Evolutionary and Physiological Adaptation of *Pseudomonas aeruginosa* to Elevated Concentrations of Sodium Chloride

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ABSTRACT

I have investigated the evolutionary response of *Pseudomonas aeruginosa* to salt (NaCl) stress, and the physiological mechanisms responsible for this adaptation. Populations of *P. aeruginosa* founded from the same ancestral genotype were selected at three different concentrations of NaCl, low, moderate and high for about 660 generations with four independent replicates for each concentration. Adaptation was measured as the fitness of the evolved populations relative to the ancestor assessed in direct, head-to-head competition experiments conducted in the same environment in which they were selected (direct response) as well as in all alternative environments (correlated response). Results suggest that selection in each salt environment led to adaptation to that environment and a modest degree of specialization that evolved because correlated responses to selection were smaller than direct responses. In order to identify the physiological mechanisms contributing to the populations' adaptation in high NaCl concentration, I chose a sample of evolved lines that showed the strongest evidence for specialization to salt and competed them against the common ancestor in KCl and sucrose. Results suggested that increased Na^+ /H^+ antiporter activity is probably the primary mechanism behind adaptation to high NaCl concentration, however alternative mechanisms cannot be excluded. Tolerance curves, which measure the performance of a genotype across a gradient of salt concentrations, suggested no change in the high salt group's ability to tolerate extreme concentrations of NaCl. We conclude that high salt evolved population showed improvements to its ionic/osmotic stress resistance strategies mainly to Na+ efflux strategies but with no changes to salt niche.

RÉSUMÉ

J'ai investigué la réponse évolutionnaire de *Pseudomonas aeruginosa* au stress salin (NaCl) et les mécanismes physiologiques responsable pour ces adaptations. Des populations réplicates de *P. aeruginosa*, avec chacune quatre réplicats indépendant ont été fondées d'un même génotype ancestral et ont été sélectionné dans trois environnements contenant des concentrations de NaCl variés; basse, moyen et élevé pour une durée approximative de 660 générations. La mesure d'adaptation utilise le fitness relatif des populations évoluées et de la population ancestrale quand ceux-ci sont en compétition directes, de type tête à tête qui se déroulent dans les environnements alternatifs (réponse corrélée), en plus de leurs environnements de sélection (réponse directe). Le protocole de sélection a produit l'adaptation chez toutes les populations réplicates des traitements contenant des concentrations de sel de basse et élevé, en plus de deux populations dans le traitement moyen. Ces résultats suggèrent que la sélection dans chaque environnement salin a mené à une adaptation à cet environnement et un modeste degré de spécialisation qui ont évolué car les réponses corrélées à la sélection ont été plus petites que les réponses directes. Afin d'identifier les mécanismes physiologiques qui contribuent à l'adaptation des populations maintenues dans des concentrations salines élevés, j'ai choisi un échantillon des lignées évolués qui démontraient la plus grande spécialisation au sel et je les ai mit en compétition avec leurs ancêtre commun dans des milieux de KCl et de sucrose. Les résultats suggèrent que une augmentation d'activité d'anti-porteurs Na^+ /H^+ est probablement le mécanisme principale responsable pour l'adaptation à l'environnement saline élevée, par contre d'autres mécanismes alternatives ne peut pas être exclus. Les courbes de tolérance, qui servent à mesurer la performance d'un génotype chez un gradient salin, suggèrent qu'il n'y a pas de changement dans l'habilité du groupe évolué dans une haute

concentration saline de toléré un milieu salin (NaCl) extrême. En conclusion, la population qui a évolué dans un milieu de NaCl élevée a démontré des améliorations dans ses stratégies de résistance au stress ionique et osmotique, ce qui principalement due à des modifications de ses stratégies de flux de Na+, mais qui n'apporte aucun changement à sa niche saline.

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Moving from an Eastern to a Western culture and from an extreme heat (50^{o}C) to an extreme cold (-40^{o}C) wasn't easy, to say the least. I remember my first session at the University of Ottawa being a very harsh experience as it coincided with my first winter in Canada and with the OC Transpo's strike. However, meeting the cheerful and kind people from the Kassen lab and the other labs made things easier and more enjoyable.

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INTRODUCTION

Studies in the field of experimental evolution often focus on the process of adaptation to a certain environment and its consequences for fitness across a range of novel environments but ignore the underlying physiological mechanisms responsible (but see Angilletta et al. 2006). Physiologists, on the other hand, focus on the physiological mechanisms responsible for stress tolerance under the assumption that these are evolved adaptations, but usually ignore the historical process of how these adaptations themselves evolve[1]. This work aims to bring these two approaches together, with the goal of providing a more comprehensive explanation for the mechanistic causes of adaptation using an experimentally tractable system. More specifically, I investigate the evolutionary response of populations of microbes selected under NaCl stress and the possible primary physiological mechanisms responsible for adaptation.

'I have called this principle, by which each slight variation, if useful, is preserved, by the term Natural Selection'. This is how Charles Darwin defined natural selection in his book *On the Origin of Species,* in 1859[2]. Natural selection can only act if genetic variation already exists among individuals from which to select. Genetic mutations are the ultimate source of this variation that natural selection needs to act on and choose from the fittest individuals. Both, mutations and natural selection are of the main processes of evolutionary change and genetic diversity[3]. Over time, the selection process and the substitution of beneficial mutations result in adaptation to the surrounding environment and increase the population's fitness. This adaptation can be quantified by measuring fitness where fitness is defined as 'the average reproductive success of a genotype in a particular environment'[4]. It is usually measured by head-to-head competitions between two genotypes or the

evolved population and its unevolved ancestor and then estimating differences in growth rates and frequencies. Competitions allow for the detection of the effect of genotypes on one another and their competitive efficiency for resources[4,5].

Costs of adaptation may emerge during adaptation to a certain environment due to the fact that beneficial mutations were substituted to increase fitness in that particular environment and they causes a drop off in fitness in other alternative environments[6]. Costs of adaptation are indications of the emergence of specialists that evolve usually in homogeneous environments, whereas evolved populations that grow equally well over a range of environments are considered as generalists and usually evolve in heterogeneous environments. The existence of a cost of adaptation is thought to set the limits for niche[6–8]. A niche can be described as the characteristic range of values of a certain environmental factor where a particular species occupy and reproduce. The niche of a given environmental factor can be presented as a humped curve with the optimal performance at intermediate value of that environmental factor and the breadth of the niche covering a range of values across the environmental axis[7,6]. The niche can evolve by changes in the position, width and optimal value of the curve during adaptation to a certain environment and due to trade-offs by costs of adaptation.

Perhaps the oldest example of niche evolution was by Dallinger (1887) who started a selection experiment using bacterial populations with optimal growth temperature between 15.5^{o}C and 18.3^{o}C and 60^{o}C as the upper thermal limit. After 7 years of slowly increasing the temperature of the cultures, bacterial populations were able to grow at 70^{o}C. However, they lost their ability to grow at

15.5°C (the founding population's optimal temperature) thus providing the first direct evidence for a cost of adaptation and changes in the evolved population's thermal niche[9].

Environmental stress is a major factor driving evolutionary changes and diversification by imposing selection[10]. According to Kohen and Bayne (1989) stress can be defined as 'a measurable alteration of a physiological (or behavioral, biochemical or cytological) steady state which is induced by an environmental change and which renders the individual more susceptible to further environmental change' or simply it can be defined as 'any environmental change that acts to the reduce fitness of an organism'[11,12]. Many previous studies have focused on studying adaptation under stressful environmental conditions (e.g. temperature, acidity, alkalinity) in different organisms (e.g. bacteria, yeast, fruit flies, fish and plants)[13–19]. However, the usage of microorganisms as a model system has many advantages over using others: large population sizes, short generation times and the ability to create a 'fossil record' of frozen stocks that can be revived at will enable long-term evolutionary experiments to be conducted and, in principle, the physiological mechanisms responsible for adaptation dissected. Also, microbial evolutionary experiments can be controlled and replicated and their environment and genetic system can be manipulated easily[3]. For these reasons, I used bacteria, *Pseudomonas aeruginosa*, as the experiment's model organism to study adaptation to stressful conditions created by the addition of salt (NaCl) to the culture medium.

P. aeruginosa, a Gram-negative rod bacterium, is an opportunistic pathogen that occupies a wide range of environments with different salinities and is able to deal with sudden and fluctuating change in ionic and osmotic pressure. *P. aeruginosa* is a frequent cause of urinary tract infections where it survives urine concentrations and dilutions and it is also considered as a soil bacterium that survives

low and high rainfall periods[20,21]. *P. aeruginosa* is capable of growing under high concentrations of NaCl (up to 1M) and known for its metabolic diversity and ability for adaptation[22,23]. All of these properties make *P. aeruginosa* suitable to use for selection experiments in saline stressed environments.

Salinity of the habitat is an important factor that affects cellular processes and the growth of microorganisms[24]. In bacteria, a sudden increase in NaCl concentration of the external environment leads to a rapid influx of Na^+ and Cl^- into the cytoplasm and efflux of water accompanied by a decrease of cytoplasmatic volume which affects proteins, biological macromolecules, nutrient uptake, deoxyribonucleic acid (DNA) replication and other physiological processes[24–28]. *P. aeruginosa* deal with a wide range of salt concentrations and continuous changes in the surrounding environmental salinity by two main strategies. The first is to increase the efflux of intracellular Na^+ to lower intracellular concentrations of Na^+, which are toxic to the cell at high levels, using mainly Na^+/H^+ antiporters that exchange Na^+ for H^+ [29–32]. The second strategy is the intracellular accumulation of compatible solutes (osmoprotectants) in order to regain osmotic balance and increase cytoplasmic volume[33]. The absence of strategies to face ionic/osmotic stress has an inhibitory and maybe a lethal effect on bacteria[3].

Chapter 1 investigates the consequences of selection at different salinities on the specificity of adaptation. To do this, I used competition experiments against the unevolved ancestor to assay the relative fitness of lines that had evolved for approximately 660 generations in the same salinity in which they had been selected (the direct response to selection) and at a range of different salinities

(the indirect, or correlated, responses to selection). To my knowledge, this is the first selection experiment using a bacterial system to investigate the different adaptive patterns to salt stress.

Chapter 2 explores the mechanisms that underlie the adaptation to high salt and the associated physiological changes of ionic/osmotic stress resistance responses and their diversity among the replicate lines of the evolved populations. It also discusses the effect of the salt selection environment on niche evolution.

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Chapter 1

Evolutionary Response and Adaptation Patterns under Stress

Introduction

Stress is an environmental factor that causes injury to biological systems and reduces fitness[10, 34]. Environmental stress can be a major factor driving evolutionary changes and diversification in nature by enforcing directional selection that affects fitness and shifts the mean of traits[10]. Investigating patterns and mechanisms of adaptation and variation under stressful conditions is needed for a better understanding of the processes driving adaptive evolution and allows testing theories about the emergence and fate of diversity.

