiy et al. 2014), although the data supporting a general pattern is still limited. Figure 3 shows how the beneficial Rif^R mutations correlate with the background fitness. We observe a strong significant negative correlation between the fitness benefit of *H526N* mutation with the fitness of the genetic background it arises. For the mutation *H526Y*, a highly frequent mutation segregating in natural populations of *Mycobacterium tuberculosis* (Kapur et al., 1994; Yue et al., 2003; Gagneux et al., 2006), its fitness benefit correlates marginally with the fitness of the genetic background. Rodriguez-Verdugo *et al.* 2013 also found rifampicin alleles conferring higher fitness improvements in more maladapted genetic backgrounds. In

the case of mutation *D516V* it is always beneficial but its effect does not correlate with the background fitness.

We have previously measured the level of pairwise epistasis for these alleles in rich medium (LB), an environment where none of these alleles individually was beneficial (Trindade et al. 2009). We can therefore compare how the level and type of epistasis changes with the environment and hence evaluate epistasis by environment interaction (Supplemental Fig 2 and 3). Flynn et al. (2013) have recently found that the sign and magnitude of epistasis between beneficial mutations in *E. coli* can vary with the environment where the cells are grown. For our sample of resistance alleles we observe a significant genotype by environment interaction for the level of epistasis, when comparing poor and rich medium. This is observed when we consider competitive fitness (GxE, $P<0.05$, ≈9% of the variance) and also the traits r (GxE, $P<0.05$, ≈35% of the variance) and K (GxE, $P<0.05$, ≈55% of the variance). Although all resistant clones have a smaller fitness, when competing with the sensitive strains in rich LB medium, some of the mutations confer beneficial effects at the level of r and K (Supplemental Fig 2 and 3).

Although the sample is small these results, together with those of previous reports (Remold and Lenski 2004; Lalic and Elena 2012; Zee et al. 2014) suggest that the type of epistasis may change as the environment changes. Our results also hint that epistasis between beneficial and deleterious resistance alleles may be more positive than that between costly resistance alleles.

Materials and Methods

The strains used were *E. coli* K12 MG1655 wild-type (ara+) and Δara. Rifampicin-resistant (Rif^R) and streptomycin-resistant (Str^R) clones were the same used in (Trindade et al. 2009), which we previously showed to exhibit epistasis in rich medium. Briefly, sets of single spontaneous clones Rif^R or Str^R were obtained by plating in LB agar medium supplemented with the appropriate antibiotic and randomly selecting clones after 24h incubation at 37ºC. Single resistant clones were exposed to a second antibiotic to select for spontaneous mutants resistant to the two antibiotics. Generalized transduction of the resistance mutations with bacteriophage P1 was performed to eliminate unknown genetic background effects (Trindade *et al.*, 2009). Four Rif^R clones were chosen for this study based on their superior fitness in minimal medium and five Str*R* clones were chosen based on their inferior fitness in

minimal medium (Trindade *et al.* 2012). All 20 pairwise combinations giving rise to double resistance were tested. The fitness effects of the double resistance mutations were measured by competitive assays. The double resistant mutants were competed against a reference strain, *E. coli* K12 MG1655 Δara, in minimal medium supplemented with 0.4% of glucose at 37°C, in an approximate proportion of 1:1, for 24 h with aeration. Accurate values of each strain initial and final ratios were estimated by plating appropriate dilutions of the mixture in Tetrazolium Agar (TA) agar plates. The fitness effect of each mutant strain—that is, the selection coefficient (*s*) —was estimated as the per generation difference in Malthusian parameters for the resistant strain and the reference strain: $s = \ln(R_f/R_i)/t$, where t corresponds to the number of generations and R_f and R_i to the final and initial ratios between resistant and reference strains, respectively. In minimal medium no cost of the Δara marker is detected. Five independent assays were done for each double resistant clone. The competitive fitness values of the double mutants in Luria-Bertani (LB) medium presented in Supplemental Fig 2 are based on Trindade et al., 2009. The traits maximum growth rate (r) and carrying capacity (K) were determined at 37ºC using a 200 µL growth assay in a Bioscreen C Microbiology Reader (Growth Curves Ltd, Finland), after two days of acclimation to the growth conditions. Growths were started with $2x10^6$ cells and a minimum of four independent assays were done for each single and double resistant clone. The OD_{600nm} of cultures in the Bioscreen was measured every 20 min and the experiments were run for 24 h with continuous shaking (aeration). In these conditions the growth curves did not display any evidence of death cell. *i.e.* a decline in the OD_{600nm}. Maximum growth rate was calculated as the maximal slope of the exponential phase using four points corresponding to 1h time interval. Assuming a logistic growth model, the carrying capacity, or yield, was determined by measuring the final OD_{600nm} after 24h of growth as commonly used as a proxy for CFUs (MacLean and Buckling, 2009). For LB a 1:4 dilution was done before measuring the OD_{600nm} (Supplemental Fig 1.)

