molecular systems biology

Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*

Martin Dragosits, Vadim Mozhayskiy, Semarhy Quinones-Soto, Jiyeon Park, Ilias Tagkopoulos

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Submission date:	6 March 2012
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Editorial Decision:	18 September 2012
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 March 2012

Thank you for having submitted a manuscript entitled "Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress adaptation in Escherichia coli" for consideration for publication in Molecular Systems Biology.

Your paper has now been seen by Editors of the Journal, and we have decided to return it to you without sending it for extensive peer review.

In this study, you allow E. coli to adapt over 500 generations to four different stress conditions (osmotic, oxidative, n-butanol, and pH, plus a control culture). Fitness tests of the resulting adapted populations reveals cross-protection, as well as cases negative cross-stress interactions (e.g. butanol- and osmostic-stress evolved cells are more sensitive to hydrogen-peroxide). You then resequence the genomes and profile gene expression from one strain for each culture, identifying a series of mutations that can be plausibly connected in some cases to stress adaptations or to gene expression changes. We acknowledge that this work provides a well-designed dataset, and shows common pathways and genes that are mutated across more than one stress condition. Nonetheless, at this time, we feel that this work remains somewhat descriptive in nature, and the novel conceptual insights into the mechanisms underlying stress-adaptation and cross-stress interactions remain somewhat modest. Moreover, the direct mechanistic role of these mutations in conferring stress resistance or modulating gene expression in these conditions remains somewhat unclear, especially in the absence of additional direct evidence. Overall, we are not convinced that this study currently provides the degree of conclusiveness and novel biological insight our audience would expect in

Molecular Systems Biology.

I am very sorry to have to disappoint you on this occasion, but I hope that this early decision will allow you to submit your work elsewhere without undue delay.

Re-submission 06 August 2012

We would like to submit a revised version of our manuscript entitled "Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in Escherichia coli" to be considered for publication in Molecular Systems Biology. This manuscript summarizes the findings of a major project in our laboratory, culminated over a period of two years, which involved laboratory evolution of microbial strains, phenotypic characterization, resequencing, transcriptional profiling, and integrative computational analysis of the resulting data.
As discussed in our personal communication, we have revised our previously submitted manuscript (MSB-12-3621) to include validation of the genotypic-phenotypic relationship for the case of bunatol-evolved strains through mutation reversals and subsequent competition assays (main text, supplementary text, Figure 4, Figure S27, Table S-XII).
In this work, we explore the emergence of cross-stress behavior during microbial adaptation under various environmental stressors. We first evolved E. coli cells (4 biological replicates) in five distinct environmental conditions (control, osmotic, acidic, nbutanol, and oxidative stress), and then we systematically characterized the Darwinian fitness of all strain pair and environment combinations through both competition assays and growth curves. Interestingly, many cases of both positive and negative cross-stress behavior were observed across the combinatorial spectrum. To identify the genetic basis of the acquired stress resistance, we re-sequenced the genomes of the adapted strains. We further performed transcriptional profiling (RNA-Seq) of all strains under a specific (osmotic) stress where cross-stress protection was most evident, and through clustering analysis we identified and further researched genes and processes that are implicated in this phenomenon. We further validated the genotype-phenotype association by creating repair mutants of all confirmed mutations in the butanol-evolved strain.
There are two main reasons why we believe this work is suitable for publication to Molecular Systems Biology:

Significance and novelty. Most studies until now have focused on reporting circumstantial or partial evidence of cross-stress protection, usually without reporting the genetic basis of this phenomenon. In addition, most studies focus on the effect that a pretreatment with a mild dose of the same stressor has to the organism's survival. A notable exception is a study that was published a few months ago by Audrey Gasch in PLoS (Berry et al., PLoS Genetics, Nov 2011), that used a deletion-library to identify the genetic basis of stress resistance under three conditions in yeast. We here present a consistent "omics" record of microbial evolution that does not exist for the abiotic stressors that we study and a comprehensive phenotypic and system-level analysis that led to the identification of the genes and processes that are implicated to cross-stress protection. Most importantly, this is the first time that the emergence of cross-stress behavior has been investigated. In addition, our study provides a link between crossstress protection and various iron-related genes.