The purpose of this work was to study evolutionary and physiological adaptation under salt stress. In this chapter I investigated the evolutionary response of microbial populations to stressful environments and the consequences this has for fitness in novel environments. More specifically, I asked after the effectiveness of selection at generating adaptation to environments that differed in their level of salt stress over the course of approximately 660 generations of selection. I was interested in both the direct response to selection, that is, how much fitness improved in the environment in which selection occurred, as well as the indirect responses to selection, which is the change in fitness in novel environments that are the pleiotropic consequence of adaptation to a 'home' or 'native' environment.

Adaptation is a two step process. In the first step, mutation (or migration or recombination) generates genetic variance in fitness among individuals. This variation is then sorted by selection, with the type having the highest fitness displacing all others. When mutation is the sole source of genetic variation, beneficial mutations are substituted sequentially in a stepwise manner, leading to increases in fitness over time. Beneficial mutations are substituted without regard to their fitness in alternative environments, and so the result of adaptation to any given environment may be a loss of fitness in other environments. This situation is known as a cost of adaptation, with increases in fitness under selection being associated with a loss of fitness, relative to the unevolved ancestral type, under novel conditions. Costs of adaptation are thought to be important in preventing the emergence of a universal superior genotype that can survive successfully everywhere.

Costs of adaptation can be due to antagonistic pleiotropy, in which mutations that are beneficial in one environment are deleterious in another, or the accumulation of mutation of neutral effect in the selection environment but harmful elsewhere[6,4]. This leads to a drop off in the correlated fitness relative to the ancestor. The consequence of a cost of adaptation is the evolution of trade-offs in fitness across environments and the emergence of highly specialized types that have high fitness in some but not all environments. Note that trade-offs can also emerge even in the absence of costs of adaptation so long as beneficial mutations tend to have environment-specific effects such that the direct response to selection is greater than the correlated responses in unselected environments. Selection in a uniform, temporally homogeneous environment should thus lead to the evolution of specialists. Generalists that maintain fitness across a broad range of environments, on the other hand, are expected to evolve in more heterogeneous environments[6].

To investigate the importance of stress in the evolution of specialization I conducted an evolutionary experiment using laboratory populations of microbes evolving at different levels of saline stress. The usage of bacterial system has many advantages, such as, the ability to control the experimental conditions, the short generation time and the large population size. Bacteria can be also kept frozen for future analysis and it is easy to measure their fitness.

The model organism in this study is *Pseudomonas aeruginosa* (Pa), a gram negative bacterium which is found mainly in soil and water and can occupy a wide range of environments that differ in salinity. *P. aeruginosa* is also considered as an opportunistic pathogen causing infections in hospitalized patients[20]. Sodium chloride (NaCl) was chosen as the stressor to be imposed as it can cause both ionic and osmotic pressure.

A single colony of *P. aeruginosa* was allowed to evolve in three different environments (low, moderate and high salt concentrations) with four replicate lines for each treatment. Each population was transferred daily to a fresh medium for a total duration of about 660 generations. Conditions of growth remained constant for all the serial-transfer experiments. Adaptation was measured as an improvement in fitness relative to the ancestor via competition experiments. If the fitness response was measured under the selective regime of the evolved population then it is called *direct fitness response*; however, if it was measured under any other non-selective regime it is called *correlated fitness response*. To my knowledge, this is the first long-term experimental evolution study to investigate the adaptation patterns of bacteria under salt stress.

More specifically, this work addressed the following questions:

1) Is there a direct response to selection? Is *P. aeruginosa* capable of adapting in all salt treatments?

2) Is the adaptation of the evolved groups specific to salt, or is it a more general adaptation to the conditions of growth in the laboratory?

3) Do direct fitness responses of the evolved groups differ from each other? Is adaptation specificity to the selection agent? **Do direct fitness responses increase as the stressor (NaCl) intensity increases?**

4) Is adaptation to one environment associated with a loss of fitness, relative to the ancestor, in other environments? Is there a cost of adaptation?

5) Is there evidence of specialization in the form of trade-offs in fitness? Are responses of direct fitness greater than correlated fitness?

Materials and Methods

Strains and media

Pseudomonas aeruginosa strain14 with a *lacZ* neutral gene insertion[35], Pa14-*lacZ*, was used as the baseline organism for the selection experiment and its isogenic wild-type, Pa14, was used as the ancestor in the competition assays. Colonies of Pa14-*lacZ* appear blue on agar supplied with 40 mg L^{-1} of 5-bromo-4-chloro-3-indolylbeta-D-galactopyranoside (X-Gal) which makes it easy to distinguish them from the white colonies of the wild-type (ancestor). Pa14- *lacZ* and Pa14 clones were stored at -80^{o}C with 16% (v/v) glycerol.

Liquid culture media consisted of M9 minimal salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 6.8 g/L Na_2HPO_4 and variable concentrations of NaCl) supplemented with 15 mg/L $CaCl_2$ and 0.5 g/L $MgS0_4$, and 1.7 $X10^{-3}$ M carbon source (glucose). Three different NaCl concentrations (low, medium and high) were used to prepare the selection experiments' treatments; 8.6 mM, 380 mM and 500 mM. Bacterial populations were cultured in 2 mL of media in each well of a 24-well plate (Corning Incorporated, Corning, NY) and it was incubated at 37^{o}C in an orbital shaker (150 rpm).

Pilot Experiments

The salt concentrations (low, moderate and high) that had been used in the selection experiments were chosen based on two pilot experiments. In the first one, Pa14-*lacZ* was exposed to a wide range of NaCl concentrations: 0, 8.6, 17, 34, 170, 250, 500 mM and 1, 1.5, 2 M for 24 h and then plated on minimal medium agar plates for cell density determination (Fig. 1). Pa14-*lacZ* was then tested for its ability to keep population growth under daily 100-fold serial dilution in minimal medium with different NaCl concentrations: 8.6, 380, 500, 600, 700 mM and 1M. The second pilot experiment

was performed by daily transferring the cultures (20 μL) to fresh medium (2 mL), incubating them at 37^{o}C and then taking the optical densities at 630nm after every 24 h for 10 days (Fig. 2).

Neutrality of *lacZ* insertion

Zhang and Rainey[35] checked for the neutrality of the *laZ* insertion in *Pseudomonas fluorescens* SBW25 with respect to fitness in different media[35]. Their results suggested the comparability of fitness levels of SBW25-*lacZ* with the wild-type. However, it was crucial to check the neutrality of the marker in Pa14-*lacZ* under the experiment's conditions of different salinities. Pa14 wild-type and Pa14-*lacZ* were competed in each treatment of NaCl (8.6, 380 and 500 mM). The fitness of the Pa14-*lacZ* was estimated relative to the wild-type (see the next section "selection experiment" for details about performing competitions).

Selection Experiment

Three different NaCl concentrations were used in the selection experiments; 8.6, 380 and 500 mM with four independent replicates (lines) founded within each NaCl treatment (3 treatments x 4 replicates within each treatment = 12). Lines (replicates) evolved independently in the sense that there is no migration or gene flow among replicates within a treatment[36]. Daily serial transfers of 20 ul of each bacterial culture into 2 ml (100-fold dilution) of fresh medium of the same NaCl treatment were performed for a total of 100 transfers of about 660 generations with 6.64 generations per day (ln100/ln2, number of doublings that increases population density 100-folds). Samples from each population were checked for contamination every 5 days, by plating them on minimal medium agar plates supplied with X-Gal to make sure there are no white colonies (contamination), and then frozen for further analysis.

Fitness Assay

The response of fitness was estimated at the end of the experiment for each evolved population by first isolating a single colony of the most abundant morphology from every evolved population of each treatment and then competing a sample of this genotype against the oppositely-marked wild type (ancestor) A stock culture of the evolved genotype was prepared from each isolate and preserved at -80^{o}C with 16% (v/v) glycerol. The competitors (the ancestor and a single evolved population isolate) were first revived separately from frozen stocks in minimal medium (8.6 mM NaCl) for 24 h. Then they were cultured separately in the competing medium in order to bring them to the same physiological state. After that, the ancestor and the evolved line were initially mixed in 1:1 ratio by volume in the competition medium and after 24 h their mixture was transferred to fresh medium of the same type of the competition environment and left to compete for another 24 h. Initial (after 24 h of the first transfer) and final (after 24 hr of the second transfer) cell densities of each competitor were measured by plating them on minimal medium agar plates contain X-Gal in order to differentiate between the blue colonies of the Pa14-*lacZ* evolved population isolate and Pa14 ancestor (wild type) white colonies. The direct response to selection was obtained from competition assays in the selective environment of the evolved population, while the correlated response is calculated from competitions in alternative environments. Estimates of fitness calculated as the rate at which the frequency of the evolved population changes relative to the common ancestor:

$$w = 1 + \frac{\ln(P\text{final}) - \ln(P\text{initial})}{\text{Doubling}}$$

where (*P*initial) and (*P*final) are the ratios of the frequency of the evolved population to the ancestor population after the first growth cycle (initial) and the second growth cycle (final) respectively.

Doubling is the number of doublings or generations (calculated for the ancestral strain) that occur between the initial and final measurements (24 hours).

Evolved populations are identified by the NaCl concentration they have been selected in; E8.6, E380 and E500 for populations evolved in 8.6 mM, 380 mM and 500 mM NaCl, respectively.

Statistical Analysis

Mean direct responses to selection were evaluated for each of the three evolved groups using one-sample *t*-tests against a null hypothesis that mean fitness is 1. The probabilities for the direct response to selection were calculated using one-tailed test since I had an *a priori* reason to expect increases in fitness due to selection. Correlated fitness responses, on the other hand, were evaluated using two-tailed *t*-tests since I had no compelling reason to expect a response in one direction or the other. The significance level of all tests was set to α=0.05. Means of direct fitness responses across the three groups were compared with one-way ANOVA. ANOVAs (two-way) were used to test for line, assay environment and line-environment interaction effect on fitness:

$$\text{Fitness} = \text{Line} + \text{Assay Environment} + [\text{Line x Assay Environment}] + \text{error}$$

Post hoc tests (Tukey HSD) were performed to check for the source of any significant results.

The overall (genotype)- by-assay environment interaction was tested by ANOVA (two-way):

$$\text{Fitness} = \text{Group} + \text{Assay Environment} + [\text{Group x Assay Environment}] + \text{error}$$

SPSS 18 was used to perform all the statistical tests.

