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E. coli populations in unpredictably fluctuating environments evolve to face novel stresses through enhanced efflux activity

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Abstract

There is considerable understanding about how laboratory populations respond to predictable (constant or deteriorating-environment) selection for single environmental variables like temperature or pH. However, such insights may not apply when selection environments comprise multiple variables that fluctuate unpredictably, as is common in nature. To address this issue, we grew replicate laboratory populations of *E. coli* in nutrient broth whose pH and concentrations of salt (NaCl) and hydrogen peroxide (H_2O_2) were randomly changed daily. After ~170 generations, the fitness of the selected populations had not increased in any of the three selection environments. However, these selected populations had significantly greater fitness in four novel environments which have no known fitness-correlation with tolerance to pH, NaCl or H_2O_2. Interestingly, contrary to expectations, hypermutators did not evolve. Instead, the selected populations evolved an increased ability for energy dependent efflux activity that might enable them to throw out toxins, including antibiotics, from the cell at a faster rate. This provides an alternate mechanism for how evolvability can evolve in bacteria and potentially lead to broad-spectrum antibiotic resistance, even in the absence of prior antibiotic exposure. Given that environmental variability is increasing in nature, this might have serious consequences for public-health.

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Introduction

In nature, organisms often face environments that fluctuate predictably or unpredictably. Such fluctuations are thought to affect a number of ecological and evolutionary processes including species coexistence (reviewed in Chesson 2000) and diversity in a community (Abele 1976; Hiltunen et al. 2008), evolution of invasive species (reviewed in Lee and Gelembiuk 2008) and co-evolution (Harrison et al. 2013). Not surprisingly therefore, the evolutionary effects of environmental fluctuations have been thoroughly investigated, both theoretically (Levins 1968) and empirically (reviewed in Kassen 2002; Hedrick 2006), leading to a number of insights. Stable environments tend to favor specialists (i.e. organisms with small niche width) whereas fluctuating environments often lead to the evolution of generalists (i.e. organisms with large niche width) (Kassen 2002). Furthermore, evolutionary outcomes may depend on the periodicity of the environmental fluctuations. For instance, environmental shifts within the life-time of an organism can favor phenotypic plasticity (Ancel 1999; Meyers et al. 2005), while environments that change over several hundreds of generations, particularly in asexual populations, can lead to evolution through selective sweeps of large-effect mutations (Cohan 2005). However, when the environment fluctuates over intermediate time scales that are not long enough to promote selective sweeps and yet not short enough to select for plasticity, the evolutionary outcomes become more difficult to predict and might depend more critically on the underlying genetic architecture (Chevin et al. 2010).

Two other factors that can potentially determine the evolution of populations under environmental fluctuations are the predictability of the fluctuations (Hughes et al. 2007; Alto et al. 2013) and the complexity of the environments (Barrett et al. 2005; Cooper and Lenski 2010). Although there are some empirical studies that explicitly compare evolution under predictable

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and unpredictable fluctuations (Turner and Elena 2000; Hughes et al. 2007), they typically concentrate on changes in a single environmental variable. However, in nature, environmental fluctuations typically happen simultaneously along multiple conditions which can potentially lead to a very different evolutionary response from a case where each of these stresses act individually. Thus, it is not clear whether the insights gained from evolutionary studies on unpredictable fluctuations in one environmental variable are relevant to cases where there are simultaneous fluctuations in multiple environmental variables. Furthermore, most environmental factors like temperature and pH are expected to vary on continuous scales, thus creating a much larger set of combinations experienced by the populations, compared to the scenario when a few discrete environmental combinations are investigated (Barrett et al. 2005; Cooper and Lenski 2010). Finally, there has been little elucidation of the mechanisms underlying adaptation to unpredictably fluctuating environments.

Here, we subjected replicate populations of *E. coli* to complex (combination of multiple values of pH, salt and H_2O_2), randomly changing environments. After ~170 generations, the selected populations did not have any overall fitness advantage over the controls in the individual components of selection environments (i.e. pH, salt and H_2O_2). Interestingly, the selected populations had greater fitness when faced with novel stresses that did not have any known correlation with the selection environments. This fitness advantage continued to exist (albeit at lower levels) even after subjecting the selected and control populations to further ~50 generations of directional selection in the novel environments. Contrary to expectations from theoretical studies, the mutation rates of the selected populations did not increase significantly, and there was no evolution of hypermutators. The ability to face novel stresses was partly

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explained by the evolution of greater efflux activity in the selected populations. To the best of our knowledge, this is the first study that demonstrates the evolution of multi-drug efflux activity as a result of exposure to fluctuating environments.

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Materials and Methods

Figure 1 shows a schematic of the various experiments performed in this study, along with the chief results for each.

2.1 Selection experiments

2.1.1 Selection under a stable environment

This study was conducted on *Escherichia coli* (strain NCIM 5547) populations. We plated this strain on nutrient agar, and created a suspension using bacteria from three randomly picked colonies. This suspension was used to initiate six bacterial cultures. Three of these were randomly assigned to a regime wherein they were grown at 37ºC, 150 rpm in 100 ml conical flasks. These three populations were sub-cultured every 24 hours (1ml in 50 ml of nutrient broth) for 30 days and will be henceforth referred to as the S (**S**table-Environment) populations. At the time of every sub-culturing, the optical density (OD_{600}; measured using a Nanodrop™, Thermo Scientific) was ~0.2, signifying 5.64 generations every 24 hours (Bennett and Lenski 1997). The other three populations were subjected to unpredictably changing complex environments for 30 days (see below) and will henceforth be referred as the F (**F**luctuating-Environment) populations. For both these sets of populations, we created glycerol stocks at the time of each sub-culturing.

2.1.2 Selection under complex, randomly changing environments

In a pilot study, we observed the growth of this strain at various concentrations of salt (sodium chloride, NaCl), hydrogen peroxide (H_2O_2) and pH (both acidic and basic) in nutrient broth (NB). These three conditions are henceforth referred to as *component environments*. Based on

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these observations, we created 72 arbitrary combinations of NaCl, pH and H_2O_2 values such that in any combination, the magnitude of one component was equal to that found in NB (i.e. pH = 7.0 or [NaCl] = 0.5g% or [H_2O_2] = 0) and that of the other two were individually expected to negatively affect growth. Thus, for example, combination # 46 denotes a NB containing 2 g% of NaCl + 9.5pH + no H_2O_2 whereas combination # 10 stands for 0.5 g% of NaCl + 9.5 pH + 0.01 M of H_2O_2 (SOM S1). Each combination was assigned a tag between 0-71 and a uniform-distribution random number generator was used to obtain a sequence of numbers (with replacement) in that range. Each replicate F population experienced 30 different combinations of the components according to this sequence. This ensured that the environments faced by these populations were not only unpredictable, but also complex, i.e. involved three different environmental variables. As a result, unlike the S populations, the F populations did not grow to similar OD_{600} values in every generation. Therefore, we arbitrarily assigned an OD_{600} value $\geq$ 0.1 as a criterion for sub-culturing to the next environment. Any F population which did not reach an $OD_{600} \geq 0.1$ was allowed to grow for another 24 hours in the same flask. If it failed to cross the threshold even after 48 hrs post-inoculation, 100 µl of glycerol stock from the previous environmental combination was revived in NB and then transferred to the next combination in the selection sequence. All other culture conditions were identical to those experienced by the S populations.