Interest by Molecular Systems Biology audience. This work is well-aligned with the systems approach to biology and identification of the genetic basis of fitness effects that MSB has pioneered and we believe that the manuscript will be well-received by its audience as it links to previous work that has been published in this journal (e.g. Atsumi et al. 2010, Conrad et al. 2011, Goodarzi et al. 2010).
Thank you for your consideration.

2nd Editorial Decision 18 September 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial

concerns on your work, which, I am afraid to say, preclude its publication in its present form.

While the last two reviewers cautiously supportive, the evaluation of reviewer #1 indicates that additional effort is needed to clarify the broader importance of these findings. In this regard, Reviewer #2's point 14 seems relevant, and some additional discussion of the environmental or bio-industrial relevance of combinations of these stresses deserves some justification and discussion.

A few additional comments:

- Reviewer #1's point 4 seems to be adequately addressed by the fact that the rpoB point mutations in each strain were distinct. Nonetheless, this should be clarified in the manuscript text.

- Reviewer #1's point 6 should probably read "I *don't* think standard deviation should be shown here," and indeed our journal policies discourage the use of error bars for two biological replicates. We suggest plotting the two datapoints directly.

- Reviewer #2's point 13. In the editor's opinion, "adaptation" is the appropriate word and is commonly used to describe genetic changes resulting from natural selection. Nonetheless, Reviewer #1's point 1 is somewhat related, and suggests that some clarification is needed regarding long term vs short term adaptation, with some possible changes in nomenclature.

- Lastly, journal policy generally requires that authors provide the data underlying all new experimental results either as supplemental data or via public community repositories. To this end, we ask that you deposit the new RNA-seq data in a public repository and include the resulting accession number or reviewer login in the Methods section of this work.

We also provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for Figures 2 & 4. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Referee reports:

Reviewer #1 (Remarks to the Author):

In this work, the authors compare and contrast E. coli populations evolved under five different regimes. One of the main conclusions is that the authors find that strains evolved under one stress regime are often tolerant to other stresses. They go on to apply genome sequencing and RNA-seq analysis of the osmolarity response to identify mutations found in specific evolved strains.

This is an interesting topic and the paper represents a lot of nice work. My biggest critique is that I think it is more appropriate for a specialized journal. While the results are interesting, it is a rather descriptive manuscript that is likely to be of most interest to other microbial scientists.

Additional specific comments:
1. The authors describe their fitness comparisons as "cross-stress protection" but to me this doesn't seem quite the same as previous descriptions of cross-stress protection, which characterize induced tolerance to one stress after pretreatment with a different stressor. I would characterize this more as

cross-stress or multi-stress tolerance evolved after selection.

2. Maybe I'm missing something, but I don't see how the authors could distinguish the two strains being competed. Unless I missed that, a clearer description of that assay would be helpful.

3. One page 7, "Surprisingly, rpoB mutations were not found in the P500 strain indicating that adaptation to low pH and to the medium may be under antagonistic pleiotropy ..." I don't see the evidence or rationale for this.

4. Since the authors did not resequence the parental strain, is it possible that the rpoB mutation found in 4 of the 5 strains is actually an error in the starting genome and represents a change in the pH-evolved strain?

5. The authors compared gene expression in the evolved strains growing in high osmolarity, for reasons that are not clear. How is this expression relevant? The authors were surprised to find low overlap in the osmo-expression response, but to me that seems largely expected since only one of these strains was adapted to this condition.

6. In some of the competition assays, the error bars represent standard deviation of the mean from two replicates - I think standard deviation should be shown here.

Reviewer #2 (Remarks to the Author):

This is a very good study on the topic of cross-stress evolution and the authors conduct a very thorough investigation.