Epistasis (ε) was calculated as in (Trindade et al. 2009), *i.e.* $\varepsilon = W_{Rif.Str} - W_{Rif} * W_{Str}$, where $W_{i,j}$ is the competitive fitness of the strains, with resistances *i* and *j*, against a sensitive strain carrying a neutral marker. The significance of an epistatic interaction was determined by error propagation: the error of the value of ε, $\sigma_\varepsilon = \sqrt{W_{ab}^2\sigma_{WAB}^2 + W_{AB}^2\sigma_{Wab}^2 + W_{aB}^2\sigma_{WAb}^2 + W_{Ab}^2\sigma_{WaB}^2}$, as in previous studies (Trindade et al. 2009; Silva et al. 2011; Borrell et al. 2013). Whenever the value of ε was within the error we considered that alleles did not show any significant epistasis.

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209 Figure Legends

210 Figure 1. (a) Fitness of each double resistant mutant against a wild-type sensitive
211 strain measured by competitive fitness assays; and resistance trait effects (b) growth
212 rates and (c) carrying capacity. *K88R, K43N, K88E, K43T* and *K43R* are the Str^R
213 backgrounds where the effect of the Rif^R alleles *D516V, H526N, H526Y* and *D516N*
214 were measured. All clones with fitness above one are expected to outcompete the
215 sensitive strain (error bars represent 2SE, n>4). 65% of the mutants with a
216 combination of the two resistance alleles are beneficial.

217

218 Figure 2. Massive Positive Epistasis between Rif^R and Str^R alleles. (a) Observed
219 competitive fitness of single and double resistant clones. (b) Maximum growth rates
220 and (c) carrying capacity. Level of pairwise epistasis at the level of competitive
221 fitness (b), growth rate (d) and carrying capacity (f).

222 Figure 3. Effect of genetic background for each beneficial Rif^R mutation. Diminishing
223 returns epistasis (Kryazhimskiy et al. 2014) for H526N, i.e. the fitness effect of
224 H526N significantly decreases with the fitness of the genetic background (*i.e.* the
225 cost of streptomycin resistance) ($P<0.001$, $F=309.3$). No significant correlation for
226 alleles *D516V*, *H526N* and *H526Y* is detected.