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2.2 Adaptation under different conditions

2.2.1 Estimating Fitness

Following a recent study (Ketola et al. 2013b), we estimated fitness as the maximum growth rate during a defined period (here 24 hours). For all growth rate measurements mentioned below, the relevant glycerol stocks were revived in 50 ml nutrient broth for 18 to 20 hours. 4 µl of this revived culture (20 µl in case of selection under deteriorating environment) was inoculated into 2 ml of medium in 24-well cell culture plates and kept under 150 rpm at 37ºC. The OD_{600} of each well was measured every two hours over a period of 24 hours on a plate reader. The maximum growth rate of the bacterial population was then computed using a QBASIC (v 4.5) script to fit straight lines on overlapping moving windows of three points on the time series of OD_{600} values. The maximum slope obtained among all the fitted lines for a given time series, was taken as an estimate of the maximum growth rate for the corresponding population.

2.2.2 Fitness under component environments

In this part of the study, we estimated the fitness of S and F populations (after ~170 generations in their respective selection regimes) under four conditions of the component environments (i.e. salt, acidic pH, basic pH and H_2O_2). The concentrations used were the extreme values of the selected ranges for these environments (see SOM S2 for details).

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2.2.3 Fitness under novel environments

In this part of the study, we assayed the fitness of the S and F populations (after ~170 generations in their respective selection regimes) under four different stressful conditions: presence of two heavy metals (cobalt and zinc), one antibiotic (norfloxacin) and a DNA intercalating agent (ethidium bromide). All these four chemicals are known to reduce the growth rate of *E. coli*, and will be henceforth referred to as *novel environments*. The concentrations used in the assays are listed in SOM S2. These four conditions were selected because we failed to find any study that reports a correlated change in fitness in any of them due to selection for any of the component environments (salt, pH or H_2O_2). Thus, it is unlikely that resistance to any of the four novel environments can evolve as a correlated response to direct selection on the component environments (see section 4.2 for discussion).

2.2.4 Fitness after selection under deteriorating novel environments

We then investigated whether the fitness advantage of the selected populations over the controls in the novel environments, persisted even after directional selection. For this, all F and S populations were further selected separately under continuously increasing concentrations of cobalt, zinc and norfloxacin for 8 days (see SOM S3 for details). Directional selection on ethidium bromide could not be done for logistical reasons. The maintenance protocol was the same as that for the selection under fluctuating environments (see section 2.1.2). After the 8th day (i.e. ~ 50 generations), populations were stored as glycerol stocks for fitness estimation.

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2.2.5 Replicates and statistical analysis

For a given environment (e.g. cobalt or pH 4.5), the growth rate of each S and F population was measured in three replicates. All 18 growth rate measurements for a given environment (6 populations × 3 replicates) were conducted simultaneously in a single 24-well plate and constituted a trial. The entire process of revival and growth rate measurement for each component and novel environment was repeated one more time on a different date, to ascertain the consistency of the results. Thus, the study consists of two trials for fitness estimation in each environment, except under deteriorating novel environment, which consists of one trial.

To compare the fitness of the F and S populations averaged over multiple environmental conditions, we performed separate 4-way mixed-model ANOVAs for fitness under component environments, novel environments, and novel environments post directional selection. In each of these ANOVAs, selection (2 levels: S and F) and assay environments (3 or 4 levels) were treated as fixed factors crossed with each other. Replicate (3 levels, nested under selection) and trial (2 levels, nested in selection × assay environment × replicate), were treated as random factors. Since there was only one trial for the deteriorating environment, it was excluded from the analysis in that case. The fixed factor "assay environment" had four (acidic pH, basic pH, salt and H_2O_2), four (cobalt, zinc, norfloxacin and ethidium bromide) and three (cobalt, zinc, norfloxacin) levels in the ANOVAs on component environments, novel environments and novel environments post directional selection, respectively.

We were also interested in comparing the performance of the F and S populations in each assay-environment. Although, this can be done by comparing the appropriate means under the selection × assay environment interaction through planned-comparisons, we refrained from this approach. This is because such a comparison would neglect factors like replicates and trials, which were

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essential parts of our study design. Therefore, we followed a previous study (Ketola et al. 2013b) and performed separate 3-way mixed model ANOVAs for each assay environment and controlled for family-wise error rates through sequential Holm-Šidàk correction of the *p*-values (Abdi 2010). This approach means that our interpretation, of a difference in fitness between the S and F populations being statistically significant, is conservative. The design of the ANOVAs was similar to the one above, except that assay environment was no longer a factor in the ANOVA.

In order to judge the biological significance of the differences in mean growth rates for F and S populations at different assay environments, we computed Cohen's *d* statistic (Cohen 1988) as a measure of the effect sizes (Sullivan and Feinn 2012). Following existing guidelines (Cohen 1988), we interpreted the effect sizes as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$ respectively.

All ANOVAs in this study were performed on STATISTICA v5.0 (Statsoft Inc.) while the Cohen's *d*-statistics were estimated using the freeware Effect Size Generator v2.3.0 (Devilly 2004).

2.3 Mechanism of adaptation

2.3.1 Mutation rates

We used a well-established variant of fluctuation test (Crane et al. 1996) to estimate the mutation rates of F and S populations (see SOM – S4 for details). This method uses a median-based estimator (Jones 1994) for the expected number of mutants and has been widely followed and recommended (Pope et al. 2008). The mutation-rate data were analyzed using a 1-way ANOVA with selection (2 levels: F and S) being a fixed factor.

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2.3.2 Efflux activity

One of the ways in which bacteria can fight multiple stresses is through active efflux of materials from the cell (Nies 1999; Poole 2005). A variant of an existing protocol (Webber and Coldham 2010) was used to quantify the efflux activities of F and S populations (see SOM – S5 for details). The data were analyzed using a 2-way mixed model ANOVA where selection (2 levels: S and F) and replicates (3 levels, nested in selection) were treated as fixed and random factors respectively.

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3 Results

A non-technical summary of the major results is presented as figure 1.

3.1 Adaptation under different environmental conditions

3.1.1 *Fitness under component environments*:

After ~170 generations of selection, the maximum growth rates of the F populations were significantly greater than the S, when measured under conditions that were components of the fluctuating selection regime, i.e. pH (4.5/10), high salt or H_2O_2 ($F_{1,4}$ = 9.34, p = 0.038, Table 1, Fig 2). However, the effect size for this difference was small (d = 0.2, Table 1) which indicated that the difference might be biologically insignificant. There was also a significant main effect of component environment ($F_{3,12}$ = 79.92, p = 3.4E-08) which is not surprising as the different conditions (i.e. pH 4.5, H_2O_2, etc.) are not expected to affect the growth rates similarly. However, the component environment × selection interaction was also significant ($F_{3,12}$ = 3.850, p = 0.038), implying that there were differences in terms of how the growth rates of F and S populations were getting affected by the various conditions. To investigate this in greater detail, we conducted separate ANOVAs for the effect of each condition of the component environment and found that the growth rates of the F and S populations did not differ significantly in any of the stresses (Table 2, Fig 2). The effect sizes of the differences were large for two conditions (pH4.5 and H_2O_2), medium for salt and small for pH10 (Table 2). Interestingly, in pH 4.5 although the difference has a large effect size, it is actually the S populations which have higher growth rates than the F. Overall, this set of analysis leads to the conclusion that in spite of a significant main effect, there is no evidence to indicate that the F populations generally

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performed better than the S under conditions that were components of the fluctuating selection regime.