Results

Neutrality of the Genetic Marker

The marker gene *lacZ* has no detectable effect on fitness in several laboratory media[35]. For the purpose of this experiment, neutrality was re-evaluated under specific salt treatments (8.6, 380 and 500mM in minimal medium). This was achieved by measuring the fitness of the Pa14-*lacZ* relative to the wild-type (Pa14) by direct competition with six replicates for each regime. Results (Table1) show no significant difference between the two marker states (no significant effect of *lacZ* marker on fitness) based on *t*-test (one- sample, two-tailed, df= 5). Thus, Pa14 with *lacZ* marker insertion can be used in this experiment under the different salt treatments without affecting fitness.

Direct Response to Selection

The direct responses of the three evolved groups (E8.6, E380, E500) are summarized in Table 2 and presented in Figure 3 and 4. Both E8.6 and E500 groups show a significant gain of direct relative fitness with the E8.6 group having the highest increase (34%) and E500 a slightly more modest increase of 24% (Table 3). The medium salt group (E380) shows less marked fitness gain (17%) with substantial variance among replicate lines (Table 3, 4).

Despite the fact that E8.6 has the highest direct fitness gain (34%) followed by E500 (24%) and E380 (17%), there is no significant difference in direct fitness across the three groups (P =0.383, df=11, one-way ANOVA). This shows that their responses to selection didn't differ from each other.

Correlated Response to Selection and the Specificity of Adaptation

The correlated fitness responses of the three groups (E8.6, E380, E500) are summarized in Table 2 and presented in Figure 3 and 4.

In general, four out of the six correlated fitnesses show significant increase in fitness relative to the ancestor, while the other two didn't differ significantly from the common ancestor (Table 5). E8.6 group shows significant gain of correlated fitness in the 380 mM environment but not in 500 mM. In comparison, E500 has a significant gain of correlated fitness at both 8.6 mM and 380 mM environments. Even though, the E380 group only shows significant increase of correlated fitness at 8.6 mM, two lines from the group have significant increase in fitness in 500mM (Table 5).

I also investigated whether the evolved populations had adapted specifically to salt concentration or whether adaptation had occurred primarily to other factors in the culture medium such as types and concentration of nutrients. If the evolved population adapted specifically to its NaCl treatment concentration, it would be the fittest in its own selective salt concentration when compared with other populations evolved in other conditions of salinity. I tested this prediction by conducting two levels for comparisons. The first is across the same group where direct fitness is compared to the groups' correlated fitness responses (e.g. E8.6 direct fitness compared to its correlated fitnesses in 380 and 500 mM). The second type of comparison is between the direct fitness of the evolved group and the correlated fitness of the other evolved groups in the same environment (e.g. E8.6 direct fitness compared to E380 and E500 correlated fitness in 8.6 mM)

E8.6 direct fitness was greater than the group's correlated fitness responses at 380 mM (33.8%) and 500 mM (9%) (Table2). Table 6 of ANOVA (two-way) indicates significant effect of the type of the assay environments on fitness of E8.6 group. Tukey's test results showed significant difference between direct fitness and correlated fitness at 500 mM ($P < 0.05$). E8.6 group's direct fitness was also compared to correlated fitness responses of E380 and E500 groups in 8.6 mM environment by

conducted student's *t*-tests (one-tailed, independent-samples) in order to compare the direct fitness of the E8.6 and the correlated fitness of the other evolved groups in 8.6 mM. Results indicated that the E8.6 direct fitness is significantly better than the correlated fitness response of E500 groups in 8.6 mM (P=0.047, df= 6) whereas it didn't differ significantly from E380 group fitness in 8.6 mM environment (P=0.3, df= 6). Theses results assume that much of the group's adaptation was low salt specific.

The direct fitness of the E380 group (17%) was lower than the group's correlated fitness in 8.6 mM (29%) and not too much higher than the correlated response in 500 mM (16.6%) (Table 2). Results (Table 7) show significant effect of the type of the assay environment on E380 group fitness where correlated fitness response at 8.6 mM is significantly different from direct fitness and correlated fitness at 500mM ($P<0.001$).

Both, line and line-assay environment interaction have significant effect on fitness of E380 (Table 7). This suggests variation in fitness of the different lines over the three assay environments. However, E380 lines' correlated fitness gains in 8.6 mM were in general higher than their direct and correlated fitnesses in 500 mM. The group's direct fitness gain was lower than E8.6 and E500 correlated fitness gains in 380 mM (Table 2). This suggests no specificity of adaptation of E380 group.

E500 group direct fitness gain was about 24% and the group's correlated fitness response in 8.6 mM was 19% whereas it was 27% in 380 mM (Table2). ANOVA's result indicates no significant effect of the type of the assay environment or the line-assay environment interaction on fitness in E500 (Table 8). Nevertheless, there is a significant effect of line on fitness. Those results suggest no

difference in the overall mean fitnesses of the E500 group in the three assay environments but at line- level there is variation in fitness over the three environments which might mask evidence of specificity. Student's *t*-tests (one tailed, independent-samples) results indicated no significant difference between the group's direct fitness again and both E380 correlated fitness and E8.6 correlated fitness in 500 mM (P=0.25, P=0.059, respectively). Variation of fitness at line-level gave some evidences to assume the much of the specificity of adaptation of E500 group is to salt but not particularly to 500 mM.

Costs of adaptation, Trade-offs, Specialists, and Generalists

Is adaptation to one environment associated with a loss of fitness, relative to the ancestor, in other environments? Such costs of adaptation can be detected by a significant loss of fitness in at least one novel environment as a result of selection in another environment. This process should lead to the evolution of specialist types with highest fitness in the environment of selection and low fitness elsewhere. However specialists can also evolve in the absence of costs of adaptation if the direct response to selection exceeds the correlated responses in novel environments. In both cases, trade-offs in fitness across environments evolve such that no one type has highest fitness in every environment.

The results showed no cost of adaptation among any of the three evolved groups when assayed in the other two non-selective environments (Table 3, 5). However, there were some evidences for specialization since population did better in their own environment than in novel environments but there was asymmetry in the responses. ANOVAs (Table 6-8) report the trade-off in fitness of E8.6 group, therefore I assumed its specialization, however, there was no significant effect of the type of

the assay environment on E380 and E500 fitness responses which indicated the absence of trade-offs and the emergence of generalists patterns. Though, E500 results showed significant effect of line on fitness and variance in responses among lines which indicated the emergence of specialists at line-level such as in the case of line 4.

Discussion

Direct Fitness and Stressor Intensity

A number of previous studies have indicated the ability of bacterial systems to adapt under different stressful conditions such as acidity, alkalinity, low and high temperature[13,16,37]. *P. aeruginosa* is known for its ability to grow in a wide range of environments that differ in salinities and an ability for adaptation[20,23]. I hypothesised that *P. aeruginosa* would be capable of adapting to the experiment's salt stress. In general, our results indicated that all the three groups showed gain of direct fitness response. The low salt evolved group (E8.6) showed the highest significant direct fitness gain 34% (Table 3). This adaptation to low salt treatment was detected in a previous work on *E. coli*, where it was selected in glucose-limited medium, the same medium I used for the low salt group, and had a better fitness response than its founding ancestor[38]. High salt group (E500) also gained significant direct fitness 24% which indicates that *P. aeruginosa* can get better in dealing with high NaCl concentrations (Table 3). However, moderate salt group (E380) gain of direct fitness (17%) was insignificant, owed to the heterogeneity in response of the groups' lines where two lines have significant gain and the other two lines direct fitness gains were insignificant (Table 4). It is also possible that the E380 insignificant overall adaptation is due to the lack of statistical power that it could have been clearer if there were more replicates of the direct fitness competition assay of the E380 evolved population.

Even though, the low salt evolved group (E8.6) has the highest direct fitness with 34% gain, followed by E500 (24%) and then E380 (17%) (Table3), there was no significant difference in the direct relative fitness gain of the three evolved groups. This shows that their responses to selection didn't differ from each other. Therefore, there is no evidence here to suggest that the direct fitness gain and the adaptation level are correlated to the stressor intensity which opposes the hypothesis

that adaptation becomes greater as the stressor intensity increases due to the increase of the selection severity[13,37,39]. However, this might be due to variation in direct fitness responses across the replicate lines.

Correlated Fitness and Specificity of Adaptation

It was notable that both E8.6 and E500 did fairly well at 380 mM but less well the farther away from their 'home' salt concentration they got. This result suggests that the advance in fitness depends quantitatively on how similar the environments are. A similar pattern was detected for *E. coli* populations propagated at 20^{o}C when assayed at temperatures ranging from 20-39.8^{o}C[39].

The E8.6 basically showed no or very little improvement at 500 mM, whereas the E500 did substantially better at 8.6 mM. This suggests that different genes were responsible for adaptation at these two extremes and support the results suggesting the specificity of their adaptation.

The specificity of a population to its selection environment can be defined as being the fittest in 'home' environment when compared with other populations evolved elsewhere.

We tested for the specificity of adaptation with respect to salt concentration of the three evolved populations by conducting two levels for comparisons. The first is across the same group where direct fitness is compared to the groups' correlated fitness responses. The second type of comparison is between the direct fitness of the evolved group and the correlated fitness of the other evolved groups in the same environment.

There were enough indications to assume that much of the specificity of adaptation was to low salt concentration of E8.6 group and to elevated concentrations of salt in the case of E500 group but may be not particularly to 500 mM. If E500 group's adaptation wasn't specific to high salt, one would

expect it to behave almost similarly to E8.6 but it didn't. I believe that the specificity of E500 group wasn't clear due to variance among lines.

The low salt evolved group's fitness gain in moderate and high salt environments might be due to the fact that lab conditions represent stressful environments that lead to mutations in genes involved in global regulation of stress as it has been shown in a number of studies[40–42].

Results of the moderate salt evolved group (E380) were very surprising. The group's best performance was almost always in 8.6 mM. Even the other two evolved groups had higher fitness gain in 380 mM than E380. One possible way to explain this pattern and the lack of specificity is by assuming that the moderate salt concentration treatment (stressor) wasn't intense enough to create adaptation that is specific to that salt concentration. Another explanation would be the need for more generations to allow for more mutation to occur and fix. I ran this selection experiment for about 660 generations since many previous works used a similar number of generations and have demonstrated costs of adaptation, trade-offs and specificity of adaptation in that time frame, at least to different carbon sources[43–45].

Costs of adaptation, Trade-offs, Specialists, and Generalists

Both, costs of adaptation and trade-offs are expected outcomes of the process of adaptation to a certain environment and their emergence suggests the specialization of the evolved populations. Previous work has reported the association of adaptation to some stressful conditions with costs of adaptation and trade-offs. For example, the trade-off of bacterial populations at high temperature (40^{o}C) after being selected at low temperature (20^{o}C), acid (pH 5.6) selected populations showed costs of adaptation in alkaline environments, as well as some antibiotic-resistance bacteria when assayed in antibiotic-free environments[13,16,46]. However, it is also assumed that the emergence of

costs depends on the type of the environmental selective factor and on the direction of the change in that environmental agent[37,39].