Main comments:
1. The abstract states "transcriptional profiling of all strains under a specific stress, n-butanol.....". The manuscript suggests that RNAseq was done for all strains under osmotic stress (not butanol).
2. There was a lot of discussion under the results section. Not sure if that can be avoided, but sometimes it is distracting.
3. The following supplementary tables S-Vii, S-VIII, S-X are not referred to in main text, and also suppl figs S1-S3, S21-26. Fig S22-25 can be referred to in the results section " effect of individual mutations on cross stress protection."
4. Two tables s8 and s11 were not in the merged supplementary data.
5. Since the manuscript relies heavily on the supplementary sections - it would make it easier to follow if the supplementary text was better organized into methods and other text, and provide better references to this material in the main text. E.g: the gene regulatory network and fig s21 do not have any reference to in the main text.
6. Page 7 states that P500 are unfit when compared to any other strain on M9 without any stressor. But from Fig 2 it seems like P500 performs similar to the H500 strain.
7. Was the pH of the media measured at the end of every dilution. At 24 hours post dilution the media changes considerably - for example the butanol containing media may become acidic. Hence the evolution may be occurring under mixed stressor conditions.
8. Methods, page 12. It is not clear to what the section maximum stressor concentration is referring to.
9. I did not find an explanation of specifically Darwinian fitness in the supplementary text (as mentioned in fig 4 legend) Please also define W once in the manuscript.
10. Fig 2 legend A. In absence of other stressors all populations showed small evolutionary tradeoffs. From figure and table sV, it looks like only H500 and P500 strains are slightly worse than G500. Also please rephrase this line for better clarity: shaded areas....500 generations.
11. Are the sequencing and Rna-seq data deposited in a public database? I missed the reference to that.
12. Also, did the authors only sequence one clone per evolved strain, and if so, how do they know that the same mutations will be there in the other 3 evolved replicates as well?
13. The words adaptation and evolution are sort of used interchangeably. This may not be accurate. To me, adaptation suggests immediate short-term effects that do not require mutations, while evolution is a more permanent change and does involve mutations.
14. As opposed to the earlier study by Tagkopoulos et al 2008, the stresses selected in this study are a somewhat artificial set - not likely to all be found associated together in nature. Thus the authors

may wish to provide a few lines in the discussions emphasizing (aside from general improvement in knowledge for such phenomenon), what the direct usefulness of this particular study was.

Minor edits:
1. Add spaces between number and units e.g. 2 mM, or 10 ml
2. Fig s22, please change araC to acrA
3. Page 10. butanol not bunatol

Reviewer #3 (Remarks to the Author):

Review of Dragostis, Mozhayskiy, Quinones-Soto, Park and Tagkopoulos: "Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in Escherichia coli."

Overview:
In this manuscript, the authors artificially evolve the same strain of E. coli in four difference stress environments and analyze the genetic and gene expression changes that drive this adaptation. They find in general that there is a surprising degree of overlap between mutations that are beneficial in one stressful environment and in others, observing a variety of adaptions including likely loss-of-functions, changes in expression, changes in copy-number and other mutations with less clear effects.

General Comments:
Overall this manuscript is a strong contribution on an interesting topic. The experimental approaches appear (to this non-expert) to be rigorous and well-planned. I have a few comments on presentation and explanation as well as one or two minor additional analyses that may improve the work.

The Discussion section could be improved by a slightly wider perspective: some of the observations made by the authors parallel nicely to theoretical expectations. For instance, amplification of gene copy number to achieve increased expression is a topic of current interest (Bergthorsson et al, 2007; Kondrashov and Kondrashov, 2006). Likewise, the observation that optimal fitness cannot always be achieved when starting from a particular point in gene space speaks to the role of contingency in evolution (Wagner, 2008).

There are also a couple of places where the authors' experimental decisions could be better motivated: particularly, why were the genome re-sequencing data assembled de novo while the expression data were mapped to a reference genome (as would be expected). De novo assembly is of course arguably preferable when possible, but with short reads, one might suspect assembly problems that the authors probably controlled for, but which are not mentioned.

In the section on expression changes, the authors note that only a few of changes in expression were shared across the strains. But given the size of the genome, it may actually be surprising that any changes are shared (e.g., should independent samples of size ~50 drawn from 4000 genes show any overlap?). The authors should address this question statistically.

On page 7, I was a bit confused by the discussion of fepA reverse mutants in the various environments. Partly, my confusion is due to poor word choice: "fepA was found to be an adaptive mutation in all environments." Clearly what is meant is that a change in fepA was found to be adaptive, but this should be made clearly. It would also help to discuss this no merely in genetic terms (e.g., mutant, wild-type, reverse mutant) but also in functional terms: how the fepA mutant differs functionally from wild-type to mutant (is the mutant giving an expression increase? This point was not totally clear from my reading). Perhaps the authors should unify the expression analysis section with this one to better elucidate the functional nature of the mutants considered.