3.1.2 *Fitness under novel environments:*

When analyzed together, the F populations had significantly higher maximum growth rates under the four novel environments that we investigated ($F_{1,4}$ = 1320.48, *p* = 3.4E-06, Table 1, Fig 3) and the effect size of this difference was large (d = 1.45, Table 1). As with component environments, there was a significant effect of the novel environment ($F_{3,12}$ = 20.38, p = 5.3E-05) and a significant novel environment × selection interaction ($F_{3,12}$ = 48.90, p = 5.3E-07). When analyzed separately, F had significantly greater growth rate than S in three (Cobalt, Norfloxacin, Ethidium bromide) of the four novel environments and the effect size in each of these cases was large (Table 2, Fig 3). In the case of zinc, F still had a higher growth rate than S, although the differences were not statistically significant and the effect sizes were small. However, crucially, the S populations never had a larger growth rate than the F populations. This suggests that the F populations have evolved to counter at least three novel environments without becoming worse than the controls in at least one other novel environment.

3.1.3 Fitness after selection under deteriorating novel environments

After directional selection for ~50 generations in three novel environments, F populations had significantly higher growth rates compared to the S and a medium effect size of the difference ($F_{1,4}$ = 24.68, p = 0.008; *d* = 0.51, Table 1, Fig 4). When analyzed separately, the difference in growth rates in all three novel environments were significant after Holm-Šidàk correction (*p* =

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0.036, $p = 0.039$, $p = 0.043$ for Cobalt, Norfloxacin and Zinc respectively, Table 2) and the effect sizes of the differences were large (Table 2).

3.2 Mechanisms of adaptation

3.2.1 Mutation rates

Although the F populations had higher mutation rates than the S with a large effect size (d = 1.65, Table 1), this difference was not statistically significant ($F_{1,4} = 4.112$, $p = 0.11$; Table 1, Fig 5A). More importantly, the mutation rates were of the order of 10^{-8} to 10^{-9} which suggests that the ability of the F populations to face novel environments is not attributable to the evolution of hypermutators (i.e. alleles that cause 100-1000 fold increase in mutation rate) (Denamur and Matic 2006).

3.2.2 Efflux rates

The F populations had significantly higher activity of the ATP dependent efflux pumps with a large effect size of the difference ($F_{1,4}$= 34.93, $p = 0.004$; $d = 3.83$, Table 1, Fig 5B). This indicates the evolution of an increased rate of efflux of toxic materials from inside the cells, which can explain the ability of the F populations to maintain a higher growth rate in novel environments.

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4 Discussions

4.1 No adaptation to the component environments

When fluctuations in the selection environment are predictable, organisms often evolve to have higher fitness in the component environments (i.e. conditions experienced during the selection process) (Leroi et al. 1994; Turner and Elena 2000; Hughes et al. 2007; Coffey and Vignuzzi 2011; Alto et al. 2013; Puentes-Téllez et al. 2013; Condon et al. 2014), although some studies report no change in fitness relative to the ancestors or controls (Buckling et al. 2003). However, when the selection environment changes unpredictably, fitness in the component environments can increase (Turner and Elena 2000; Ketola et al. 2013b), show no change (Alto et al. 2013), increase w.r.t some life-history traits and decrease w.r.t others (Hallsson and Björklund 2012) or increase in some of the environments but not all (Hughes et al. 2007). In this study, although we observed an overall statistically significant increase in fitness under component environments (Fig 2), the magnitude of this increase was not biologically meaningful (Table 1).

There can be multiple (and non-exclusive) reasons for the above observations. Firstly, it is known that the response to selection depends on the rate at which the environment is changing (Venail et al. 2011) and some evolutionary outcomes are possible only when the environments change relatively slowly (Lindsey et al. 2013). In our experiment, the environment changed every 24 hours i.e. every ~6 generations, which might be too fast for the bacteria to adapt. Secondly, earlier studies on effects of unpredictable environmental fluctuations have mostly been in the context of simple conditions like temperature (Hallsson and Björklund 2012; Alto et al. 2013; Ketola et al. 2013b), pH (Hughes et al. 2007) or two sugar combinations (Cooper and Lenski 2010). However, our study looked at unpredictable combinations of multiple values of

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three conditions (pH, salt and H_2O_2) leading to a total of 72 possible environments, out of which 24 were actually experienced during the course of this study (SOM S1). This means that, on an average, our bacteria were forced to adapt to a novel combination almost every day. When the direction and target of selection changes stochastically, alleles that experience positive selection in a given environment might end up being neutral or negatively selected in the next environment. If the changes in environment happen sufficiently fast (as was the case in our populations), alleles with positive effects on fitness may not get sufficient time to get fixed before the environment changes. As a result of this, at least in the short run, a population might keep on evolving continuously, without really improving in terms of fitness in any of the component environments.

Another reason for the insignificant change in growth rate in component environments might be the relatively short duration of the selection experiment (30 days, ~170 generations). This appears to contradict a recent study demonstrating that within three weeks of selecting *Serratia* populations under randomly fluctuating temperatures, the population growth rates can increase significantly (Ketola et al. 2013b). However, when we estimated the effect sizes from the fitness data of the previous study (Ketola et al. 2013a), it was found that the Cohen's *d* for the differences in fitness of the control and the selected were small to medium (SOM S6) and never large. It is evidently difficult to compare effect sizes across model organisms. However, if we adopt the same criteria for interpreting Cohen's *d* statistics for both studies, then the conclusions are identical: selection for short durations lead to statistically but not biologically significant increase in fitness under component environments. Interestingly, when *E. coli* are subjected to long term selection (~2000 generations), the magnitude of increase in fitness under unpredictable environments is typically less than that under predictably fluctuating environments (Hughes et al.

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2007). This suggests that somehow, stochastic fluctuations hinder the evolutionary increase of fitness more than deterministic fluctuations, which is consistent with similar experiments on viruses (Alto et al. 2013).

To summarize, given the fast rate of change of environment, large number of possible states that selection environment could take and the relatively short duration of the selection experiment, it is not surprising that the F populations in general failed to increase fitness in the component environments. By that line of reasoning, there was no reason to expect any changes in fitness under novel environments either. However, that was not the case.

4.2 Short-term and long-term increase in fitness under novel environments

When exposed to novel environments, the F populations had significantly higher fitness in three of the four environments and suffered no disadvantage compared to the controls in the remaining one (Fig 3, Table 2). This suggests that selection under fluctuating environments can increase the ability of populations to face completely novel stresses, which agrees with the results of a previous study (Ketola et al. 2013b). This is also consistent with observations from the literature on invasive species that organisms inhabiting disturbed habitats are often able to cope better with novel environments, thus becoming better invaders (reviewed in Lee and Gelembiuk 2008).