I predicted that both moderate and high salt evolved groups would have a negative correlated fitness while the low salt group's correlated fitness would drop off in high salt regimes. I also expected the trade-off of populations evolved in moderate and high salt treatments in low salt regime and the other way around since specialization is usually associated with selection in homogenous environments while generalists are associated with heterogeneous conditions[6,47]. Results indicated the absence of any costs for adaptation across the three evolved groups. However, evidences were provided for specialization (trade-off) of E8.6 population while E380 and E500 groups seem to behave as generalists. E500 group generalist pattern might be due to the variation among replicate lines where results showed the specialization at line-level (line 4).

Conclusion

Selection resulted in adaptation by all replicate populations in low and high salt conditions and in two of the four replicate lines in the intermediate salt condition. Evolved lines also tended to display higher fitness in environments in which they were not selected, although this was not true for all lines. It was also found that quantitative similarity of the environments played a role in the advance in fitness. No cost of adaptation had accompanied fitness improvements, however, some populations showed evidence of a trade-off in fitness across environments, with the direct responses to selection generally exceeding the correlated responses. There was some evidence for specialization, although the strength of the trade-offs detected varied depending on the underlying environment: lines evolved at low salinities showed strong evidence of specialization when tested at high salinities, whereas the reverse was not true. Lines evolved at high salinities tended to improve in fitness substantially at lower salinities. These results thus suggest, in the first place, that adaptation to salt was specific to the particular salt environment, and secondly that three lines selected at high salt came to resemble generalists whereas those selected at low salt became more highly specialized.

Chapter 2

Physiological Adaptation to Stress

Introduction

Extreme environmental conditions force organisms and populations to deal with those challenges by evolving adaptive mechanisms in order to survive and reproduce. Those environmental stresses can lead to an increase in genetic and phenotypic variation at the physiological, morphological, biochemical and or behavioural levels[10,48–50]. For instance, wing sizes, ratios of wing dimension and ethanol vapor resistance are morphological and physiological changes in *Drosophila* in response to stresses of ethanol vapor and poor culture medium[51, 52]. Any stress-induced changes have to be inherited in order to have evolutionary significance[53–55].

Chapter 2 discusses the primary physiological mechanism of adaptation, the physiological divergence and changes in the salinity niche of evolved bacterial populations under salt stress.

Identifying the primary physiological mechanisms that are responsible for adaptation is crucial for our understanding of adaptive evolution, as it provides more detailed insight into how adaptation actually happens. Unfortunately, few studies are able to provide insight into both the evolutionary and physiological changes responsible for adaptation, at least on microevolutionary time scales. Here I address the physiological adjustments and the physiological basis of adaptation to salt stress that can help shed light on the basis of adaptation patterns.

Exposing bacteria to high concentrations of salt causes a rapid influx of both Na^+ and Cl^- ions into the cytoplasm. Any increase in the intracellular salt concentration would affect protein function

and the maintenance of proper trans-membrane potentials[56]. In addition, water efflux through the bacterial semipermeable membrane decreases the volume and water activity of the cytoplasm and result in inhibiting a number of physiological activities such as nutrient uptake, DNA replication, and biofilm formation in some bacteria species[24, 57]. However, bacteria have the ability to respond to changes in the osmolarity of their surrounding environment in order to survive and reproduce [21, 58]. In order to restore the level of its cytoplasmic volume and to avoid ionic and osmotic stress, the bacterial cell has two main tolerance mechanisms. The first strategy is to remove the intracellular sodium ions using mainly sodium (Na^+) / proton (H^+) antiporters and cation/ H^+ antiporters can also be used[59]. The second mechanism is the de novo synthesis or the uptake of compatible solutes/ osmoprotectants from the surrounding medium with specific transport systems. Osmoprotectants are small organic, non-toxic molecules that are highly soluble. They lead to an osmotic balance with the outside medium when accumulated in the cytoplasm. They do not interfere with the central metabolism of the cell even if accumulated to high concentrations[24,60–63]. *P. aeruginosa* , our experiment model organism, has the ability to survive stressful environments and the sudden increase in the salinity by activating either or both of these adaptation mechanisms[21, 58, 64].

In this study I aimed to determine the physiological adaptation strategy responsible for salt resistance. In particular I ask the question: did the evolved population adapt to salt stress by resisting ionic stress (Na^+ efflux) or did they adapt primarily to the osmotic pressure (via the intracellular accumulation of osmoprotectants) or to both? To achieve this, I chose a number of genotypes isolated from populations that had evolved in low and high salt for further study. I assayed the competitive fitness of these evolved lines in two novel environments where one of the regimes creates osmotic pressure (where Cl^- is the common ion between the novel environment and NaCl environment) and the other causes both osmotic and ionic stress.

Evolved populations may diverge genetically despite selection in a common environment when chance and contingency are the dominant features of adaptive evolution and when the same selection pressure leads to different genetic and phenotypic 'solutions'[7,36,50].

I investigated the extent to which selection in a common environment led to divergence in the genetic responses to selection in salt environment by assaying the performance of evolved lines across a wide range of environments that create different kinds of ionic or osmotic pressure. I would expect variation in response of the replicate evolved lines in the various salt treatments assuming that there are different adaptive genetic and phenotypic 'solutions' in the common selection environment.

I also asked how did the salt concentrations of the selection environment in the previous experiment (Chapter 1) impact the salinity tolerance of the evolved lines. In order to do so, I examined the evolution of tolerance curves, which are often used by physiologists to describe how performance changes as a function of variation in some underlying abiotic variable such as temperature, pH, or salinity. Tolerance curves often have a characteristic shape, with peak performance at some intermediate value of the environment. Although the physiological processes governing the shape of a tolerance curve are often well understood, we know comparatively little about how tolerance curves actually evolve (but see Knies *et al*, Bennett and Lenski)[65,66]. Here I ask how selection at each of the salt concentrations in our previous experiment impacted the salinity tolerance of our evolved lines.

I investigated the ability of the evolved lines to tolerate extreme salt concentrations and the effect of the selection environment on niche. To do this I compared shifts in the optimal concentration and the upper limit of the salt niche of the three evolved groups. Salt niche can be defined here as the range

of salt concentrations where the population is capable of respiration when other experimental conditions are held constant with salt optimum is where the population has its best performance.

Materials and Methods

Fitness Assay-Adaptation Mechanism

To determine the physiological adaptation strategy responsible for salt resistance four lines, from the previously described selection experiment in Chapter 1, were chosen based on their direct fitness gain to compete with the common ancestor in two novel environments. From low salt evolved group both line 1 and 4 were chosen and lines 3 and 4 were picked from populations evolved at high salt since their direct relative fitness responses are the highest (Table 2).

I used two novel treatments. The first was minimal medium supplied with KCl (500 mM, 1 Osm/L) with almost the same osmolarity as the 500 mM (Osm/L) NaCl regime and the second treatment was minimal medium with 1M sucrose (1 Osm/L).

The ancestor and the chosen evolved lines were first revived separately from frozen state in minimal medium (8.6 mM NaCl) for 24 h. Then they were cultured separately in the competing medium in order to bring them to the same physiological state. After that, the ancestor and each line were initially mixed in 1:1 ratio in the competition medium and after 24 h their mixture was transferred to fresh medium of the same type of the competition environment and let them compete for another 24 h. Initial and final cell densities of each competitor were measured by plating them on minimal medium agar plates containing X-Gal. Relative fitness was then calculated as described in Chapter 1. Relative fitness responses were then assessed using *t*-test with two-tailed probabilities. ANOVA (two-way) was used to test for line, assay environment and line-environment interaction effect on fitness across the three environments (NaCl, KCl, sucrose):

$$\text{Fitness} = \text{Line} + \text{Assay Environment} + [\text{Line x Assay Environment}] + \text{error}$$

Biolog Assays

Biolog panels were used to measure ionic/osmotic response and monitor physiological variation between evolved populations and the common ancestor and shifts in NaCl niche. Biolog PM9 96-well microplates (Biolog, Hayward CA) were used for this purpose. Each well contains different type and concentration of dried ionic, non ionic substrates as well as cationic and anionic solutes. Tetrazolium redox dye chemistry was used to measure cell respiration. Following the supplier's (Biolog, Hayward, CA) protocol for Gram-negative bacteria, about 20 µl of the evolved populations isolates (E8.6, E380, E500) and ancestor (Pa14-*lacZ*) were grown from frozen stocks in 2 ml of the selection medium (minimal medium with 8.6 mM, 380 mM, or 500 mM NaCl) for 24 h. Then, broths were streaked for isolated colonies on minimal medium agar plates and incubated overnight at 37°C. Cells were picked from the agar surface with a sterile cotton swab and suspended in 1 ml of the inoculation fluid IF-0 (0.5% NaCl). Cell densities were brought to 42% T (about 0.375 OD) using a spectrophotometer. 10 µl of the 42%T cell suspension was added to 50 µl of IF-0+dye (85% T). 60 µl of the 85% T cell suspension was transferred into a sterile vial containing 12 ml IF-10+dye (IF-10 supplied by Biolog Inc.). PM 9 plates were then inoculated with this cell suspension, 100 µl / well with two replicate plates for each line. Plates were incubated at 37°C for 48 h. Their absorbance was taken using automated Bio-Tek Elx800 microplate reader at 630 nm.

Physiological Divergence

I used PM9 profiles to check for the evolved groups' physiological variation and their ability to withstand various novel sources of ionic/ osmotic stress by comparing their performance under different substrates at different concentrations (60 wells) relative to the common ancestor. However, I had excluded a total of 36 wells of the different concentrations of NaCl (used for niche analysis)

and wells which test for organism's performance to ionic and osmotic stress with different osmoprotectants since I was interested in responses to ionic pressure and osmotic stress of the evolved populations without the interference of osmoprotectants which might improve performance. The absorbance data of the evolved groups of each substrate were normalized to (divided by) the ancestor's mean performance in the corresponding well and then these normalized data for all the substrates were averaged for each evolved group. The normalized mean of the overall performance of each evolved group was compared to the ancestor's mean performance (1) by conducting two-tailed *t*-tests (null hypothesis: normalized mean performance =1).

Salt Niche

I used 12 different NaCl concentrations (0.1, 0.3, 0.5, 0.68, 0.85, 0.94, 1.02, 1.1, 1.9, 1.3, 1.5 and 1.7 M) from Biology PM9 plates to test for changes in niche optimal concentration and the upper limit across the three evolved populations. Two ANOVAs (one-way) were conducted to test for the effect of selection environment on the optimal salt concentration (peak location) and on the highest salt concentration that can be tolerated (niche limit).

