Deciphering the genetic and transcriptional basis of cross-stress responses in *Escherichia coli* under complex evolutionary scenarios

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24 **Abstract**

25 A tantalizing question in microbial physiology is the inter-dependence and evolutionary potential of
 26 cellular stress response across multiple environmental dimensions. To address this question, we
 27 comprehensively characterized the cross-stress behavior of wild-type and evolved *Escherichia coli*
 28 populations in five abiotic stresses (n-butanol, osmotic, alkaline, acidic, and oxidative) by performing
 29 genome-scale genetic, transcriptional and growth profiling, thus identifying 18 cases of cross-stress
 30 protection and one case of cross-stress vulnerability. We identified 18 cases of cross-stress protection
 31 and one case of cross-stress vulnerability, along with core and stress-specific networks. We tested
 32 several hypotheses regarding the effect of the stress order, stress combinations, mutation reversal and
 33 varying environments to the evolution and final cellular fitness of the respective populations. Our results
 34 argue of a common systems-level core of the stress response with several crucial implicated pathways
 35 that include metal ion binding and glycolysis/gluconeogenesis that is further complemented by a stress-
 36 specific expression program.

37 **Keywords: cross-stress protection / evolutionary trade-offs / microbial evolution / stress**
 38 **adaptation**

39

40 Introduction

41 Regardless of their complexity, organisms have developed a rich molecular and behavioral repertoire to
 42 cope with environmental variations in order to maintain homeostasis and cellular function. The term
 43 *stress* is used to describe conditions where environmental parameters differ substantially from an
 44 organism's optimal conditions for growth and for bacteria it has been an active area of research for
 45 decades¹, given its industrial and medical importance. Most studies have focused on responses to single
 46 stressors, such as pH²⁻⁴, temperature⁵⁻⁷, oxidation^{8,9} and UV^{10,11}, thus providing an important insight
 47 on stress-related cellular responses and their underlying mechanisms. More recently, several studies
 48 have investigated cases of stress combinations and particularly of *cross-stress protection*, where
 49 exposure in a given stressor confers a fitness advantage against an exposure to a second stress¹²⁻²³.

50 Evidence of cross-stress behavior has been mostly circumstantial so far and documented throughout the
 51 microbial kingdom. Early work on glucose- and nitrogen-starved *Escherichia coli* cells showed increase
 52 survival rates after heat shock or hydrogen peroxide-mediated stress when compared to non-stressed
 53 cells²⁴, with the alternative sigma factor *rpoH* a crucial link for heat shock protein production during
 54 starvation stress²⁵. *E. coli* cells adapted to high ethanol concentrations had decreased growth under
 55 acidic stress²⁶ and high temperature environments induce a similar transcriptional program to that
 56 observed under low oxygen²⁷. Pre-adaptation to elevated temperature can reduce both cell death rate
 57 and mutation frequency caused by hydrogen peroxide in *Lactobacillus plantarum*²⁸. Osmotic stress was
 58 found to confer inducible heat tolerance in *Salmonella typhimurium*¹⁵, while trehalose synthesis, which
 59 is important for osmoprotection, was shown to feature a heat-inducible component that increases heat
 60 tolerance of osmo-adapted *Salmonella enterica* cells¹⁴. More recently, gene-deletion libraries were used
 61 to investigate hydrogen peroxide (H₂O₂) tolerance in yeast²⁹.

62 The emergence of cross-stress behavior and the impact of the environmental correlation-structure during
 63 its evolution remain tantalizing questions that need to be further investigated. In a recent study, the
 64 fitness of *E. coli* populations in five stressors was measured after adaptation for 500 generations under
 65 various other stressors and cross-stress dependencies were found to be ubiquitous, highly interconnected
 66 and emerging within short timeframes^{30,26,28}. While studies like that provide valuable insights on the
 67 evolutionary potential of cross-stress behavior, our knowledge of static cross-stress dependencies as well
 68 as the effect of specific environmental characteristics to their dynamic, evolutionary potential remains
 69 limited. Environmental structure is known to be important for the trait evolution during periodic

70 fluctuations³¹⁻³³, co-evolution in microbial communities³⁴ and can influence the rate and direction of
 71 evolution. Additionally, cross-stress responses may be dependent on the type of co-evolution or the
 72 order of exposure to the pair of stresses, while it is not clear if and in which cases, evolved mutants
 73 revert to wild-type phenotypes in the prolonged absence of the stressor.

74 In this study, we have constructed a comprehensive map of cross-stress behavior by measuring the
 75 population fitness in pair-wise stress combinations for five stressors (osmotic, oxidative, alkaline, acidic,
 76 *n*-butanol) that are biotechnologically and biologically important^{3,9,35-37}. By performing genome-wide
 77 transcriptional profiling for each case, we identified key genes and pathways that are implicated in this
 78 phenomenon (**Figure 1A**). We then compared the observed cross-stress response arising from a short-
 79 term exposure to sequential combination of stresses to the response resulting from a longer term
 80 evolution of several *E. coli* lineages over 500 generations under the respective stressors. We identified
 81 the genetic basis of these differences by genome-wide re-sequencing of the mutants. Several hypotheses
 82 related to the stress order, stress combinations, mutation reversal and evolution in varying environments
 83 were tested through further evolving cells already adapted to *n*-butanol and osmotic to a total of 1000
 84 generations under the respective conditions (**Figure 1B**). Fitness assessment and re-sequencing of the
 85 evolved strains provide insight on the genetic basis of acquired stress resistance and cross-behavior,
 86 while functional analysis highlight pathways that play a central role in this phenomenon.