The simplest explanation for the better performance of the F populations under novel environment could be the presence of cross-tolerance / correlated response to selection for pH,

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salt and H_2O_2. However, we failed to locate any study in the literature that has observed an increased growth rate under any of our novel stresses as a result of selection for any of the three stresses that we used as components for the fluctuating environment. This is perhaps not surprising since the four novel stresses that we used had very different modes of action. For example, while norfloxacin is a DNA gyrase and topoisomerase IV inhibitor (Drlica and Zhao 1997), ethidium bromide is a DNA intercalating agent which can cause frame-shift mutations (Singer et al. 1999). Cobalt affects the activity of iron-sulfur enzymes (Ranquet et al. 2007) but zinc acts primarily by preventing manganese uptake by cells (McDevitt et al. 2011). Although, in principle, it is possible that the F populations had evolved four different mechanisms to fight each of these stresses through correlated response(s), the likelihood of such an event was low, particularly given the relatively short duration of the selection. While this lack of evidence from the literature cannot be construed as a proof, it at least forced us to explore the possibility of other mechanisms that might be at work to confer a growth advantage to the F populations upon exposure to novel environments.

Some theoretical studies suggest that environmental fluctuations spanning across a few generations (as in our study) can promote the maintenance of genetic variation in a population (Turelli and Barton 2004), presumably leading to advantages under novel conditions. A different modeling approach predicts that when environments fluctuate rapidly, organisms of intermediate fitness are selected, which can tolerate a multitude of conditions, but none too well (Meyers et al. 2005). Intuitively, it is expected that any mechanism which allows a population to attain higher growth rates upon first exposure to a novel stress, will also be beneficial under directional selection for that stress. This is because a higher growth rate during the initial stages of exposure

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to a stress allows the bacterial population to undergo more divisions and hence an increased probability of acquiring a suitable beneficial mutation which can then spread in the population. Thus, the F populations were expected to have greater fitness under directional selection in deteriorating environments, which was indeed found to be the case (Fig 4). After ~50 generations, the F populations retained an overall higher growth rate than the S populations in all three novel environments (Table 2). However, the magnitude of difference in fitness between F and S populations reduced after 50 generations of directional selection (*cf* Fig 3 and Fig 4). This observation is consistent with a scenario where the selected populations have more standing genetic variation but do not have an increased mutation rate. Greater standing genetic variation makes the existence of a favorable mutation in the population more likely, thus providing an early growth advantage. On the other hand, an increased mutation rate in the F populations is expected to increase the difference in fitness vis-à-vis the controls, at least in the short run (Wagner 1981), which was not found to be the case.

4.3 No change in mutation rates

Fluctuating environment are expected to favor an increase in the rate of generation of genetic variation through an increase in mutation rates of the populations (Leigh Jr 1970; Ishii et al. 1989). Although the average mutation rate of our F populations was larger than that of the S (Fig 5A), the difference was not statistically significant. There can be multiple (not mutually exclusive) reasons for this observation. Firstly, ~170 generations of selection might have been insufficient for the mutation rates of the selected populations to have diverged enough to be statistically distinguishable from those of the controls. The fact that the magnitude of the difference is ~6 folds and the effect size of the differences is large (Table 1) is consistent with

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the notion that mutation rates in the selected populations are increasing. Secondly, constitutively-expressed mutator alleles are typically thought to spread in a population by hitch-hiking with a beneficial allele (Sniegowski et al. 1997; Taddei et al. 1997; Gentile et al. 2011), although see (Torres-Barceló et al. 2013). However, as in our experiments, if the direction of selection keeps on changing very often, then it is unlikely that a particular mutation would be favorable for long. This will also make it difficult for an attached mutator allele to hitch-hike to fixation with the selected mutation.

Thus, although it is difficult to pin-point the reason, the chief result here is that the difference between the mutation rates of the selected and the control populations was relatively small and not statistically significant. Therefore, it is unlikely that the observed growth rate differences in the novel environments are due to the selected populations generating genetic variation faster than the control populations. This prompted us to look at another class of mechanisms that can possibly lead to broad-based stress resistance in bacteria, namely, multidrug efflux pumps.

4.4 Evolution of increased efflux activity

Over the last two decades, bacteria have become resistant to a large number of antibiotics due to, *inter alia*, the action of a variety of multidrug efflux pumps (MEPs) (Li and Nikaido 2009; Nikaido and Pagès 2012). As the name suggests, MEPs are protein systems that throw out a wide range of antibiotics and biocides from the cells and can also play a role in combating environmental stresses like bile salts (Thanassi et al. 1997) or organic solvents (Fernandes et al. 2003). Our results indicate that the F populations have evolved significantly higher efflux activity as compared to the controls (Fig 5B). Increased activity of MEPs can lead to resistance

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to norfloxacin (Morita et al. 1998; Nishino et al. 2009) and ethidium bromide (Ma et al. 1993; Nishino et al. 2009) in *E. coli*, both of which were observed here (Fig 3). Multiple drug resistance is known to be associated with higher MEP activity in different kinds of bacteria (Li and Nikaido 2009; Nishino et al. 2009; Nikaido and Pagès 2012 and references therein) including *E coli* (Pena-Miller et al. 2013). However, to the best of our knowledge, this is the first laboratory experimental evolution study in *E. coli* that demonstrates the evolution of increased energy-dependent MEP activity and the concomitant change in growth rate in the presence of an antibiotic (norfloxacin) and a mutagen/biocide (ethidium bromide), due to an exposure to fluctuating complex environments.

An increased efflux activity could have evolved in our F populations in at least two ways. Firstly, it could have been accidentally fixed in the population due to genetic drift. However, the chances of such an event are low due to the relatively large population sizes involved in bacterial systems and the observation that the increase in efflux happened in all three replicate F populations. Increased efflux might also evolve as a result of a direct or a correlated response to selection. Prima facie, this appears counter-intuitive because multi-drug efflux pumps are often studied in the context of drugs or biocides, although of late they have been shown to confer resistance to components of the *E. coli* environment like bile salts (Thanassi et al. 1997) or steroid hormones (Elkins and Mullis 2006). Since none of these stressors were present during the selection process, it is hard to see how increased efflux activity could have been directly selected for. This inconsistency gets resolved if we take into account the suggestion made by some authors that MEPs have more functions than removing drugs, including clearing out different kinds of secondary metabolites (Poole 2005) and virulence (Piddock 2006). Recently it has been shown

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that a MEP called *norM* can reduce the intracellular levels of reactive oxygen species and increase the ability of *E. coli* cells to survive H_2O_2 (Guelfo et al. 2010). Since H_2O_2 was a constituent of our fluctuating environment, all else being equal, any change leading to increased levels of *norM* should be favored. Incidentally, among all the component environments, the effect size of the difference between S and H was the maximum for H_2O_2 (Table 2). This is again consistent with the expectation that resistance to H_2O_2 has experienced positive selection. Moreover, the resistance to low pH in *E. coli* is mediated by a regulator called GadX (Ma et al. 2002) which has been shown to elevate the levels of another MEP called mdtEF (Nishino et al. 2008). Finally, the levels of acrAB (a well-studied MEP), are increased by higher concentrations of NaCl (Ma et al. 1995). Although it is not possible to make a definitive statement from our data, all these facts are consistent with the notion that there could be direct or indirect selection for increased efflux activity through MEPs in our populations.