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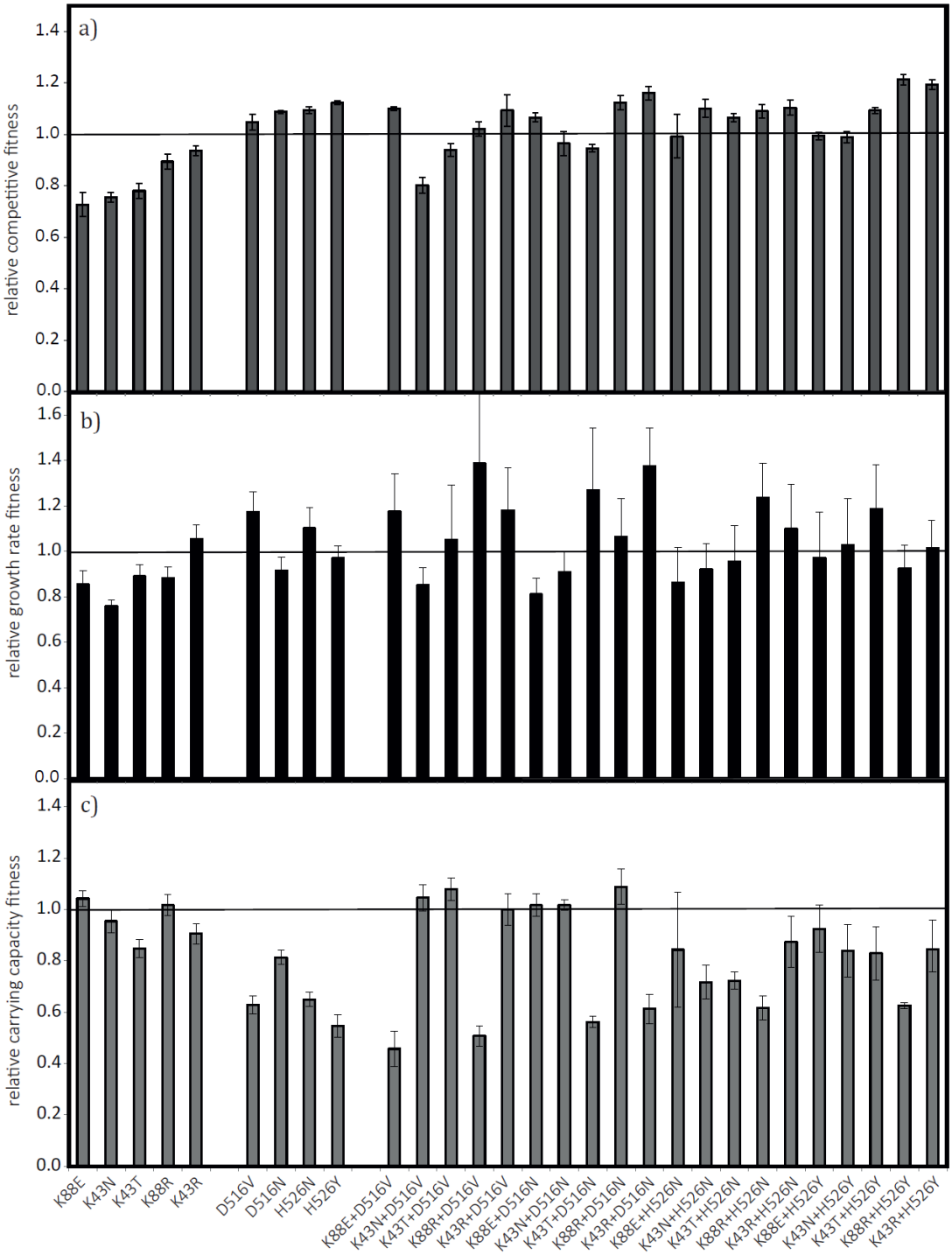
Figure 1


