Cross-stress protection in bacteria: an evolutionary perspective

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Abstract

Outside the laboratory, microbes rarely live in conditions of optimum growth as environmental parameters constantly fluctuate. “Stress” is the term used when these fluctuations vary severely from optimum, and the ability of microbes to sense and respond to environmental stress is critical to their survival. Due to their importance to medical, agricultural and industrial fields, the underlying response mechanisms have been studied extensively over the past decades and as a result we now understand their key components.

Interestingly, despite previous work on the adaptive potential of *E. coli* to temperature (2-4), UV radiation (5), high ethanol (6), isobutanol (7), and various carbon sources (8-10), little is known about the genetic basis of microbial adaptation to abiotic environmental factors. Even less is known what the effect of stress adaptation is on microbes regarding their capacity to be protected under other stresses. In this work, we summarize a methodology to comprehensively characterize the genetic basis of cross-stress dependencies and acquired stress resistance during adaptation in the bacterium *Escherichia coli*. We present an integrative approach that identifies the genetic basis and evolutionary potential of E. coli adaptation to six environmental stressors, systematically investigates cross-stress protection or vulnerabilities in *E. coli* strains and finally elucidates evolutionary trade-offs related to this complex phenomenon.

A. Introduction

Aim 1. Characterize the genetic basis of cross-stress dependencies in *E. coli*
Hypothesis: Pre-exposure of E. coli to stress A can alter resilience to a second stress B.

Challenge: Determine the regulatory network that underlies various stress-response pathways. Identify common sub-networks within responses to different stresses. Evaluate when and why exposure to stress A confers an advantage/disadvantage for its survival under stress B.

Approach: We will expose *E. coli* to pairs of stresses (acidic, alkaline, osmotic, oxidative, temperature, n-butanol) in a sequential manner. Transcriptional profiling will be used to characterize molecular responses to exposure. Survival curves, growth curves, and competition assays will be used to measure fitness. We will use machine learning (network inference, clustering) for network reconstruction and development of a cross-stress resistance model.

Impact: The collected data and performed analysis will elucidate the stress-specific network of *E. coli*, and its characteristics (nexus points, hubs, common pathways). Validated cross-stress dependencies data will provide a base upon which new

processes of biotechnological, medical and agricultural interest (sterilization or stress resistance) can be developed.

Aim 2. Identify the evolutionary potential, cross-stress dependencies, genetic and epigenetic basis of *E. coli* adaptation to environmental stress

Hypothesis: Genetic and epigenetic changes are responsible for E. coli adaptation to stresses.

Challenge: Identify mutations, expression and methylation patterns that are responsible for *E. coli* adaptation under six stressors. Identify cross-stress behaviors that are different after adaptation and draw causal links to underlying genetic or epigenetic changes.

Approach: We will subject *E. coli* to adaptation in six stress environments for 2,000 generations. Fitness and cross-stress protection or vulnerability after evolution will be measured with growth curves and competition assays. We will identify the genetic basis of any adaptation by sequencing, differential expression by whole-genome transcriptional profiling (RNA-Seq), and DNA methylation patterns by methylated DNA immunoprecipitation (MeDIP-Seq). We will validate fitness effects associated with candidate mutations by reverting them to wild-type.

Impact: Studying microbial adaptation to stressful environments will improve our understanding of the evolution, plasticity, and organization of bacterial genomes which in turn has relevance to human health and industrial applications.

Strong **preliminary analysis in support of this project (beginning investigator)** reveals a set of adaptive mutations, differential expression in anticipated and new targets, and evidence of a broad spectrum of behaviors under the cross-stress protection/vulnerability hypothesis.