Results

Adaptation Mechanism

I investigated the physiological mechanism of adaptation to high salt by comparing relative fitness responses in NaCl, KCl and sucrose environments of a selection of lines evolved under high salt concentrations and compared them to the relative fitness responses of low salt group lines (control) under the same environments (Fig. 5)

In general, low salt evolved lines (control) performance in KCl and sucrose is as good as the ancestor, with the exception of line four in sucrose (Table 9), which has a significant reduction in relative fitness. Fitnesses of the lines evolved in high salt are better than the ancestors' in NaCl, KCl and sucrose, except for line three in sucrose whose fitness is similar to the ancestor's (Table 9). These results indicate that line 4 (E500) had improved physiologically in dealing with both ionic and osmotic stress whereas line 3 (E500) was well adapted to deal with ionic stress.

Relative fitness was significantly affected by the type of the assay environment and the line tested (Table 10). In order to figure out the source of this significant result, I ran a post-hoc tests (Tukey's) and pairwise comparisons. Results indicate a significant difference in relative fitness of the E500 lines in NaCl and KCl, as well as in NaCl and sucrose. Relative fitness of the two lines of E8.6 group didn't differ significantly in the three regimes except in sucrose for line four.

Physiological Divergence

The physiological divergence of the evolved populations was tested by comparing each group's overall performance in the 60 wells of PM9 to the ancestor's. Groups doing better than the common ancestor will have normalized mean values higher than 1. Results showed no significant difference

in the performance in both E8.6 and E380 groups compared to the ancestor's (*P*=0.89, *P*= 0.647,df= 3 , respectively). However, E500 group's performance differed significantly (P=0.027, df=3).
In order to test for variation in performance between independent replicate lines of the same evolved population, ANOVAs (one-way) were used. The results indicate significant difference in performance between the lines of E380 group (*P*=0.03, df=3, 4) where line 2 differ significantly from all the other three lines (Tukey's test) and also differed significantly from the ancestor (*t*-test, two-tailed, *P*=0.012). On the other hand, we could detect no variation in fitness among lines evolved at E8.6 nor among those evolved at E500. Figure 6 shows the normalized performance of evolved groups' lines in PM9 plates and the means of their performance.

Salt Niche

I tested for the effect of selection environment on the optimal salt concentration and on the highest salt concentration that can be tolerated. Figure 7 represents the curves of tolerance of the three evolved populations across 12 different NaCl concentrations in PM9 profiles. Table 11 presents the optimal NaCl concentrations of the evolved populations as well as the highest salt concentration that can be tolerated. The highest tolerable concentration is the highest salt concentration at which the OD is significantly different from zero (*t*-tests, two-tailed). Results indicated that neither the peak location nor the upper limit of the niche was significantly affected by the selection environment (*P*=0.495, df= 2, 9, *P*=0.354, df =2, 9, respectively).

Discussion

Adaptation Mechanism

Bacteria under salt stress experience two types of strains: ionic stress and osmotic pressure. Ionic stress results when the ions influx rapidly into the cytoplasm increasing the intracellular concentration of these ions which would have toxic effect and inducing water efflux. Water moves from higher-solute concentration to lower (osmosis) which leads to the shift of the osmolality of the cytoplasm from optimum and then osmotic stress is generated[3]. *P. aeruginosa* can resist high salinity by removing the intracellular Na^+ ions using Na^+ / H^+ antiporters as well as cation/ H^+ antiporters. The second strategy is the accumulation of osmoprotectants in order to regain osmotic balance and increase cytoplasmatic volume[33].

I have investigated the primary mechanism that was responsible for adaptation to high salt in an evolution experiment conducted for 660 bacterial generations. The hypothesis was, if the evolved populations adapted to high salt primary by improvements in Na^+ / H^+ antiporters then we would expect the highest relative fitness in NaCl, and if it was induced mainly by developments in cation / H^+ antiporters then we would have almost the same gains in relative fitness under both NaCl and KCl. However, if adaptation resulted from the accumulation of osmoprotectants, we would expect the highest relative fitness to be under the sucrose regime.

The results support the hypothesis that improvements in Na^+ / H^+ antiporters are the main reason behind adaptation of the high salt evolved population. However, they high salt evolved population also showed relative fitness gain under KCl while the low salt evolved lines (control) did not which suggests that cation / H^+ antiporters could be involved in the adaptation process but to a lesser degree or that the populations had adapted by improving also mechanisms to deal with Cl^- ions.

In addition, there was evidence that osmoprotectants played a role in adaptation to salt, at least in the case of line 4 of E500 group since it gained relative fitness under sucrose. Whereas relative fitness of E8.6 lines and line 3 of E500 either didn't differ from the ancestor, or was worse.
These findings don't exclude the possible involvement of other processes and strategies such as changes in the permeability of the outer membrane porin[67].

Chapter 1 results indicated that line 4 of E500 is a high-salt specialist, since it had low fitness under low salt concentrations. This would indicate that this line might need salt for better performance. Results also indicated that line 4 also evolved to face osmotic pressure (in sucrose) and functioned well under KCl (might be due to Cl^- adaptation). It is interesting to note that these responses are very similar to what is observed among some halophiles where the intracellular accumulation of KCl allows proteins to function normally under high salt conditions. Also, other types of halophiles have adapted by the intracellular accumulation of high concentrations of osmoprotectants which allows them to grow over a remarkably wide range of salt concentrations[68].

Further work is needed in order to confirm the results of the Na^+/H^+ antiporters being the primary mechanism responsible for the physiological adaptation and salinity resistance of the high salt evolved population. This could be achieved by investigating levels of genes expression related to ionic/osmotic stress regulation, such as genes associated with osmoprotectants synthesis, Na^+/H^+ and cation/H^+ antiporters activities or the genes regulating them. Another possibility is the measurement of the intracellular osmoprotectants concentrations within the evolved populations (specially line 4 of E500) and compare them to the ancestor's which would exclude or suggest adaptation by osmoprotectants accumulation.

Physiological Divergence

Adaptive evolution might result in divergence in the genetic response in a common selection environment[7,69,70]. Here I have tested to what extent selection can lead to divergence between replicate lines within a treatment by testing each line's overall response in a range of novel substrates that create different kinds of ionic or osmotic pressures.

Results of lines' overall performance in the 60 wells of PM9 compared to the ancestor's suggest that evolved lines differed from the ancestor only in the case of high salt evolved populations. This result suggests that the E500 lines varied in the physiological mechanism by which they adapted to salt, indicating that adaptation has occurred through different genetic routes in comparison to the other two treatments' evolved lines.

Results also showed that independent lines of high salt group did not vary from each other in terms of performance in novel environments that create ionic/ osmotic pressure. One possible explanation is that there are limited adaptive solutions to the salt stress, therefore, there are limited physiological strategies to deal with high salinity. E8.6 groups' lines did not differ under PM9 stressful environments. However, the moderate salt evolved group did (Fig. 6).

Salt Niche

Very few studies attempt to characterize the tolerance curve of evolved lines in an experimental setting. Here, I have investigated the effect of selection at different salt concentrations on the upper limits to salt tolerance and the optimal salt concentration.

Particularly, I was interested if the moderate and high salt evolved groups would show extensions in their niches' upper limit so they would show preadaptation to more extreme salt concentrations of their adaptation to moderate/high levels of salinities. Even though previous results (Chapter 1) of low salt group showed the lack of any cost of adaptation when assayed in moderate (380 mM) and high (500 mM) NaCl environments there might be still a chance that it would trade-off in higher concentrations and would suffer from niche shrinkage as a consequence of its prolonged growth in low salt. As for the peak location, the expectations were that they would be located near each group's selected salt where they should have their best performance. Nevertheless, this might not be the case for the optimal peak location, given that experiments in Chapter 1 suggested that the E380 and E500 are generalists. E8.6 selective regime wasn't tested here since it wasn't part of the 12 concentrations (100 mM- 1.7 M) provided by PM9 plates.

Results indicated limited effect of the selection environment on both peak location and the upper limit of the niche. This means that neither the moderate/high evolved groups became more salt tolerant nor the low salt group became more salt sensitive which suggests that the upper limit of the salt niche is strictly conserved and it is difficult to go beyond which might be due to the conservative force acted by natural selection inside the niche[71]. Similar patterns of no change in the upper niche and optimal peak location have been seen in temperature adapted populations of *E. coli* (42^{o}C) when assayed in a range of different temperatures (12-44^{o}C). However, the thermal optima of populations adapted at 32^{o}C diverged by about 10^{o}C[65]. Also, in another study of high temperature adapted

baceriophage G4, results showed changes in optimal temperature and niche width across a range of temperatures[66].

One possible way of pushing the upper limits of salt tolerance curve is the slow increase in salt concentrations. This was achieved by Samani and Bell where after a stepwise increase in NaCl concentrations, yeast populations adapted to lethal salt concentration as a result of indirect response to selection under lower salt concentrations[15].

Conclusion

I conclude that Na^+ efflux strategies and more specifically Na^+/H^+ antiporters might be the primary mechanism responsible for the physiological adaptation and the salinity tolerance of the high salt evolved population. However, this does not exclude the involvement of other mechanisms such cation/ H^+ antiporters or the accumulation of osmoprotectants. Further work, such as measuring levels of expression of genes related to salt tolerance or detecting the intracellular concentrations of osmoprotectants is needed to help confirming theses findings. Results also provided another piece of information about tolerance curves that salt niche upper limit is conserved.

Chapter 2

Conclusion

The objective of the study was to bring together evolutionary and physiological approaches to study stress tolerance with the aim of providing a more comparative description of adaptation which provides more detailed insight into how adaptation actually happens. Unfortunately, few studies are able to provide insight into both the evolutionary and physiological changes responsible for adaptation.