a)
relative competitive fitness
b)
relative growth rate fitness
c)
relative carrying capacity fitness
K88E
K43N
K43T
K88R
K43R
D516V
D516N
H526N
H526Y
K88E+D516V
K43N+D516V
K43T+D516V
K88R+D516V
K43R+D516V
K88E+D516N
K43N+D516N
K43T+D516N
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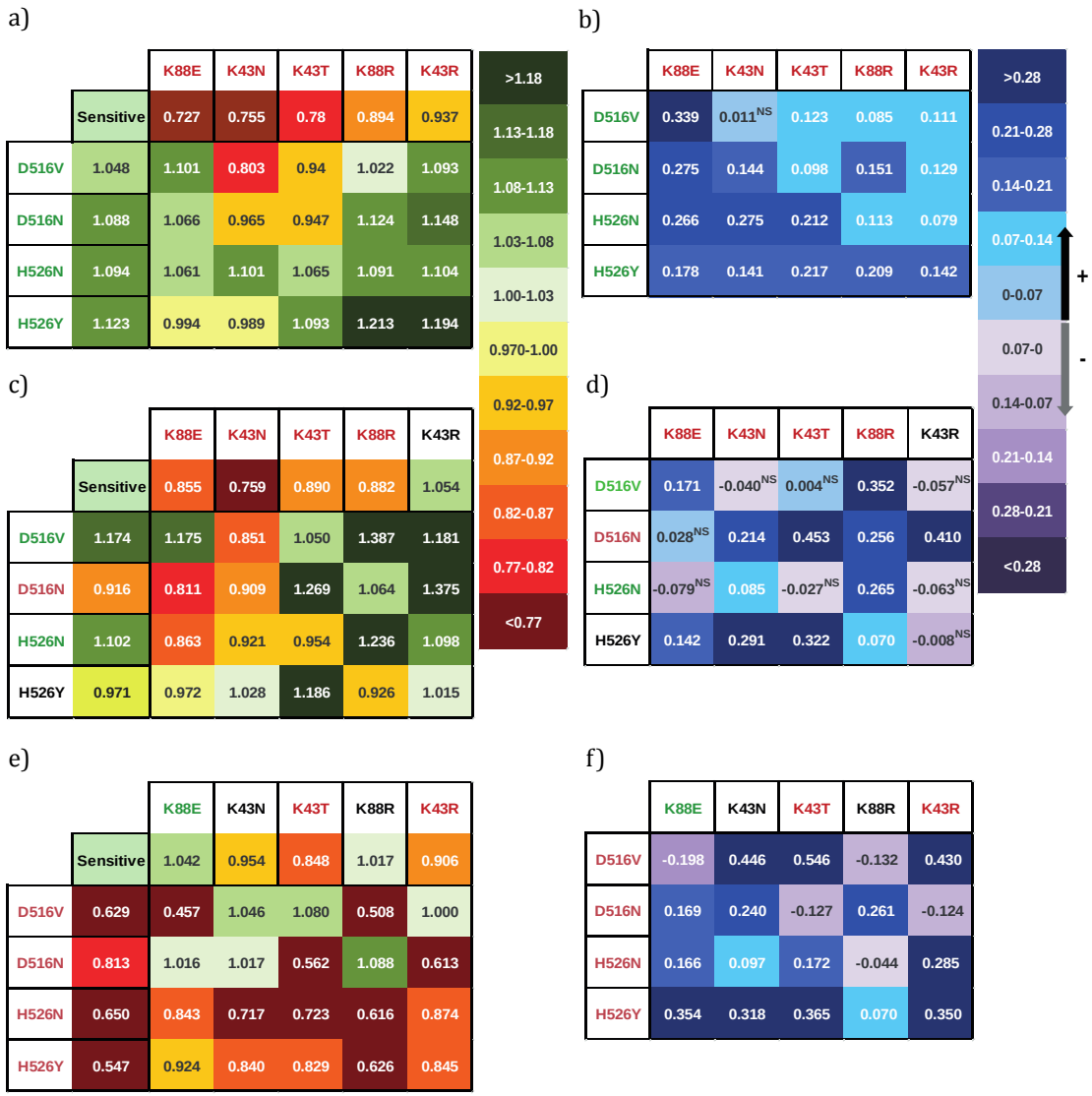
K43R+H526Y