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5. Conclusion

Since bacterial MEPs are often broad-spectrum (Nikaido and Pagès 2012), it is likely that our F populations would show greater fitness against several other kinds of stresses, including antibiotics, and this advantage might be maintained even after directional selection in the novel environments. This suggests that these populations have evolved greater evolvability, i.e. ability to respond to selection pressure (Colegrave and Collins 2008). Generally, evolvability is thought to increase through elevated mutational supply (Taddei et al. 1997), or increased rates of genetic exchange (Colegrave and Collins 2008) or increase in the neutral space of the selected populations (Wagner 2005). Here we show that evolvability can also evolve in bacteria through the evolution of broad-spectrum stress resistance mechanisms such as MEPs, with potentially serious consequences for the society. Over the last three decades, the day-to-day variability in climate has increased across the globe (Medvigy and Beaulieu 2012). Our results indicate that unpredictable environmental fluctuations at this time scale can lead to the evolution of enhanced efflux activity, which in turn can foster multi-drug resistance in bacteria. This is a scary scenario from a public health perspective, and no amount of judiciousness in drug-use schedules can prevent this kind of evolution. Moreover, due to the extremely broad substrate ranges for such pumps, it is hard to predict what other traits might evolve when bacteria are subjected to such stochastic fluctuations, particularly under natural conditions. Taking a broader perspective, it would be interesting to know how more complex multi-cellular organisms respond to increased environmental unpredictability. These questions directly relate to the immediate future of the living world, and therefore, merit further investigation.

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Assay	Mean S	Mean F	ANOVA F(1,4)	ANOVA p values	Effect Size±95% CI	Inference
Fitness in Component Environments	0.109	0.121	9.34	0.038	0.20±0.33	Small
Fitness in Novel Environments	0.028	0.058	1320.48	3.4E-06	1.45±0.37	Large
Fitness Post Deteriorating Environments	0.076	0.086	24.68	0.008	0.51±0.54	Medium
Mutation Rate	1.0E-09	6.4E-09	4.11	0.11	1.65±1.85	Large
Efflux potential	0.468	0.647	34.93	0.004	3.83±1.55	Large

Table 1: Summary of the main effects of selection in the pooled ANOVAs.

Effect size was measured as Cohen's *d* statistic and interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$ respectively.

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Assay	Environment	Mean S	Mean F	ANOVA F(1,4)	p values (Holm-Šidàk corrected)	Effect Size ±95% CI	Inference
Fitness in component environments	pH 10	0.143	0.152	2.20	0.21	0.34±0.66	Small
	pH 4.5	0.0678	0.0455	3.22	0.27	0.82±0.68	Large
	Salt	0.0658	0.0939	4.19	0.29	0.56±0.67	Medium
	H_2O_2	0.161	0.193	6.54	0.23	1.22±0.71	Large
Fitness in novel environments	Cobalt	0.019	0.079	308.37	2.4E-04	2.71±0.90	Large
	Norfloxacin	0.024	0.070	68.02	1.3E-03	3.34±1.01	Large
	EtBr	0.037	0.055	111.88	2.3E-03	1.14±0.70	Large
	Zinc	0.030	0.029	0.63	0.47	0.20±0.65	Small
Fitness post selection in deteriorating environments	Cobalt	0.083	0.093	9.56	0.03	2.08±1.14	Large
	Norfloxacin	0.092	0.104	17.81	0.03	1.91±1.11	Large
	Zinc	0.054	0.060	13.15	0.04	1.20±1.00	Large

Table 2: Summary of the main effects of selection in the ANOVAs under individual environments. This table shows three set of fitness measurements, namely, component environments, novel environments and novel environments after directional selection. Effect size was measured as Cohen's *d* statistic and interpreted as small, medium and large for $0.2 < d < 0.5$, $0.5 < d < 0.8$ and $d > 0.8$ respectively.

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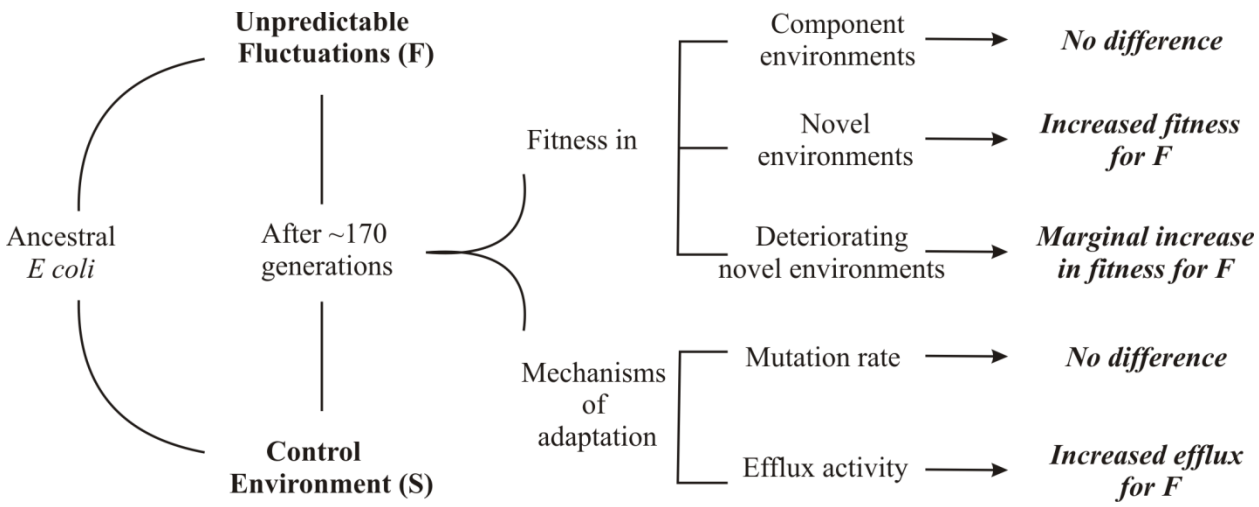

Fig 1: Schematic of the study along with the chief results for each experiment.