87

88 Results

89 Comprehensive map of the cross-stress behavior in *E. coli*:

90 We tested the cross-stress behavior of *E. coli* MG1655 cells in all the possible pair combinations across
 91 five stressful environments (*n*-butanol, osmotic, acidic, alkaline and oxidative, four biological and two
 92 technical replicates). Out of the 25 possible combinations, 18 pairs (72%) have significant cross-stress
 93 behavior which is in accordance with the presence of a general stress response in *E. coli* (**Figure 2A**,
 94 **Supplementary Table S2, Supplementary File S1**). The highest cross-protection was observed when
 95 bacteria were exposed to acidic stress prior to *n*-butanol stress ($DF = 1.15 \pm 0.04$, $p\text{-value} < 1.46 \cdot 10^{-3}$).
 96 We only detected one significant case of cross vulnerability, in the case of bacteria moved from
 97 oxidative to acid stress ($DF = 0.97 \pm 0.01$, $p\text{-value} < 1.91 \cdot 10^{-4}$). We found a significant cross-protection
 98 effect between acidic media and high salt stress ($DF = 1.10 \pm 0.02$, $p\text{-value} < 5.17 \cdot 10^{-4}$), which is a
 99 cross-stress behavior that previous studies found to be inconclusive in *E. coli* cells^{16,38} but is known to
 100 occur in *S. typhimurium*¹⁸. We also detected a cross-protection similar to that described for *S.*
 101 *typhimurium* and *Listeria monocytogenes*^{13,15} between osmotic and oxidative stresses ($DF = 1.14 \pm$
 102 0.02 , $p\text{-value} < 6.41 \cdot 10^{-3}$).

103
 104 A cross-protection was observed in bacteria adapted to either high salt concentration or *n*-butanol and
 105 then exposed to oxidative stress ($DF = 1.14 \pm 0.02$, $p\text{-value} < 6.41 \cdot 10^{-3}$ and $DF = 1.14 \pm 0.02$, $p\text{-value} <$
 106 0.02 , respectively). A recent study has identified cross-stress vulnerability for these two combinations³⁹,
 107 however in that case, *E. coli* was evolved for 500 generations in the first stress, hence accumulating
 108 mutations that can alter its cross-stress profile with respect to the ancestral line. Indeed, we found that
 109 evolved and un-evolved cell lines had significantly different behaviors in both the original growth media
 110 and the stress-induced M9 media supplemented with 100 mM H₂O₂ (**Supplementary Figures S1 and**
 111 **S2**).

112
 113 In both presence of *n*-butanol and oxidative stresses, a first exposure to 4 out of the 5 stresses gives a
 114 selective advantage to the bacteria. Pre-adaptation to other stresses except acidic provides a fitness
 115 advantage in the oxidative environment, partially analogous to what has been described for *L.*
 116 *monocytogenes*¹⁹. Surprisingly, in acidic stress we have observed the least cross-stress protection from
 117 all the other conditioned strains and we also observed the sole case of cross-stress vulnerability in the

case of the oxidative-adapted strains (**Figure 2A**). On the other hand, bacteria adapted in low pH are well-positioned to compete in all other stresses. To quantify the degree of positive or negative cross-stress behavior, we introduce *cross-stress plots* (**Figure 2B**) where the acid-conditioned cells possess the highest area of cross-stress protection (0.922), while oxidative-conditioned cells have the lowest area (0.411) that also harbors the sole case of cross-stress vulnerability.

Transcriptional profiles associated to cross-stress behavior

To analyze the underlying mechanism of cross-stress behavior we performed two types of transcriptional analysis. First, we performed a transcriptional profiling for each individual stressor at samples harvested 12 hours after exposure. Then we analyzed the transcriptional profiles after exposure to the second stressor (24 hours) to identify the differently expressed (DE) genes in the various cross-stress samples (**Supplementary Tables S3-5, Supplementary File S2**).

In the case of the genes differentially regulated in each stress, we identified 41, 203, 111 and 21 DE genes (p -value < 0.05 after Bonferroni correction) for *n*-butanol, osmotic, oxidative and acidic stress respectively. For each stress we merged results of the four different analysis (**Supplementary Figure S3**) and then data for each stress was used to make a new analysis that identifies DE found in more than one stress (**Figure 3A**). Interestingly, in acidic environments 18 of the 21 DEGs found (86%) overlap with other stresses that correlates well with the fact that this specific environment has the highest cross-stress area.