Figure 2


a)
K88E K43N K43T K88R K43R
Sensitive 0.727 0.755 0.78 0.894 0.937
D516V 1.048 1.101 0.803 0.94 1.022 1.093
D516N 1.088 1.066 0.965 0.947 1.124 1.148
H526N 1.094 1.061 1.101 1.065 1.091 1.104
H526Y 1.123 0.994 0.989 1.093 1.213 1.194
>1.18
1.13-1.18
1.08-1.13
1.03-1.08
1.00-1.03
0.970-1.00
0.92-0.97
0.87-0.92
0.82-0.87
0.77-0.82
<0.77
b)
K88E K43N K43T K88R K43R
D516V 0.339 0.011NS 0.123 0.085 0.111
D516N 0.275 0.144 0.098 0.151 0.129
H526N 0.266 0.275 0.212 0.113 0.079
H526Y 0.178 0.141 0.217 0.209 0.142
>0.28
0.21-0.28
0.14-0.21
0.07-0.14
0-0.07
0.07-0
0.14-0.07
0.21-0.14
0.28-0.21
<0.28
+
-
c)
K88E K43N K43T K88R K43R
Sensitive 0.855 0.759 0.890 0.882 1.054
D516V 1.174 1.175 0.851 1.050 1.387 1.181
D516N 0.916 0.811 0.909 1.269 1.064 1.375
H526N 1.102 0.863 0.921 0.954 1.236 1.098
H526Y 0.971 0.972 1.028 1.186 0.926 1.015
d)
K88E K43N K43T K88R K43R
D516V 0.171 -0.040NS 0.004NS 0.352 -0.057NS
D516N 0.028NS 0.214 0.453 0.256 0.410
H526N -0.079NS 0.085 -0.027NS 0.265 -0.063NS
H526Y 0.142 0.291 0.322 0.070 -0.008NS
e)
K88E K43N K43T K88R K43R
Sensitive 1.042 0.954 0.848 1.017 0.906
D516V 0.629 0.457 1.046 1.080 0.508 1.000
D516N 0.813 1.016 1.017 0.562 1.088 0.613
H526N 0.650 0.843 0.717 0.723 0.616 0.874
H526Y 0.547 0.924 0.840 0.829 0.626 0.845
f)
K88E K43N K43T K88R K43R
D516V -0.198 0.446 0.546 -0.132 0.430
D516N 0.169 0.240 -0.127 0.261 -0.124
H526N 0.166 0.097 0.172 -0.044 0.285
H526Y 0.354 0.318 0.365 0.070 0.350

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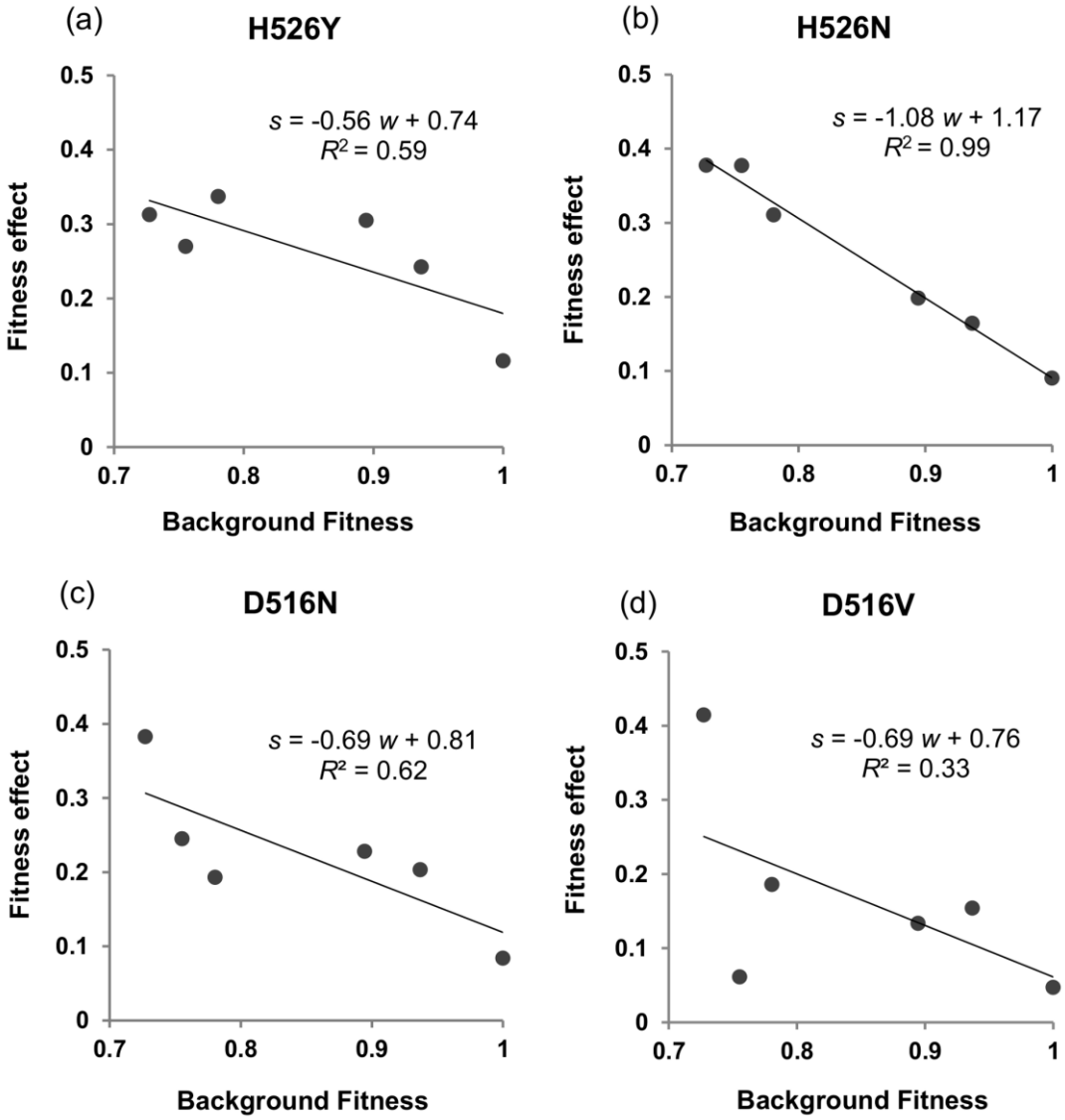
357 Figure 3