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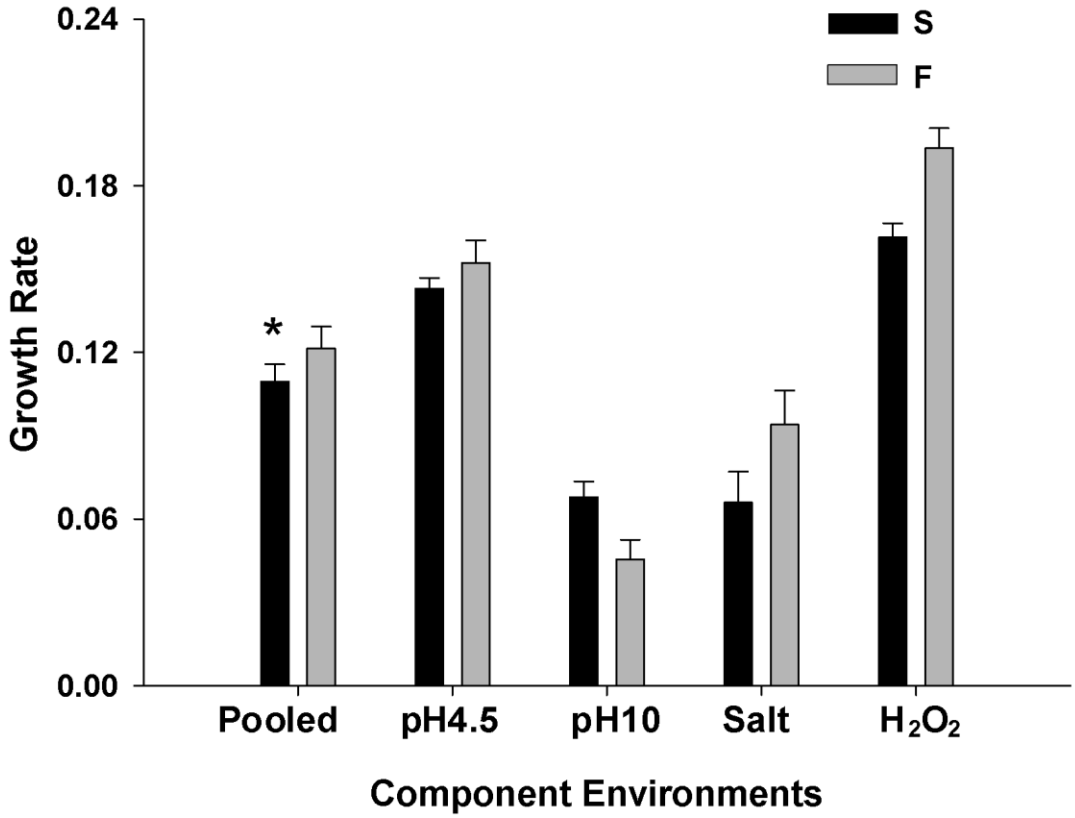

Fig 2: Mean (±SE) fitness in component environments. Fitness was measured as maximum slope of the growth trajectory over 24 hours. In the first comparison of the pooled means over all four component environments, the selected (F) populations show significantly higher fitness than the control (S) populations. The next four comparisons are for the individual component environments with no significant difference (individual ANOVAs) in the fitness of F and S populations. * denotes $p < 0.05$.

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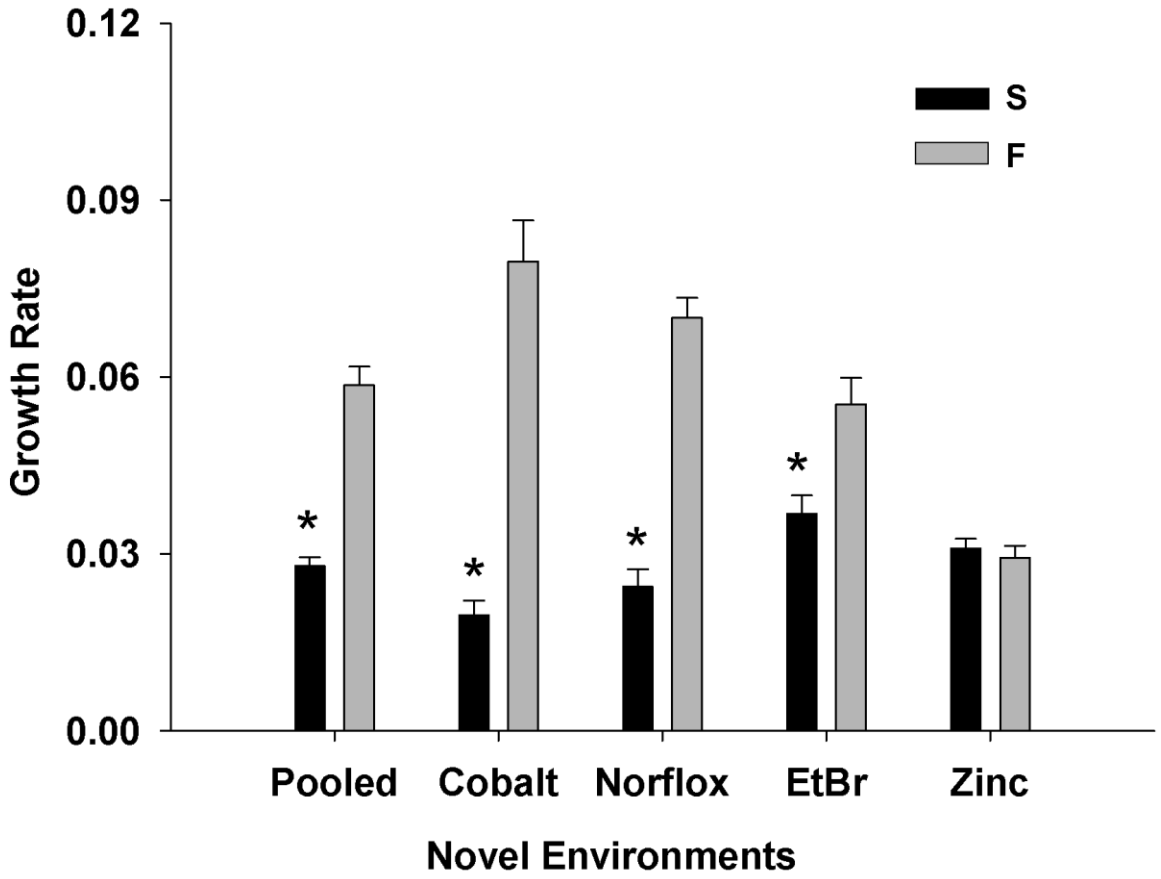

Fig 3: Mean (±SE) fitness in novel environments. Pooled over the four novel environments, F populations show significantly higher fitness than S populations. When compared separately for each novel environment, F populations show significantly higher fitness in cobalt, norfloxacin and ethidium bromide and similar fitness in zinc. * denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