The figures are generally of high quality. But for figure 1, I could imagine a pentagram with each bar chart projecting from the edges of that pentagram. The area covered by connecting the bars of each strain would then give a sense of the "cross-environment" fitness of each strain: if being highly fit in one environment is costly in others, the shape of that strain's inscribed area would differ from one with reasonable fitness across all environments. This idea is of course only a suggestion.

Minor points:

Could the authors give us a sense of the minimum numerical fitness difference between two strains detectable with their experiments?

On page 5, the discussion of rpoB's role in growth rate could be expanded and clarified. This could also connect to the discussion of the same gene on page 7.

There are a few infelicities of language here: e.g., "From these, the ynfL..." on page 5, "mutations contributing significantly lower..." on page 7 and "can attribute to..." on page 10.

References:

Bergthorsson U, Andersson DI, Roth JR (2007) Ohno's dilemma: evolution of new genes under continuous selection. Proc Natl Acad Sci U S A 104: 17004-17009.

Kondrashov FA, Kondrashov AS (2006) Role of selection in fixation of gene duplications. J Theor Biol 239: 141-151.

Wagner A (2008) Neutralism and selectionism: a network-based reconciliation. Nat Rev Genet 9: 965-974.

1st Revision - authors' response 08 November 2012

We would like to submit a revised version of manuscript MSB-12-3957, entitled "*Evolutionary potential, cross-stress behavior and the genetic basis of stress resistance in E. coli*", which addresses all points that have been raised by the reviewers and the editorial office. We would like to thank both the reviewers and the editor for the thoughtful and clear comments that have helped us to improve the structure and contents of this manuscript. Before we proceed to point-by-point replies to comments, a summary of the main changes in the manuscript's structure is the following:

- The discussion has been extended to include the relevance of this work to different scientific fields and in the context of recent theoretical work.
- The transcriptional profiling data have been moved after the genome sequencing section
- The supplemental text was re-organized and is now separated into Supplemental Methods and results.
- RNA-seq data were deposited in the GEO repository under the accession no. GSE39926. A private link to allow the Reviewer's access to the whole dataset prior to publication: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vtojhmwmocecwvg&acc=GSE39926
- We provide the source data to be associated with Figures 2, 4, and 6.

Please find in the next pages our reply and corrections made for each individual reviewer comments. Thank you for your consideration and we are looking forward for the final publication of our manuscript.

Reviewer #1 (Remarks to the Author):

In this work, the authors compare and contrast E. coli populations evolved under five different regimes. One of the main conclusions is that the authors find that strains evolved under one stress

regime are often tolerant to other stresses. They go on to apply genome sequencing and RNA-seq analysis of the osmolarity response to identify mutations found in specific evolved strains.

This is an interesting topic and the paper represents a lot of nice work. My biggest critique is that I think it is more appropriate for a specialized journal. While the results are interesting, it is a rather descriptive manuscript that is likely to be of most interest to other microbial scientists.

Response: Cross-stress behavior is a phenomenon that is not particular for a specific taxa or an organism but it is abundant across the Tree of Life. In addition, its emergence and dynamics have never been studied in this context before. The fact that we used *E. coli* as the model organism for studying this behavior does not diminish the broader implications of this study, which provides an insight to microbial physiology and has relevance to industrial applications. We cannot think of a better journal than Molecular Systems Biology to host the integrative systems biology approach that we have adopted to study this important phenomenon. We acknowledge these points might not been clear from our previous discussion, something that was also suggested by Reviewer 2 (point 14), and hence we extended our discussion section to make this connection clear. (See additional discussion on pages 10-11, lines 278-94 and pages 12-13, lines 337-57).

Additional specific comments:

1. The authors describe their fitness comparisons as "cross-stress protection" but to me this doesn't seem quite the same as previous descriptions of cross-stress protection, which characterize induced tolerance to one stress after pretreatment with a different stressor. I would characterize this more as cross-stress or multi-stress tolerance evolved after selection.