I conclude that *P. aeruginosa* was able to adapt and evolve under NaCl stress primary through improvements to Na^+ efflux strategies and more specifically Na^+/H^+ antiporters, yet I believe that more work is needed to confirm these findings, such as levels of expression of certain genes. The benefit of the physiological approach taken here with a more genomic one is that it provides suggestions about what genes to look at first. Our results also showed that high salt population adapted as generalist without changing its salt niche upper limit and to the maximum NaCl concentration that it can handle

Table 1. Neutrality of the genetic marker insertion *lacZ* under salt concentrations in Pa14

Assay Environment (NaCl) [1]	Fitness of Pa14-*lacZ* relative to wild-type	
	Mean (± SE) [2]	*P* value [3]
8.6 mM	0.978 (±0.109)	0.853
380 mM	0.98 (±0.036)	0.612
500 mM	0.989 (±0.022)	0.636

[1]Minimal medium with different salt concentrations

[2] Mean (and standard error) of six assays of competitions in each treatment

[3] Based on *t*-test for one-sample, 2-tailed, df= 5, α=0.05, null hypothesis mean fitness=1

Table 2. Means of relative direct and correlated fitness responses of evolved groups and lines

		Assay environment		
Genotype group	Line	8.6m M	380 mM	500 mM
8.6	1	1.476(±0.124)	1.253(±0.435)	1.11882(±0.019)
	2	1.334(±0.019	1.436(±0.082)	1.21078(±0.034)
	3	1.237(±0.029)	1.396(±0.093)	0.98901(±0.028)
	4	1.329(±0.003)	1.267(±0.103)	1.05872(±0.065)
Group means		1.344 (±0.049)	1.338 (±0.458)	1.094(±0.047)
380	1	1.196 (±0.054)	1.083(±0.03)	1.42(±0.103)
	2	1.527 (±0.052)	1.517(±0.048)	1.04(±0.038)
	3	1.237 (±0.049)	1.042(±0.022)	1.091(±0.056)
	4	1.21 (±0.075)	1.072(±0.011)	1.114(±0.023)
Group means		1.292(±0.0787)	1.179 (±0.113)	1.166(±0.086)
500	1	1.062 (±0.056)	1.137(±0.069)	1.06(±0.042)
	2	1.292(±0.044)	1.347(±0.063)	1.23(±0.0414)
	3	1.29(±0.135)	1.309(±0.108)	1.345(±0.0791)
	4	1.124(±0.041)	1.3(±0.015)	1.327(±0.0701)
Group means		1.192(±0.0587)	1.273(±0.0466)	1.24 (±0.065)

Table 3. Means of relative direct fitness of the evolved groups and line response patterns

Selective environment[1]	Mean fitness (±SE) [2]	*P* values[3]	Line responses[4]
8.6	1.344 (±0.049)	0.006*	$4^a,0^b,0^c$
380	1.179 (±0.113)	0.213	$2^a,2^b,0^c$
500	1.24 (±0.065)	0.034*	$3^a,1^b,0^c$

[1] Values indicate NaCl (mM) concentration in each selective environment of minimal medium
[2] The mean of direct relative fitness (±Standard Error) for each genotype group calculated from four lines means of 3 replicates for each line.
[3] *P* values based on Student's *t* test, one tailed, α=0.05 and df=3.
[4] Number of lines in each genotype group with significant gain [a], no change [b] and significant loss [c] of direct fitness.
* Significant at $P<0.05$

Table 4. Means of relative direct fitness at the line level

	Evolved groups		
	E8.6	E380	E500
Line	*P* values[1]		
1	0.031*	0.054	0.143
2	0.001*	0.004*	0.015*
3	0.007*	0.09	0.024*
4	<0.000*	0.0125*	0.021*

[1] *P* values based on Student's *t* test, one tailed, α=0.05 and df=2
* Significant at $P<0.05$

Table 5. Means of relative direct fitness of the evolved groups and some adaptation patterns

Selective environment[1]	Assay environment[2]	Mean fitness (±SE) [3]	*P* values[4]	Line responses[5]
8.6	380	1.338 (±0.458)	0.005*	$3^a,1^b,0^c$
	500	1.094 (±0.047)	0.139	$2^a,2^b,0^c$
380	8.6	1.292 (±0.0787)	0.034*	$4^a,0^b,0^c$
	500	1.166 (±0.086)	0.148	$2^a,2^b,0^c$
500	8.6	1.192 (±0.0587)	0.047*	$2^a,2^b,0^c$
	380	1.273 (±0.0466)	0.010*	$4^a,0^b,0^c$

[1, 2] Values indicate NaCl (mM) concentration in each environment of minimal medium
[3] The mean of direct relative fitness (±Standard Error) for each genotype group calculated from four lines means of 3 replicates for each line.
[4] *P* values based on Student's *t* test, one tailed, α=0.05 and df=3.
[5] Number of lines in each genotype group with significant gain [a], no change [b] and significant loss [c] of correlated fitness.
* Significant at $P<0.05$

Table 6. Two-way ANOVA treating line and assay environment as factors for 8.6-adapted population (E8.6)

Source	SS	d.f.	MS	*F*	*P*
Assay environment	0.487	2	0.243	10.755	<0.0001*
Line	0.085	3	0.028	1.258	0.311
Line x Assay environment	0.158	6	0.026	1.163	0.358
Error	0.543	24	0.023		

* Significant at $P<0.05$

Table 7. Two-way ANOVA treating line and assay environment as factors for 380 -adapted population (E380)

Source	SS	d.f.	MS	*F*	*P*
Assay environment	0.116	2	0.058	17.081	<0.0001*
Line	0.333	3	0.111	32.670	<0.0001*
Line x Assay environment	0.617	6	0.103	30.295	<0.0001*
Error	0.081	24	0.003		

* Significant at $P<0.05$

Table 8. Two-way ANOVA treating line and assay environment as factors for 500 -adapted Population (E500)

Source	SS	d.f.	MS	*F*	*P*
Assay environment	0.040	2	.020	2.695	0.088
Line	0.285	3	.095	12.743	<0.0001*
Line x Assay environment	0.070	6	.012	1.568	0.2
Error	0.179	24	.007		

* Significant at $P<0.05$

Table 9. Means of relative fitness responses in NaCl, KCl and sucrose of certain lines of E8.6 and E500 evolved groups

	Mean (± SE) *P* values[2] Assay Environment[3]					
Line[1]	NaCl		KCl		Sucrose	
E8.6 L1	1.119 (±0.011)	0.008*	1.062 (±0.010)	0.104	1.022 (±0.013)	0.340
E8.6 L4	1.059 (±0.038)	0.259	1.05 (±0.049)	0.487	0.858 (±0.008)	0.038*
E500 L3	1.345 (±0.079)	0.049*	1.114 (±0.005)	0.026*	0.93 (±0.082)	0.551
E500 L4	1.327 (±0.07)	0.043*	1.139 (±0.006)	0.029*	1.152	0.002*

[1] Lines from (E8.6) 8.6 mM NaCl evolved group, (E500) 500 mM NaCl evolved group

[2] Based on *t*-test for one-sample, 2-tailed, df= 2,1 , α=0.05, null hypothesis mean fitness=1

[3] Minimal medium with 500 mM of NaCl, 500 mM of KCl and 1M of sucrose

* Significant at $P< 0.05$

Table 10. Two-way ANOVA treating line and assay environment as factors for E8.6 and E500 groups

Source	SS	d.f.	MS	*F*	*P*
Assay environment	0.242	2	0.121	20.272	<0.001*
Line	0.172	3	0.057	9.619	<0.001*
Line x Assay environment	0.095	6	0.016	2.656	0.055
Error	0.095	16	0.006		

* Significant at $P<0.05$

Table 11. Optimal and upper salt limits for the different evolved groups across 12 NaCl concentrations tested in PM9 plates.

Evolved Group	Line	Optimal NaCl concentration [1]	Upper NaCl limit [2]
E8.6	1	0.3	0.94
	2	0.5	0.94
	3	0.3	1.10
	4	0.3	0.94
E380	1	0.1	1.02
	2	0.1	0.94
	3	0.3	1.02
	4	0.68	1.02
E500	1	0.68	1.02
	2	0.68	1.02
	3	0.5	1.02
	4	0.1	1.1

[1] Concentration at which OD is the highest across 12 concentrations of NaCl (M)
[2] Highest tolerable concentration at which the OD is still significantly different from zero, *t*-tests, two-tailed

Figure 1. Cell density of *P. aeruginosa* under different concentrations of NaCl in minimal media. Standard error bars based on three replicates.

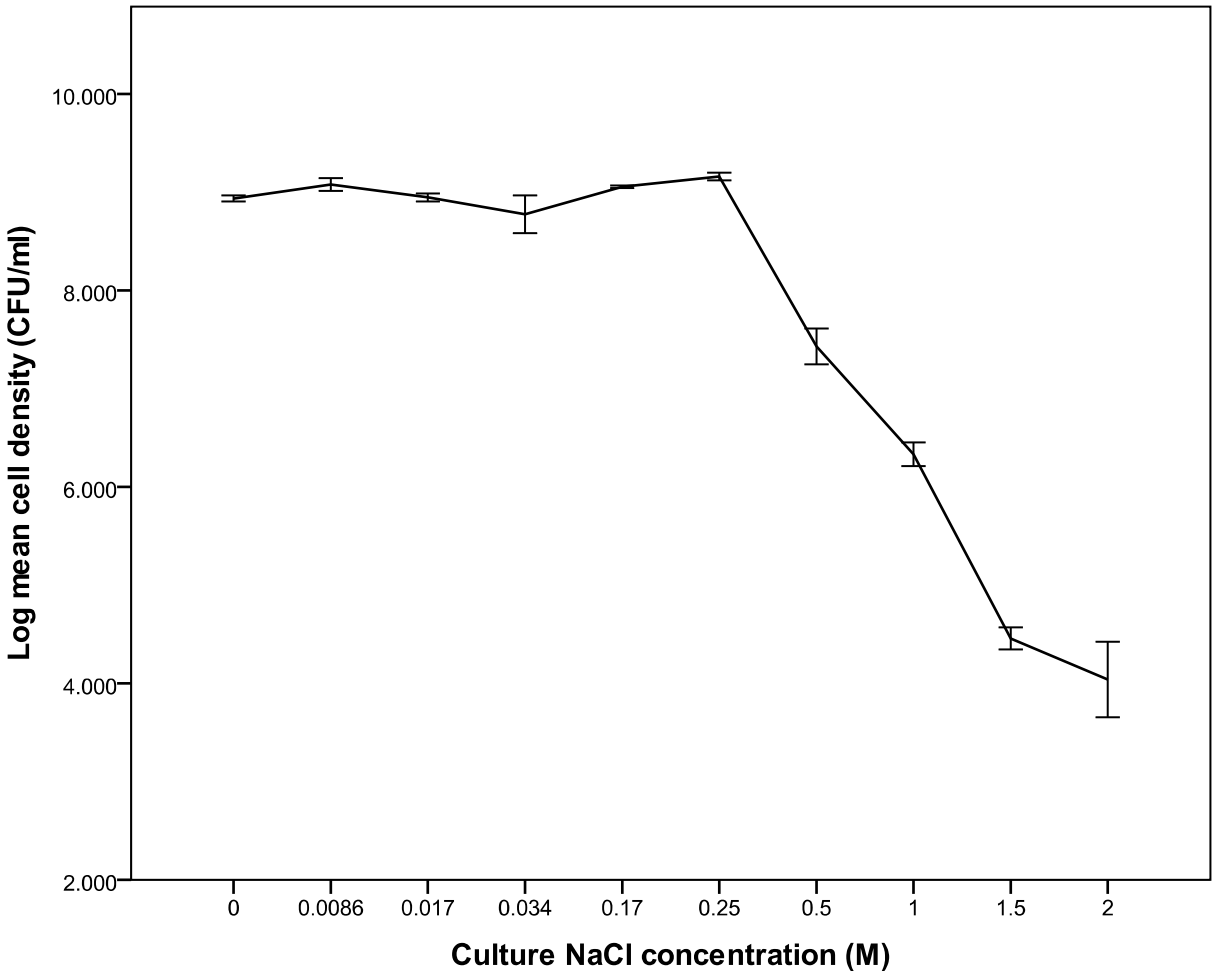


Figure 2. Optical densities of *P. aeruginosa* under different concentrations of NaCl in minimal media. Standard error bars based on two replicates.