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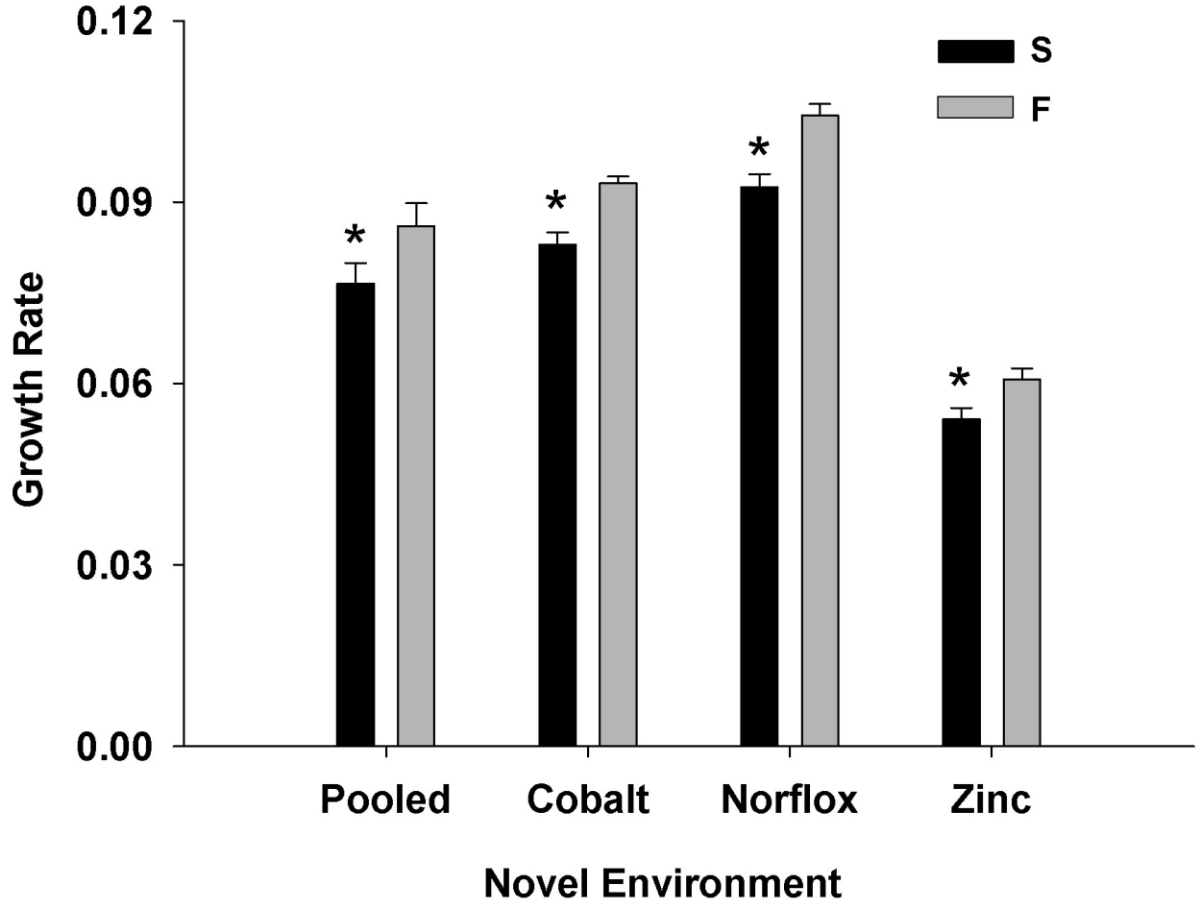

Fig 4: Mean (±SE) fitness in novel environments after facing deteriorating environments. Pooled over the three novel environments, populations selected under fluctuating environments (F) show significantly higher fitness than the control (S) populations. When compared separately for each novel environment, the F populations still had significantly higher growth rate than the S populations. This indicates that the fitness advantages of the F populations were retained after ~50 generations of directional selection. * denotes $p < 0.05$ (after Holm-Šidàk correction in the case of comparisons under individual environments).

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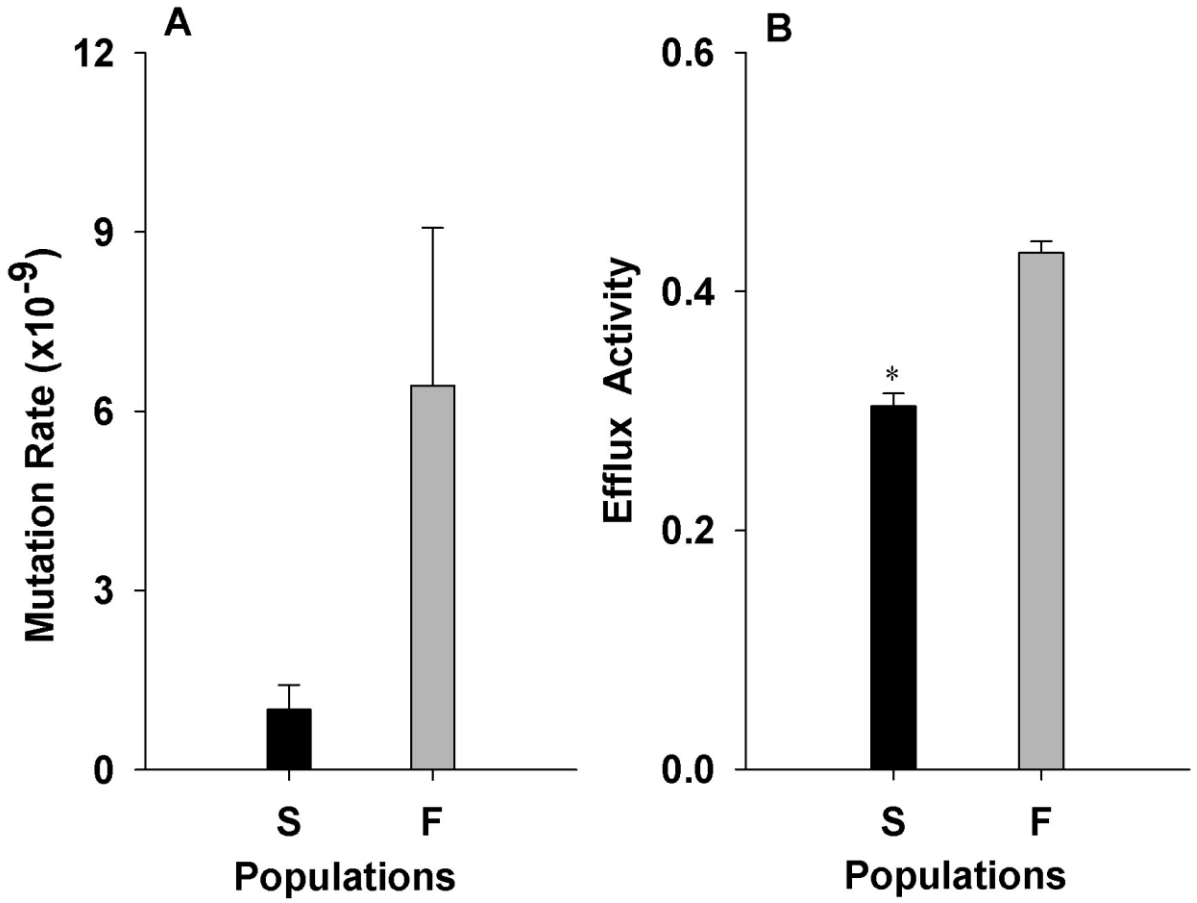

Fig 5: Mechanisms of adaptation. A. Mean (±SE) mutation rate for S and F populations estimated in the Rifampicin background. Although the F populations had slightly increased mutation rates than the S populations, the difference was not statistically significant. More importantly, the mutation rates are of the order of 10^{-9}, which indicates that hypermutators did not evolve in either of the populations. **B.** Mean (±SE) energy dependent efflux in S and F populations. F populations had significantly greater efflux activity than the S populations, which might have led to their greater fitness in novel environments.