Response: We agree with the Reviewer that the term is loosely defined. The term cross-stress protection has been used before to describe the process in which bacterial cultures exposed, short or long term, to one stress may develop resistance against other stresses. For example in a recent review on stress induced cross-protection [1], the authors provide the example of *Bacillus* spores isolated and thus adapted in desert soil being more tolerant to UV radiation than laboratory strains. What is different between the various case studies is the timescale of exposure of the first stress. To clarify this point, to set precedence and to avoid misleading the reader, we have changed several references to “acquired cross-stress protection” or “evolutionary cross-stress protection”. Affected text sections were modified throughout the manuscript.

2. Maybe I'm missing something, but I don't see how the authors could distinguish the two strains being competed. Unless I missed that, a clearer description of that assay would be helpful.

Response: From the four biological replicates, two replicates were the MG1655 strain and the other two replicates where the MG1655 strain that harbored the Δ*lacZ* mutation. First, we confirmed that the *lacZ* deletion can be used as a neutral genetic marker in our experimental setup (X-gal/competition assay, additional text in the main text page 4, lines 95-98, Suppl. Results, Suppl. Table S-I, Suppl. Figures S2 and S3). Then, we competed MG1655 replicates that adapted under a specific environment with *ΔlacZ* populations that adapted in a different environment and vice versa. Text was added on page 4, lines 95-100 to clarify this point.

3. One page 7, "Surprisingly, rpoB mutations were not found in the P500 strain indicating that adaptation to low pH and to the medium may be under antagonistic pleiotropy ..." I don't see the evidence or rationale for this.

Response: *rpoB* mutations are known to be identified early on in adaptive laboratory evolution experiments [2-3] which is consistent to what we observed in the strains that evolved in all other four environments. As we did not find such a mutation in the strain that adapted in low pH, this argues for the hypothesis that antagonistic pleiotropy might exist in acidic, M9-based environments. We rewrote this part of the manuscript in order to clarify this point (page 6, lines 150-58) and included a few sentences in the discussion section (page 11, lines 289-94).

4. Since the authors did not resequence the parental strain, is it possible that the rpoB mutation found in 4 of the 5 strains is actually an error in the starting genome and represents a change in the pH-evolved strain?

Response: Similar to the Reviewer, we were surprised at first to find *rpoB* mutations in 4 out the 5 sequenced strains. However, we can exclude an error in the starting genome of the pH evolved strain, for the following reasons: (A) The *rpoB* mutations in the various strains were distinct from each other (different amino acid changes for each *rpoB* mutation and strain where it occurred), (B) we were able to re-construct the ancestral genome from the evolved genomes (please also see Suppl. Results, page 6, lines 150-55) and found that the *rpoB* mutation was not present in the ancestral strain, (C) the parental strain was subsequently sequenced independently and published by the Tavazoie lab which confirmed our reconstruction analysis and the absence of an *rpoB* mutation (see [4]).

5. The authors compared gene expression in the evolved strains growing in high osmolarity, for reasons that are not clear. How is this expression relevant? The authors were surprised to find low overlap in the osmo-expression response, but to me that seems largely expected since only one of these strains was adapted to this condition.

Response: Transcriptional profiling under osmotic stress was performed after conclusion of competition assays, which showed that this is the environment with the most profound cases of cross-stress protection. In addition, the *n*-butanol and osmotic strains exhibited similar fitness profiles across the different environments. This, in conjunction with the fact that common protection mechanisms for different stresses exist in microbes, leads to the logical hypothesis that the overlap in gene expression can be substantial. Although there is a higher overlap than what it would be expected by chance, the observed overlap is not staggering, and our results and sentiment is on par with those published recently in [5]. We added additional text in the results section (page 8, lines 202-07) to clarify the rationale behind this decision.

6. In some of the competition assays, the error bars represent standard deviation of the mean from two replicates - I think standard deviation should be shown here.

Response: Thank you for pointing this out. We follow the recommendation of the Editor to depict individual data points instead. Please see revised Fig. 6 and Suppl. Fig. S22-S27. Figure captions have been changed accordingly.

Reviewer #2 (Remarks to the Author)

This is a very good study on the topic of cross-stress evolution and the authors conduct a very thorough investigation.