B. Background

Outside the laboratory, microbes rarely live in conditions of optimum growth as environmental parameters constantly fluctuate. “Stress” is the term used when these fluctuations vary severely from optimum, and the ability of microbes to sense and respond to environmental stress is critical to their survival. Due to their importance to medical, agricultural and industrial fields, the underlying response mechanisms have been studied extensively over the past decades and as a result we now understand their key components (1) Interestingly, despite previous work on the adaptive potential of *E. coli* to temperature (2-4), UV radiation (5), high ethanol (6), isobutanol (7), and various carbon sources (8-10), little is known about the genetic basis of microbial adaptation to abiotic environmental factors. Even less is known what the effect of stress adaptation is on microbes regarding their capacity to be protected under other stresses. “Cross-stress” protection, i.e. acquired stress resistance to a specific stress after pretreatment cells with a mild dose of the same or other stressor, has been documented for various stress combinations and it has been observed in many species across the Tree of Life, ranging from microbes (11-17, 17-24), to plants (25, 26) and humans (27, 28). For example in *E. coli,* pre-adaptation to glucose or nitrogen limitation increases survival rates after a heat shock or oxidative stress (22), and a possible link was suggested through RpoH, a heat shock regulator which was found to have an important role in protein synthesis under carbon starvation (29). Similarly, increased survival was observed after osmotic shock, when cells were pre-treated with mild

increase in osmolarity (21). Transcriptional profiling in *E. coli* revealed a high degree of overlap (~140 genes) between starvation, osmotic and acidic stress (30), and it was later found that high osmolarity or high temperature induces oxidative-stress regulons (SoxRS and OxyR) that can partially explain the cross-stress protection between these stresses (31, 32). Responses to n-butanol were recently found to share the same high overlap with those in heat shock, oxidative and acidic stress (33). Interestingly, recent work with gene- deletion libraries in yeast shows that acquired H_2O_2 tolerance after three different pretreatments occurs by different mechanisms depending on prior cellular experiences (34).

C. Research Questions

Given the importance of cross-stress protection in bacterial physiology, it is interesting to investigate tantalizing questions regarding (a) the genetic basis of cross-stress protection or vulnerability in *E. coli*, (b) the evolutionary potential, genetic and epigenetic basis of acquired resistance in stressful environments, (c) the identification of evolutionary trade-offs that re- shape the cross-stress behavioral landscape after adaptation, (d) the degree that network topology and prior biological information can be used to predict mutation targets and fitness in the case of cross-stress protection. Towards these goals, we will comprehensively characterize the cross-stress protection behavior of *E. coli* MG1655 strain under six stress environments and we will identify its genetic basis. We will then perform laboratory evolution of *E. coli* cells over 2000 generations in minimal media and six stresses (acidic, alkaline, osmotic, oxidative, temperature, n-butanol). Whole genome re-sequencing (35-37), transcriptional profiling (RNA- Seq), and MeDIP-Seq of the evolved strains will be used to reveal the genetic, epigenetic and transcriptional changes that underpin the acquired stress resistance.

D. Results

In a recent study we showed that responses to environmental factors such as oxygen and temperature reflect an ecological correlation structure, and we found common subset of genes that are differentially expressed under various abiotic factors (38). Over the past year, we characterized *E. coli* MG1655 (2 MG1655 strains and 2 MG1655 strains with the ΔlacZ mutation) over 500 generations in four stressful environments (acidic, oxidative, osmotic and n- butanol) and in glucose-limited M9 salt media as control. All cultures were grown with M9 medium plus glucose (0.4% w/v) as carbon source at 37℃. We introduced the ΔlacZ mutation into two of the experimental replicates so we can perform competition assays with X-Gal and visually count colonies (mutation was found to be neutral in the ancestral strain). For direct competition assays, the evolved strains were inoculated to approximately equal cell densities in the appropriate growth medium and samples were taken at regular intervals (0, 8, 24, and 48h). To estimate the growth of the competing strains, samples were diluted to yield 80 to 400 colonies per selective agar plate (LB agar plates with 0.5mM IPTG and 40μg mL^{-1} X-Gal). After systematically performing competition assays and measuring growth curves for all strain- environment combinations, we were able to create a fitness map for each combination.