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Supplementary Online Material for

E. coli populations in unpredictably fluctuating environments evolve to face novel stresses through enhanced efflux activity

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Running title: **Efflux activity evolves due to random environments**

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S1 – Selection regime faced by F populations

Transfer #	Combination #	pH	Salt (g%)	H_2O_2 (M)
1	46	9.5	2	0
2	54	9.5	4	0
3	31	7	5	0.014
4	64	4.5	5	0
5	31	7	5	0.014
6	30	7	4.5	0.014
7	26	7	1	0.014
8	13	7	2	0.012
9	10	9.5	0.5	0.01
10	68	8	5	0
11	51	10	3	0
12	20	6	0.5	0.012
13	22	8	0.5	0.012
14	15	7	4	0.012
15	55	10	4	0
16	1	7	5	0
17	16	7	4.5	0.012
18	53	5	4	0
19	71	10	5	0
20	54	9.5	4	0
21	8	4.5	0.5	0.01
22	29	7	4	0.014
23	38	9.5	0.5	0.014

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24	37	9	0.5	0.014
25	32	4.5	0.5	0.014
26	68	8	5	0
27	15	7	4	0.012
28	49	5	3	0
29	49	5	3	0
30	51	10	3	0

Individual component values –

Salt (g%) - 1, 2, 3, 4, 4.5, 5

pH - 4.5, 5, 6, 7, 8, 9, 9.5, 10

H_2O_2 (M) – 0, 0.01, 0.012, 0.014

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S2 – Novel and component environment concentrations used for fitness assays after fluctuating selection

	Assay Environment	Concentration
Novel environments	Cobalt chloride	14.2 mg%
	Zinc sulfate	18.4 mg%
	Ethidium bromide	2.5 mg%
	Norfloxacin	0.004 mg%
Component environments	Salt	5 g%
	Acidic pH	4.5
	Basic pH	10
	Hydrogen peroxide	0.058 M

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S3 - Selection under deteriorating environment

Environment	Concentrations		
	Day I	DayVIII	Step size
Cobalt	6 mg%	13 mg%	1 mg%
Zinc	5 mg%	12 mg%	1 mg%
Norfloxacin	0.4 ug%	1.1 ug%	0.1 ug%

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S4 – Protocol for estimation of mutation rate

We used the method suggested by Crane et al (Crane et al. 1996) for estimating mutation rates. One control population and one selected population were processed with a given batch of media. Glycerol stocks of control S and F populations were revived in NB for 18 hours. The revived culture was then diluted 10 fold. Eleven replicate flasks, each containing 50 ml of nutrient broth, were inoculated with 10 µl each of this diluted suspension. These replicate cultures were then allowed to grow for another 18 hours and then plated on nutrient agar with and without 100 micrograms/ml Rifampicin (Torres-Barceló et al. 2013) for estimating mutant counts and total numbers respectively. Colonies were counted manually, after 24 hours of incubation at 37ºC. Median estimator (λ_{med}) was estimated using following formula (Jones 1994):

$$\lambda_{med} = \frac{r_m/s - 0.693}{\ln\left(r_m/s\right) + 0.367}$$

where r_m = Number of mutants found in the median culture

s = Proportion of culture plated

Mutation rate, $\mu = \lambda_{med} / N$

where, N = Total number of living bacteria in 50 ml culture (as counted on Nutrient Agar).

Crane, G. J., S. M. Thomas, and M. E. Jones. 1996. A modified Luria-Delbrück fluctuation assay for estimating and comparing mutation rates. Mutat. Res.- Fund. Mol. M. 354:171-182.

Jones, M. E. 1994. Luria-Delbrück fluctuation experiments; accounting simultaneously for plating efficiency and differential growth rate. J. Theor. Biol. 166:355-363.

Torres-Barceló, C., G. Cabot, A. Oliver, A. Buckling, and R. C. MacLean. 2013. A trade-off between oxidative stress resistance and DNA repair plays a role in the evolution of elevated mutation rates in bacteria. Proc. R. Soc. B 280:20130007.

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S5 – Protocol for estimation of energy dependent efflux activity

For characterization of efflux properties of our populations, we modified an existing protocol (Webber and Coldham 2010) for fluorescent-based estimation of active efflux in Gram negative bacteria. Glycerol stocks of S and F populations were revived for 18 to 20 hours in NB. OD_{600} was adjusted, by diluting with NB, in the range of 0.03 to 0.06 OD units on Nanodrop (Thermo scientific 2000c). 2 ml of the OD_{600} adjusted cultures were then centrifuged, supernatant was discarded and the pellet was re-suspended in the PBS buffer (pH 7.4). Part of each suspension was then boiled at 60^0C for 10 minutes to be used as positive controls. The live cells and corresponding positive controls were then loaded in 96 welled plates in triplicates (168 µl and 180 µl respectively). 20 µl of Bis-benzimide (Excitation λ 355 nm and Emission λ 465 nm) was then added to all the wells. Bis benzimide is a small molecule which can easily enter the cell and fluoresce after intercalating with DNA. Live cells were also supplied with glucose since we wanted to look at the ATP dependent active efflux (8 µl of 1% glucose solution). The total volume of each well with live cell was 196 µl and with dead cells was 200 µl.

Fluorescence was measured on a plate reader (Tecan Infinite M200 Pro) at 37^0C for forty minutes. By 35 minutes the fluorescence levels in all wells reached their steady state. The level of fluorescence at 35 minutes was taken as the total fluorescence. The reader was then paused and 4 µl of Carbonyl Cyanide m-Chlorophenylhydrazone (C2759 Sigma) was added to all the wells with live cells. Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP) is a non-specific inhibitor of active efflux in Gram negative bacteria (Webber and Coldham 2010). Fluorescence measurement was continued for another 30 minutes for steady state to reach and the reading at 70th minute was taken as fluorescence without efflux.

The contribution of efflux was measured as –

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$$\frac{Fluorescence\ of\ live\ cells\ at\ 70\ minutes - Fluorescence\ of\ live\ cells\ at\ 35\ minutes}{Fluorescence\ of\ live\ cells\ at\ 35\ minutes}$$

Efflux contribution was arcsine square-root transformed for statistical analysis.

Webber, M. A., and N. G. Coldham. 2010. Measuring the activity of active efflux in gram-negative bacteria *in* S. H. Gillespie, and T. D. McHugh, eds. Antibiotic Resistance Protocols. Humana Press.

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S6- Effect size computed for the growth rates and yield measured in the three temperature environments from Figure 1 of Ketola et al. 2013. Means and ANOVA p-values are as reported in that paper.

	Assay Environment	**Mean C**	**Mean F**	**ANOVA p values**	**Effect Size±95% CI**	**Inference**
Growth rate	24^0C	0.31	0.34	<0.001	0.43±0.25	Small
	31^0C	0.46	0.51	<0.001	0.53±0.25	Medium
	38^0C	0.37	0.41	<0.001	0.61±0.25	Medium
Yield	24^0C	0.086	0.089	<0.001	0.21±0.25	Small
	31^0C	0.1	0.11	0.478	0.12±0.25	Small
	38^0C	0.075	0.079	<0.001	0.45±0.25	Small

Ketola, T., L. Mikonranta, J. Zhang, K. Saarinen, A.-M. Örmälä, V.-P. Friman, J. Mappes, and J. Laakso. 2013. Fluctuating temperature leads to evolution of thermal generalism and preadaptation to novel environments Evolution 67:2936-2944.