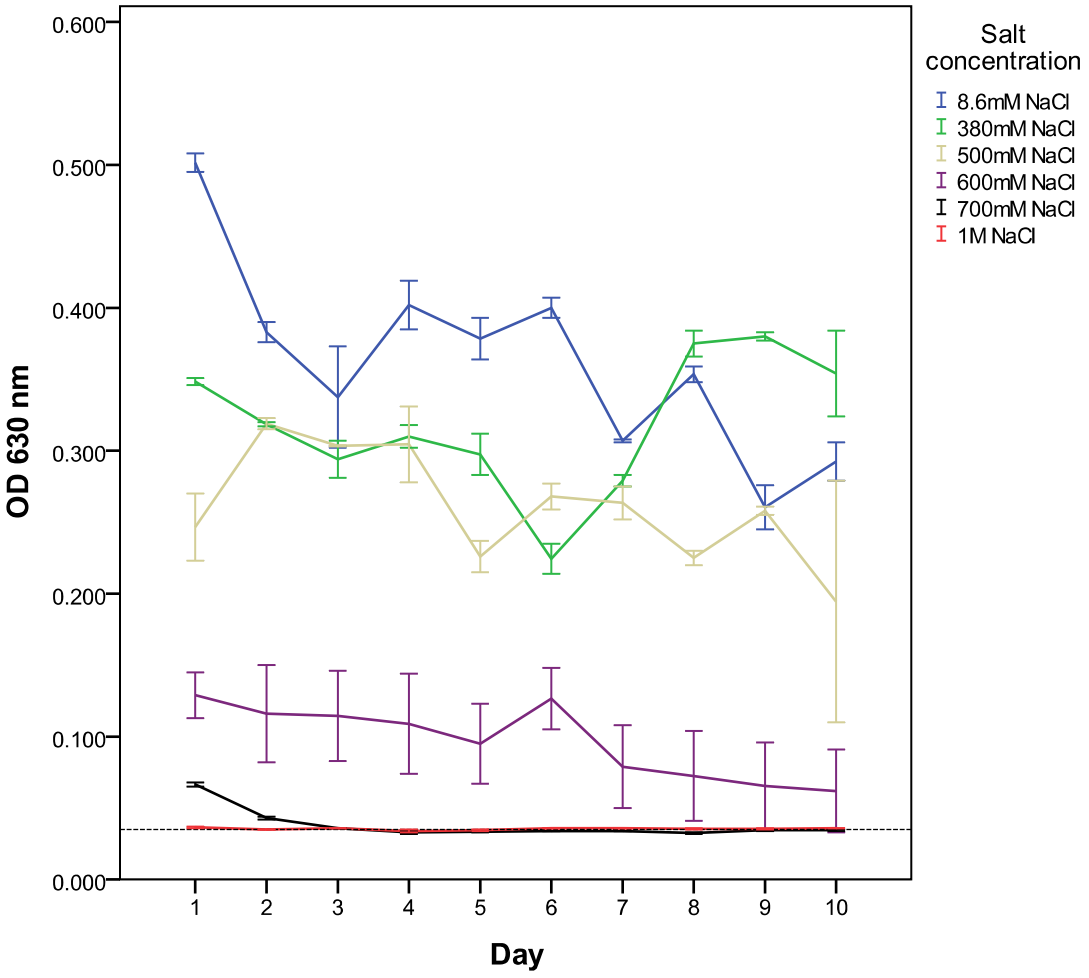


Figure 3. Means of direct and correlated relative fitness responses of the three evolved groups: E8.6 evolved in 8.6 mM NaCl, E380 evolved in 380 mM NaCl and E500 evolved in 500 mM NaCl. Error bars are standard error based on four independent replicates (lines).

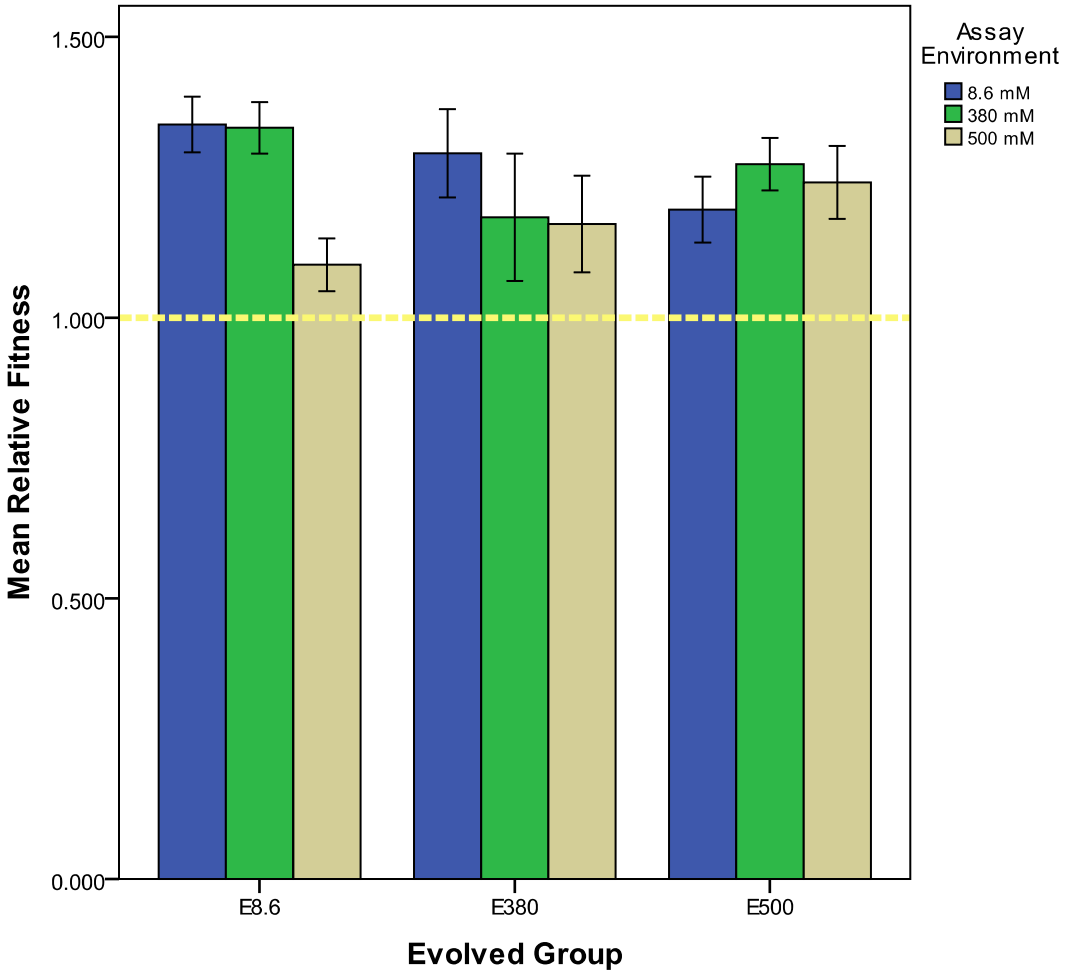


Figure 4. Means of direct and correlated relative fitness responses of the four independent replicates (lines) of each evolved population. E8.6 evolved lines in 8.6 mM NaCl, E380 evolved lines in 380 mM NaCl and E500 evolved lines in 500 mM NaCl. Error bars are standard error based on three replicates.

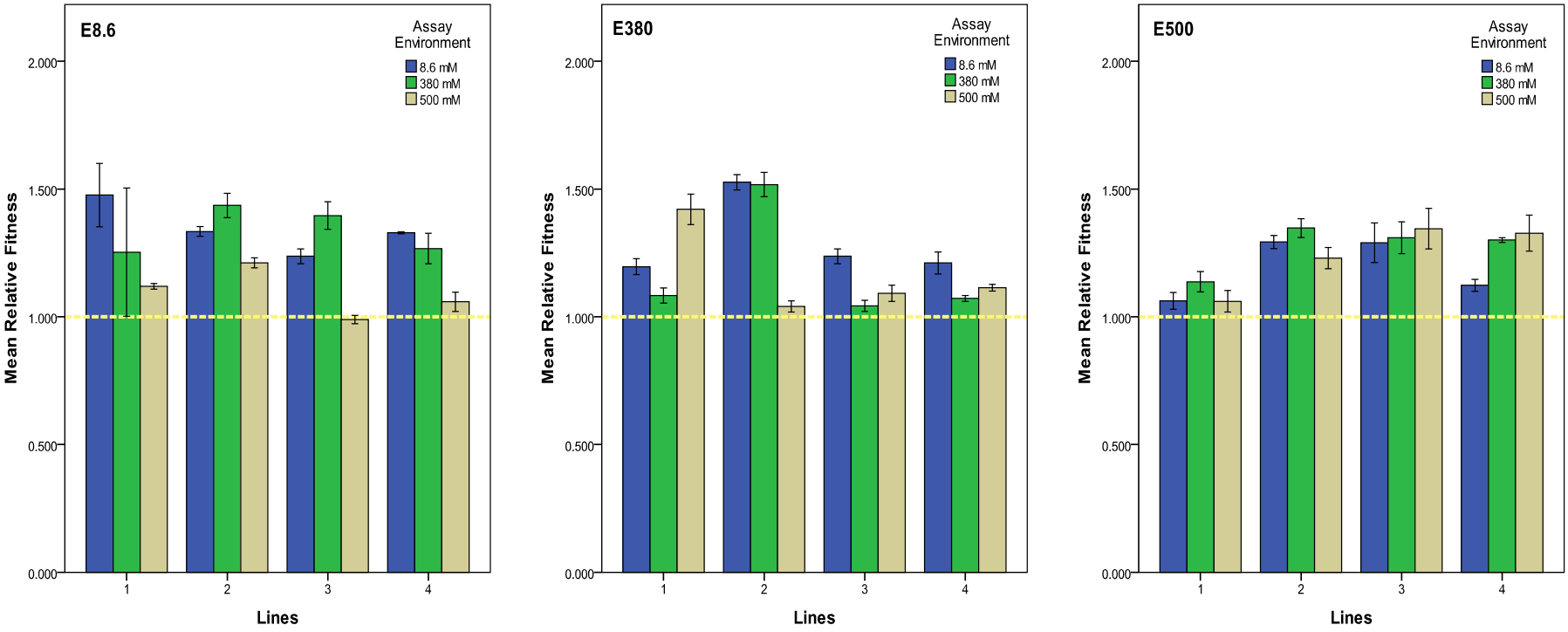


Figure 5. Means of relative fitness responses (± standard error) of the two independent replicate lines evolved in low salt and another two independent replicate lines evolved in high salt. Lines were assayed in NaCl (500 mM), KCl (500 mM) and sucrose (1M).