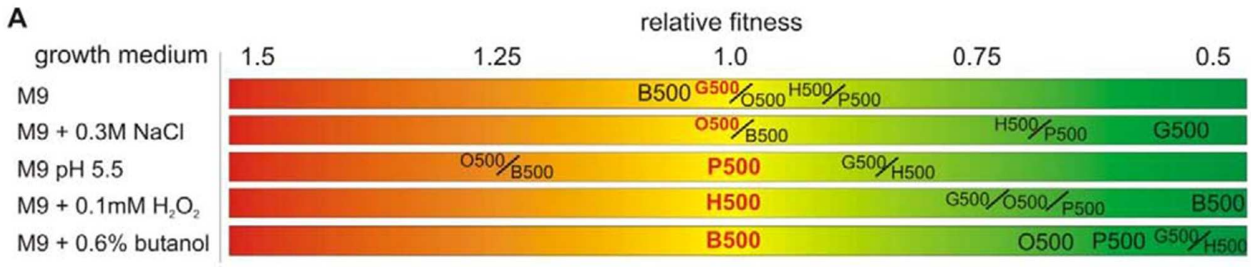


Figure 1. Fitness results from competition assays for all possible combinations of evolved strains and stress conditions (four biological replicates). Relative fitness refers to Darwinian fitness. Strain names are relative to the medium that they adapted: G500 (no stress; M9 salt glucose only), O500 (osmotic, 0.3M NaCl), P500 (acidic, pH 5.5), H500 (oxidative, 0.6% H_2O_2), B500 (n-butanol, 0.6%). Two or more strains were classified as having the same fitness index (e.g. G500/H500) based on p-values higher than 0.01.

Identifying the genetic basis of adaptation: We selected clones that performed the best under in each condition, re-sequenced them, and then mapped the Illumina pair-end reads to the *E. coli* genome. We used stringent statistical tests to identify SNPs and amplifications, and we have verified mutations and amplifications (qRT-PCR, 18 total).

Transcriptional profiling: We used RNA-Seq to perform transcriptional profiling (12 samples total) in reference to the MG1655 genome using the BWA toolkit (39). Genes differentially expressed (DE) relative to their expression levels in the reference G500 strain were identified in all of the stress-adapted strains: 43 in H500, 19 in O500, 58 in B500, and 139 in P500.

E. Discussion

All evolutionary and profiling experiments will be performed with four and three biological replicates respectively. For transcriptional analyses we will employ RNA-Seq (42). Validation of sequencing and RNA-Seq data for genes of interest will be carried out by using repair mutants (site-directed reversal of mutation) and qRT-PCR.

Aim 1. Characterize the genetic basis of cross-stress dependencies in *E. coli*

We will comprehensively characterize pair-wise stress responses for the *E. coli* MG1655 strain in each of the seven stressful environments that were selected based on (a) prior knowledge of gene expression overlap, (b) their occurrence in the natural habitat of *E. coli*, (c) potential industrial interest (e.g. butanol is a potential biofuel). A delay of 5-10 generations between the first and the second stress will be applied. Cultures will be kept out of saturation via dilution to avoid additional responses associated with growth during stationary phase. We will determine survival rates upon exposure to a second stressful condition by taking samples and plating them onto solid medium in regular intervals after the second stress is applied. Non-stressed cells grown in the M9 salt+glucose medium and cells exposed to only one stress (8 controls, 3 biological replicates each) will be used as controls. This design will result in 21 combinations and 8 controls (87 experiments) and another 21 combinations to test the null hypothesis of bilateral cross-protection (we will test it by changing the stress order). From the total 300 possible samples (42 combinations, 8 controls, 3 biological replicates, 2 samples per replicate), we will only profile cases of strong cross-dependency.