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Supplemental Data

Supplemental Figure 1

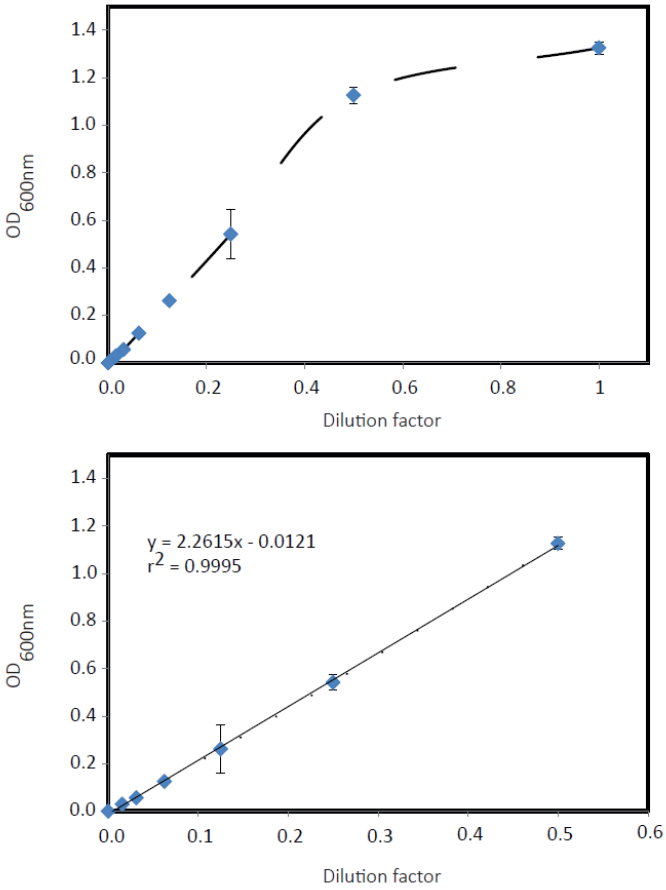


Figure S1 – Determination of the linear range of the Bioscreen. Using a 24h growth overnight in LB we performed a series of 1:2 dilutions to determine the linear range of the BioScreen. The correlation is lost when OD_{600nm} > 1 and thus, dilutions were performed to determine the correct carrying capacity of the strains in LB. In the growths performed in MM, the carrying capacity was always below 1 and thus, dilutions were not necessary.