Main comments:
1. The abstract states "transcriptional profiling of all strains under a specific stress, n-butanol.....". The manuscript suggests that RNAseq was done for all strains under osmotic stress (not butanol).

Response: We corrected the abstract to reflect the experiment performed - changed to: '…under a specific stress, NaCl-induced osmotic stress, and integration…' (page 2, line 35)

2. There was a lot of discussion under the results section. Not sure if that can be avoided, but sometimes it is distracting.

Response: We tried to reduce the discussion in the results section. However, due to the large amount of sequencing data and the wide variety of mutations that were identified, we found it important to explain some implications of the obtained results where the genes are first introduced. We felt that the readers will benefit when some analysis related with gene function was presented directly under the result section.

3. The following supplementary tables S-VII, S-VIII, S-X are not referred to in main text, and also suppl figs S1-S3, S21-26. Fig S22-25 can be referred to in the results section "effect of individual mutations on cross stress protection."

Response:

- Table S-VII and Table S-VIII are now referred to on page 6, line 138-139.
- Suppl. Table S-X is not discussed in the main manuscript, but it is discussed and referenced in the Suppl. text page 6, line 153.
- Figure S1 is now referred to in the introduction on page 3, line 70.
- Figure S2 and S3 are now referred to on page 4, line 98.
- Figure S21 is not part of the results or discussion of the main manuscript but is discussed in the Suppl. material on pages 9 and 10, lines 256-266.
- As suggested by the Reviewer, Figures S22-25 are now referred to on page 9, line 247.
- Figure S26 is now referred to on page 10, line 254.

4. Two tables s8 and s11 were not in the merged supplementary data.

Response: Tables S-VIII and S-XI represent very large datasets which are not included in the main supplements file due to their size. Tables S-VIII and S-XI are provided as individual MS Excel spreadsheets. We hope that this becomes clear by mentioning this in the Supplemental material on pages 14 and page 15, respectively, where we added the following text for clarification: 'Please see the supplementary file Table_S-VIII/Table_S-XI.xlsx, respectively'.

5. Since the manuscript relies heavily on the supplementary sections - it would make it easier to follow if the supplementary text was better organized into methods and other text, and provide better references to this material in the main text. E.g: the gene regulatory network and fig s21 do not have any reference to in the main text.

Response: Supplementary text is now organized into Methods and Results sections. We have included more references from the main text to the appropriate Supplemental Material. All figures and tables in the Supplementary material are now referenced in the main or Supplementary texts.

6. Page 7 states that P500 are unfit when compared to any other strain on M9 without any stressor. But from Fig 2 it seems like P500 performs similar to the H500 strain.

Response: The Reviewer is correct, we now changed the corresponding passage to: '…compared to most of the other strains on M9…' on page 8, lines 199-200. This change is now in line with the data presented in Fig. 2 and consistent throughout the manuscript.

7. Was the pH of the media measured at the end of every dilution. At 24 hours post dilution the media changes considerably - for example the butanol containing media may become acidic. Hence the evolution may be occurring under mixed stressor conditions.

Response: We did not measure the pH for every single daily transfer, however we measured the pH for the ancestral as well as the evolved population in all environments. The relevant information can be found in the Supplemental results section on page 5, lines 111-121. A pH decrease was observed in all stress conditions in the M9-based medium, with the same significant but small decrease being observed in all conditions (from pH of 7 to pH of 6), with an exception under the acidic stress condition (pH 5.5 to 3.9)

8. Methods, page 12. It is not clear to what the section maximum stressor concentration is referring to.

Response: We rewrote this part in order to clarify this point. The updated section now can be found on pages 14 lines 390-396.

9. I did not find an explanation of specifically Darwinian fitness in the supplementary text (as mentioned in fig 4 legend) Please also define W once in the manuscript.

Response: Darwinian fitness is calculated as described by Lenski and co-workers as a ratio of the Malthusian parameters of 2 competing populations. We now include details on the calculation in the supplementary methods sections, page 3, lines 72-80. We now also refer to this section in the main text on page 14, lines 388-89.

10. Fig 2 legend A. In absence of other stressors all populations showed small evolutionary tradeoffs. From figure and table sV, it looks like only H500 and P500 strains are slightly worse than G500. Also please rephrase this line for better clarity: shaded areas....500 generations.