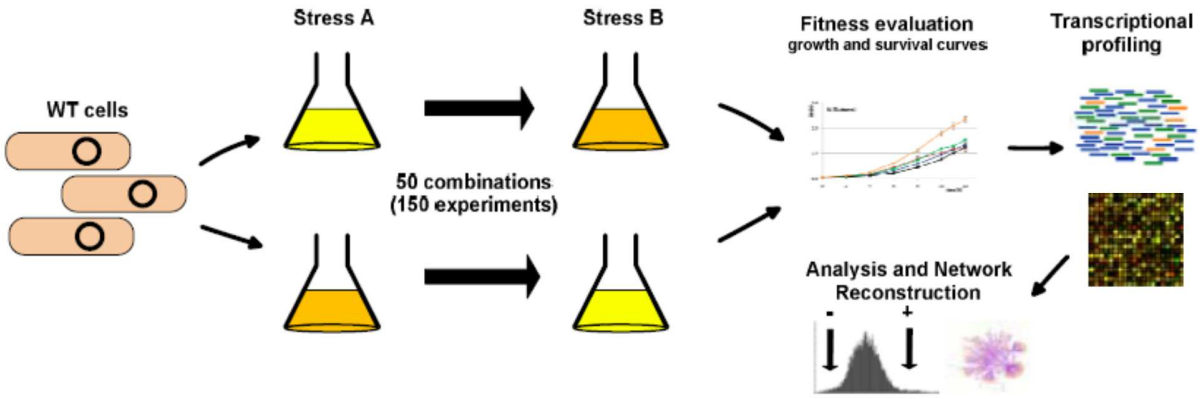
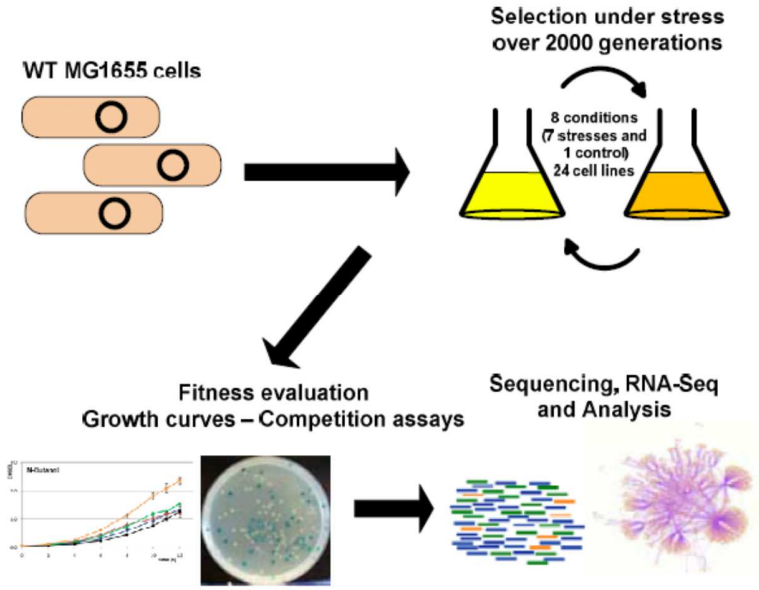


Figure 3. Overview of the experimental setup for Aim 1. WT *E. coli* cells will be exposed to two stresses in a serial manner. After measuring survival rates under both conditions, transcriptional profiling will be applied and the results will be analyzed in order to identify differentially expressed gene groups and reconstruct the underlying pathways.

<u>Aim 2: Identify the evolutionary potential, cross-stress dependencies, genetic and epigenetic basis of *E. coli* adaptation to environmental stress</u>

We will continue our adaptation study as described in the preliminary results (Fig. 4, right). For sequencing (at least x50 coverage), we will select one clone from each biological replicate at the end of the evolution experiment (28 total samples). Repair mutants, where the mutation has been reversed, will be screened as a means of validating a mutation's association with its fitness effect. Competition assays will assess cross-stress phenotype. We will conduct transcriptional profiling of one adapted clone per environmental condition (7 samples), selected based on its fitness profile and mutations.



F. Team qualifications

Our laboratory (3 postdocs, 3 PhD students) has expertise both in computational and evolutionary biology. We have developed and applied machine learning techniques in biological and medical problems related to gene expressions, sepsis and breast cancer (43-51), integrated computational and experimental techniques in synthetic gene circuit design (52-61), performed multi-scale modeling and simulations of microbial communities (62-67), as well as development of custom hardware (68, 69). Finally, our most recent work is related to the evolutionary potential of cross-stress protection, providing a glimpse of the associations present and paths possible during adaptation in multiple stresses (70).

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