Supplemental Figure 2

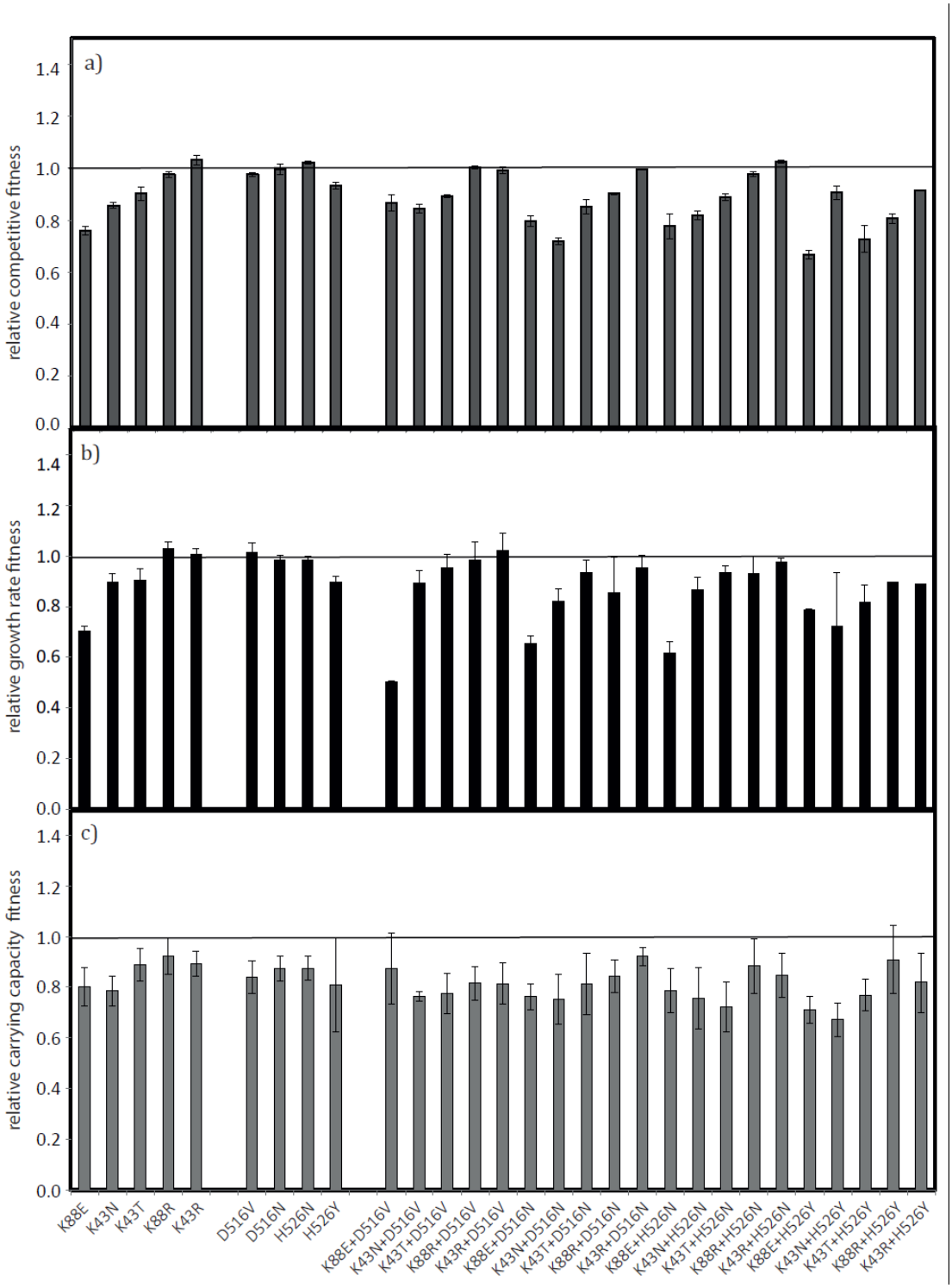
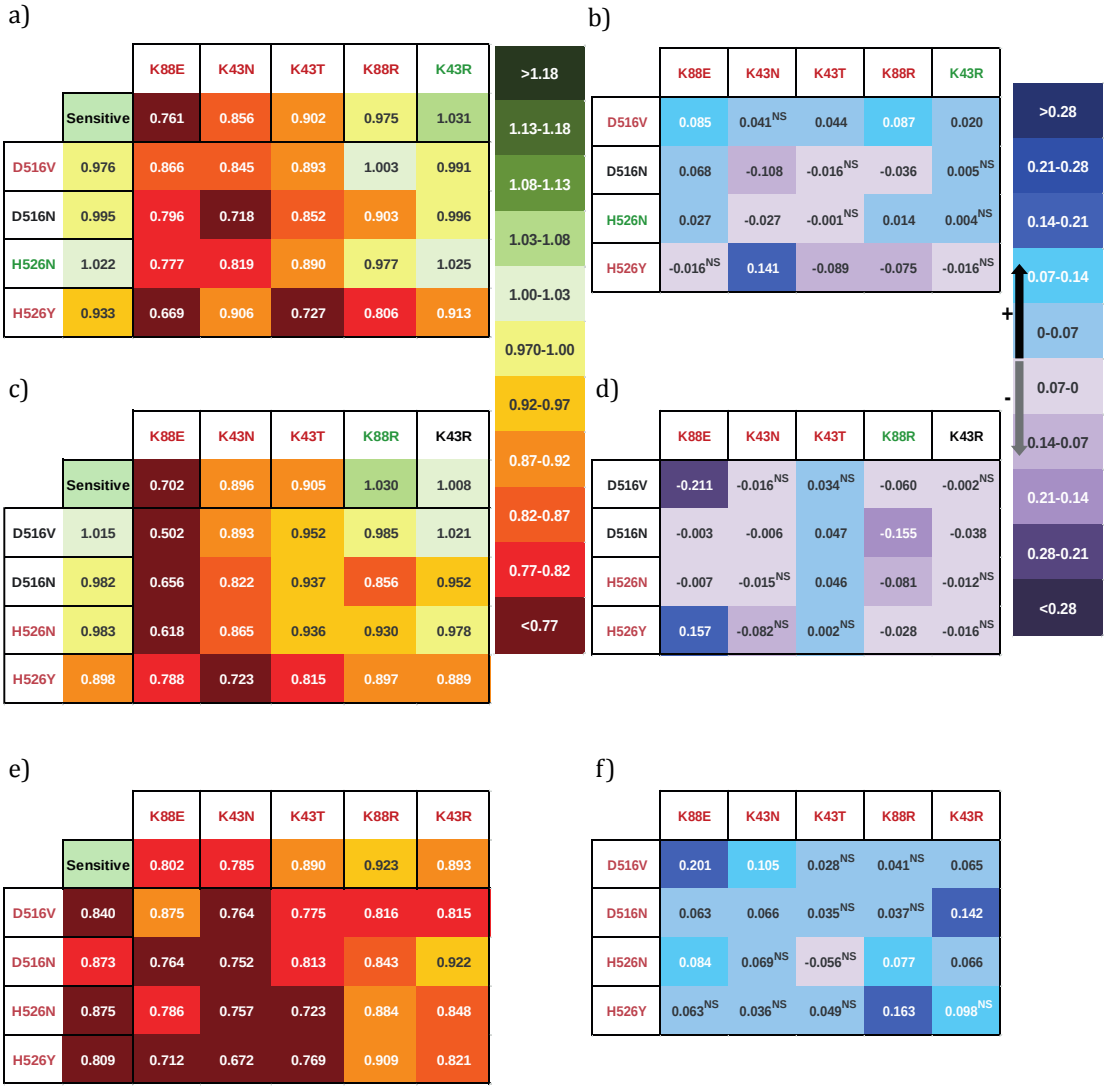


Fig S2 – Relative fitness of single and double mutants in LB as measured by (a) competitive fitness assays, (b) growth rates and (c) carrying capacity.

Suplemental Figure 3



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380 Fig S3 –Level of pairwise epistasis between Rif^R and Str^R alleles in LB for (a)
381 competitive fitness, (b) maximum growth rate and (c) for carrying capacity.

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