Response: The Reviewer is correct and to accurately reflect the data, we changed this section to: '…In the absence of other stressors, all populations showed only small fitness differences…', page 24, lines 722-23. The description of the shaded areas has been changed to better explain the figure.

11. Are the sequencing and Rna-seq data deposited in a public database? I missed the reference to that.

Response: Yes, RNA-seq data were deposited in the GEO repository under the accession no. GSE39926. Also we now mention the accession number of the dataset in the main text body on page 16, lines 433- 434. As the dataset is not publicly available yet, we provide a private link to allow the Reviewer's access to the whole dataset prior to publication:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vtojhmwmocecwvg&acc=GSE39926

12. Also, did the authors only sequence one clone per evolved strain, and if so, how do they know that the same mutations will be there in the other 3 evolved replicates as well?

Response: The Reviewer is right that only one clone was sequenced. We sought to find mutations that could explain the fitness effects observed in our study. Although we cannot claim that the same mutations appear in all replicates, we can draw valid conclusions from the obtained dataset. Furthermore, recent literature shows that although the specific mutations might be different in individual populations, populations can show convergent growth profiles within 500 generations and also, to some extent, overlapping expression profiles [6].

13. The words adaptation and evolution are sort of used interchangeably. This may not be accurate. To me, adaptation suggests immediate short-term effects that do not require mutations, while evolution is a more permanent change and does involve mutations.

Response: We acknowledge that it might be confusing to the potential reader. We made appropriate changes throughout the manuscript to avoid any potential confusion. We now use the terms 'evolutionary adaptation' or 'selection' instead of adaptation in order to point out that we looked for mutations that were fixed during the long-term stress experiment.

14. As opposed to the earlier study by Tagkopoulos et al 2008, the stresses selected in this study are a somewhat artificial set - not likely to all be found associated together in nature. Thus the authors may wish to provide a few lines in the discussions emphasizing (aside from general improvement in knowledge for such phenomenon), what the direct usefulness of this particular study was.

Response: In the current study we thought to explore to which extend specialization to a specific environment leads to trade-offs in other unrelated stresses. Considering the fast pace at which microbes, including potential pathogens, can evolve due to short doubling times and clonal growth, we think that a deeper understanding of adaptive changes on an evolutionary scale are of tremendous importance, not only as simple gain in knowledge of microbial physiology but such information is also of great importance for clinical microbiology as well as biotechnology. Extended knowledge on the impact of adaptation to a certain trait can have implications in sterilization and aseptic treatment methods. Furthermore, in biotechnological production processes (e.g. biofuels, organic acids, whole cell biocatalysis systems), the products themselves as well as other non-optimal process conditions can heavily impact the performance of such systems. As such it seems important to us to investigate to which extend such traits, which may seem unrelated to each other considering the natural habitat of bacteria, are compatible with each other.

We agree that extending the discussion to this direction will benefit the paper. As suggested by the Reviewer we now extended the discussion section to put more emphasis on this point (pages 12-13, lines 337-57).

Minor edits:
1. Add spaces between number and units e.g. 2 mM, or 10 ml
2. Fig s22, please change araC to acrA
3. Page 10. butanol not bunatol

Response: Corrected accordingly

Reviewer #3 (Remarks to the Author)

Review of Dragostis, Mozhayskiy, Quinones-Soto, Park and Tagkopoulos: "Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in Escherichia coli."

Overview:
In this manuscript, the authors artificially evolve the same strain of E. coli in four difference stress environments and analyze the genetic and gene expression changes that drive this adaptation. They find in general that there is a surprising degree of overlap between mutations that are beneficial in one stressful environment and in others, observing a variety of adaptions including likely loss-of-functions, changes in expression, changes in copy-number and other mutations with less clear effects.

General Comments:
Overall this manuscript is a strong contribution on an interesting topic. The experimental approaches appear (to this non-expert) to be rigorous and well-planned. I have a few comments on presentation and explanation as well as one or two minor additional analyses that may improve the work.