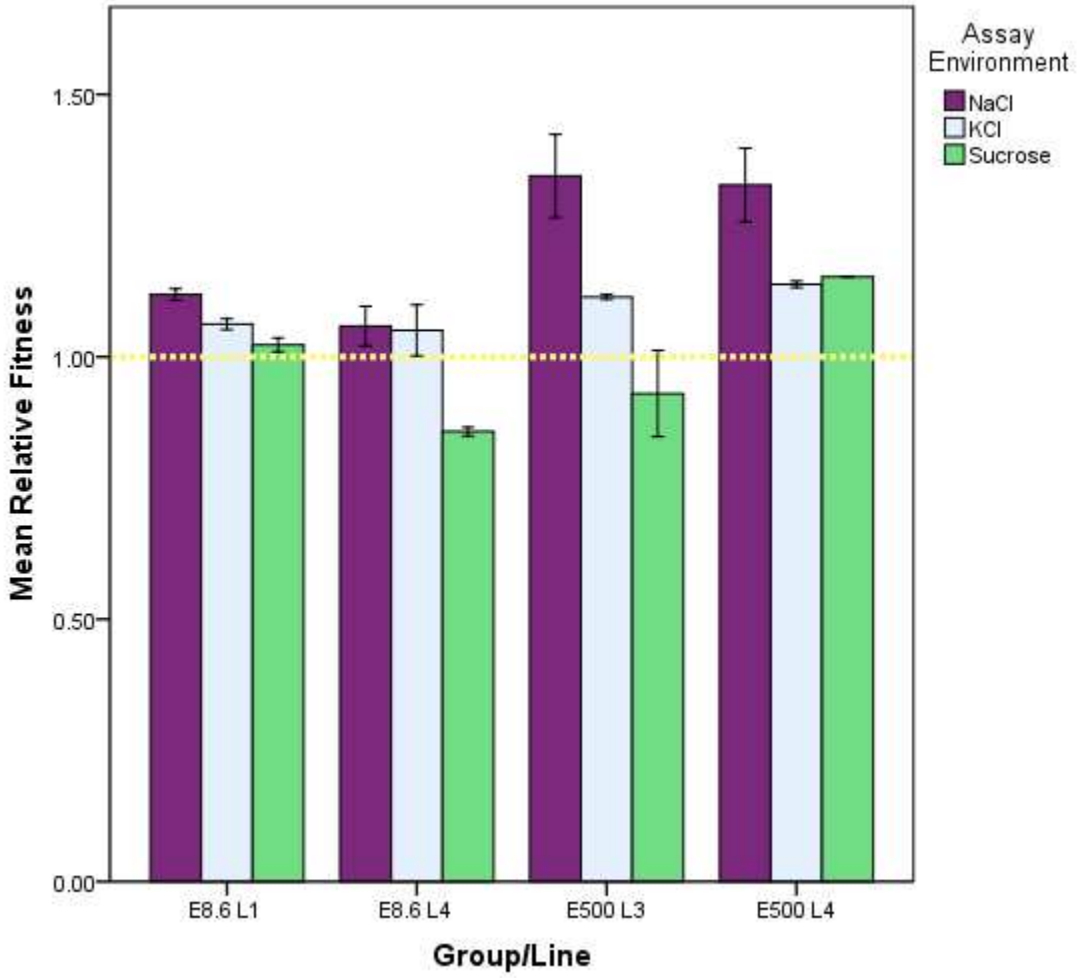


Figure 6.Normalized performance of four independent replicate lines of each evolved group (E8.6, E380, E500) in PM9 profiles. Hollow circles represent lines performance and filled circles are the means of the four lines performance.

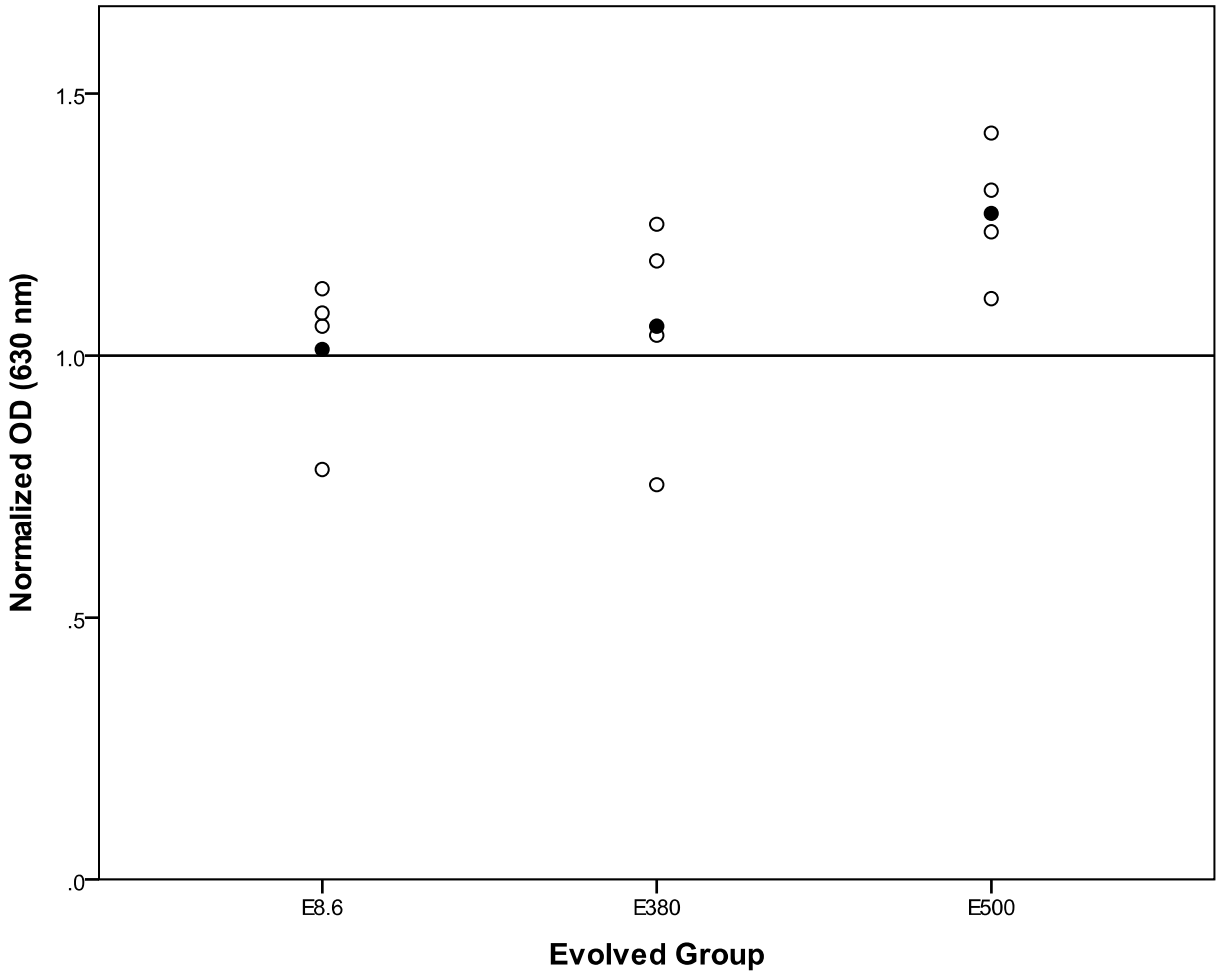
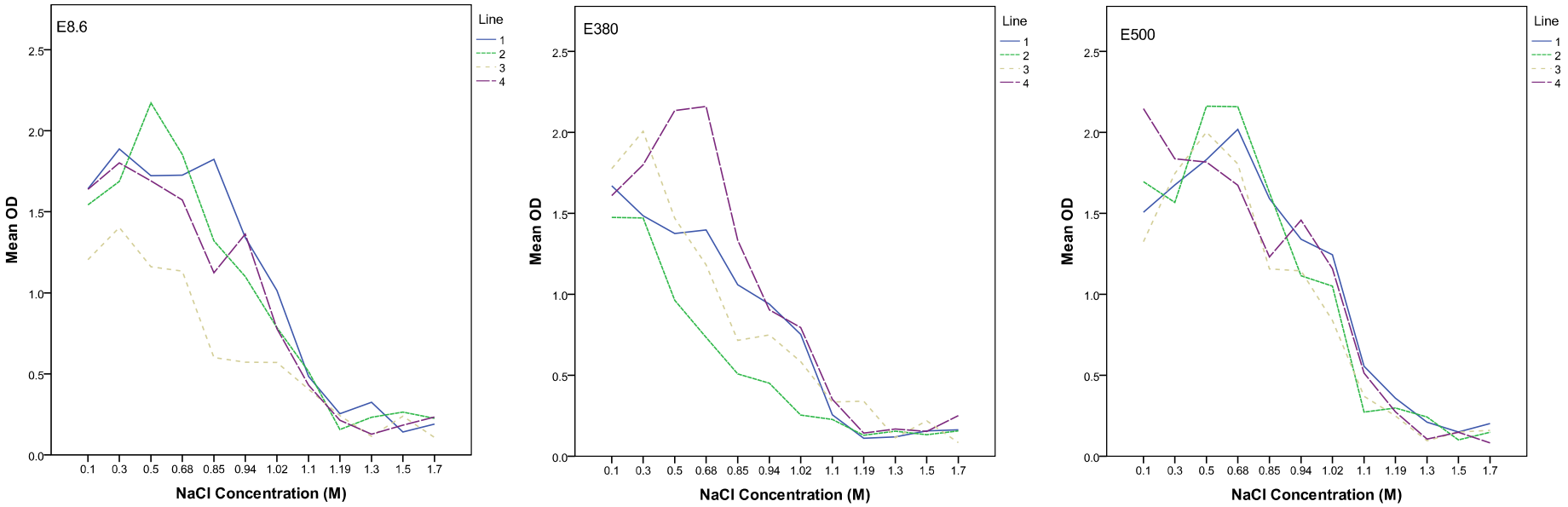


Figure 7. The mean performance (OD 630 nm) of the different evolved groups (E8.6, E380, E500) across 12 NaCl concentrations tested in PM9 plates.



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