1. The Discussion section could be improved by a slightly wider perspective: some of the observations made by the authors parallel nicely to theoretical expectations. For instance, amplification of gene copy number to achieve increased expression is a topic of current interest (Bergthorsson et al, 2007; Kondrashov and Kondrashov, 2006). Likewise, the observation that optimal fitness cannot always be achieved when starting from a particular point in gene space speaks to the role of contingency in evolution (Wagner, 2008).

Response: We now also include such a perspective in the discussion section in order to address this issue. Please find the revised section on pages 10-11, lines 278-94.

2. There are also a couple of places where the authors' experimental decisions could be better motivated: particularly, why were the genome re-sequencing data assembled de novo while the expression data were mapped to a reference genome (as would be expected). De novo assembly is of course arguably preferable when possible, but with short reads, one might suspect assembly problems that the authors probably controlled for, but which are not mentioned.

Response: Genome re-sequencing data was both mapped and assembled *de novo*, as these approaches complement each other. As described in the main text (Methods, page 15, lines 411-414) and Supplementary materials (Supplementary Results, page 6, lines 138-148 and lines 157-167) we used short read mapping to the reference genome to identify SNPs and gene amplifications; and we also performed *de novo* assembly in order to verify longer indels. We list assembly breaks in Table S-VIII as referenced *ibid*.

3. In the section on expression changes, the authors note that only a few of changes in expression were shared across the strains. But given the size of the genome, it may actually be surprising that any changes are shared (e.g., should independent samples of size ~50 drawn from 4000 genes show any overlap?). The authors should address this question statistically.

Response: The Reviewer has correctly noted that the observed frequency of genes differentially expressed (DE) simultaneously in multiple clones is significantly higher, than what would be expected by a random chance. A basic statistical discussion is added. We kept our note (but

rephrased it for clarity), that a small overlap between DE genes in different evolved strains (by the absolute gene count) signifies the uniqueness of stress-response pathways adaptation and function under different stresses. The corresponding changes can be found on page 8, lines 207-19. We also added additional discussion in Supplemental Results which is referred to from the changed paragraph of the Main text. (Supplemental results, page 8 and 9, line 230-37)

On page 7, I was a bit confused by the discussion of fepA reverse mutants in the various environments. Partly, my confusion is due to poor word choice: "fepA was found to be an adaptive mutation in all environments." Clearly what is meant is that a change in fepA was found to be adaptive, but this should be made clearly. It would also help to discuss this no merely in genetic terms (e.g., mutant, wild-type, reverse mutant) but also in functional terms: how the fepA mutant differs functionally from wild-type to mutant (is the mutant giving an expression increase? This point was not totally clear from my reading). Perhaps the authors should unify the expression analysis section with this one to better elucidate the functional nature of the mutants considered.

Response: In order to improve the readability and clarity of the results section we clearly state up- or down-regulation. Additionally, we re-arranged the manuscript and now present the transcriptional data before the repair mutants' discussion.

The figures are generally of high quality. But for figure 1, I could imagine a pentagram with each bar chart projecting from the edges of that pentagram. The area covered by connecting the bars of each strain would then give a sense of the "cross-environment" fitness of each strain: if being highly fit in one environment is costly in others, the shape of that strain's inscribed area would differ from one with reasonable fitness across all environments. This idea is of course only a suggestion.

Response: We are grateful for this suggestion to have an intuitive visual representation of the cross-stress experiments. We have now updated Fig. 2, as we thought that this representation would be more appropriate there. As such, Fig. 2A is now an environment-based bar plot, and Fig. 2B is strain-based cross-stress visualization.

Minor points:

Could the authors give us a sense of the minimum numerical fitness difference between two strains detectable with their experiments?

Response: The minimal numerical fitness difference that is detectable between two strains or populations heavily relies on the number of technical replicates. From the current experimental setup we can judge that a difference in W of at least 0.05 (5% fitness difference) can be reliably detected.

On page 5, the discussion of rpoB's role in growth rate could be expanded and clarified. This could also connect to the discussion of the same gene on page 7.

Response: As also suggested by the Reviewer 1 we updated the section dealing with *rpoB*. The revised section can be found on page 6, lines 152-58.

There are a few infelicities of language here: e.g., "From these, the ynfL..." on page 5, "mutations contributing significantly lower..." on page 7 and "can attribute to..." on page 10.

Response: Corrected throughout the text.

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