In osmotic and *n*-butanol stresses there is an under-expression of two operons, *nar* and *hya*, both reported to be over-expressed in anaerobiosis^{40,41}. The *hya* operon was also down-regulated under oxidative stress when compared to its expression under control conditions. The operon encodes for the synthesis of a hydrogenase that is implicated in pH stabilizing responses⁴¹. The *gadE*, *gadB* genes from acidic resistance system AR2, the *adiA* gene from AR3 and the *hdeAB* acidic response⁴²⁻⁴⁵ were found to be under-expressed under the osmotic and *n*-butanol stressor when compared to their expression in the absence of a stressor. As expected, the acidic resistance gene *gadB* is overexpressed under acidic stress conditions. We have identified both a common core of DEG for stress combinations and top ranked genes for individual stresses (**Supplementary Table S6, Supplementary File S2**). In osmotic stress, *proX* that belongs to the operon that encodes for osmoprotectant transportation is significantly

overexpressed^{46,47}, while in oxidative stress we have high expression of the *yhjA* and *pfkA* genes. The first is a the *oxyR*-regulated peroxidase⁴⁸ while the second is of unknown function and we have recently showed that it is implicated with H₂O₂ sensitivity⁴⁹. Surprisingly, the highest ranked DEGs for *n*-butanol are the whole *hya* operon and the *gadB* gene, which are both related to acidic resistance. Indeed, culture in *n*-butanol was found to lower the pH to 5.9 after 12h, which explains the up-regulation of the acidic-stress cluster and the resulting cross-stress protection that was observed in this study.

To measure transcriptional changes in the profiles for both cross-stress protection and vulnerability cases, we performed RNA-Seq on four stress combinations: cells adapted to osmotic stress and then exposed to *n*-butanol (cross-stress protection) and cells adapted in oxidative stress that were subsequently exposed to acidic stress (cross-stress vulnerability) and their complementary pairs (from *n*-butanol to osmotic stress and from acidic to oxidative) to identify whether the observed difference in Darwinian Fitness can be attributed to the enactment of a dissimilar transcriptional program. Two controls were used as reference: cells grown for 12 hours in M9 and then 12 hours in the second stress and a population that has been exposed to the second stress continuously for 24 hours (**Figure 3B**). In all the analyses DEGs are similar when compared to both references, except from acidic to oxidative stress (**Supplementary File S2**). When bacteria were exposed to *n*-butanol stress after being exposed to osmotic stress, there is only a small variation of expression compared to the bacteria that have been only exposed to *n*-butanol (9 DEGs only) showing that exposure to high salt conditions triggers a response similar to the response of bacteria exposed to *n*-butanol and no rewiring is needed giving the bacteria an advantage. Most of these 9 DEGs are acidic-response genes as found in the *n*-butanol samples like *gadB* and *gadE* (**Supplementary Table S7, Supplementary File S2**). In contrast, when bacteria that has adapted in *n*-butanol face osmotic stress, more than 100 genes are differentially expressed when compared to the expression under osmotic stress exposure only. A similarly large differentially expressed gene pool is observed in the case of cells exposed to oxidative stress prior to acidic stress, which also is the only cross-vulnerability observed, where the list of DEGs goes over 750 genes, arguing for the cell's need to enact a different expression program than the one used in the first stress in order to survive, which concords with a fitness disadvantage. In the 20 most significant DEGs (**Supplementary Table S8, Supplementary File S2**) there are several up-regulated genes previously described to be differentially expressed different stresses like acidic (*yjeI*,⁵⁰), manganese (*hflX*,⁵¹), cobalt (*iscR*,⁵²) cadmium (*tyrA*,⁵³) and oxidative (*pfkA*,⁴⁹). In the inverse case, there is a cross-stress protection from

acidic to oxidative and transcriptional profiling shows only 10 DEGs compared to the acidic grown bacteria that contains acidic related genes (*hdeB*, *gadB*, *hyaB*).

Dissecting the memory imprint after adaptation to sequential stress combinations.

Osmotic and *n*-butanol adapted cells demonstrate strong cross-stress protection both in the case of a short 12h exposure (**Figure 2A**) and adaptation after 500 generations³⁹. However, contrary to past results on adapted cells, where cross-protection has been found to be symmetrical, only bacteria exposed to *n*-butanol are protected from osmotic stress which argues for the existence of directionality of this phenomenon. To assess this hypothesis, we evolved osmotic and *n*-butanol adapted strains for an additional 500 generations in five independent experiments, all combinations of these environments, to further address questions related to cross-stress behavior after sequential stress adaptation (**Supplementary Table S9**). Growth rates of all the populations in media-only, osmotic stress and *n*-butanol showed that there was no growing defects after evolution (**Supplementary Figures S4-6, Supplementary Table S10, Supplementary File S3**).

The first hypothesis we tested is whether exposure to two stresses sequentially provides an advantage in an environment where both are present. We performed competition assays in an environment with both stresses (0.3mM NaCl and 0.6% *n*-butanol) between populations evolved for 1000 generations in a single stress (O1000, B1000) against those evolved in both stresses sequentially for 500 generations (O500B500, B500O500). Results show a significant fitness increase of the strains that have been exposed to both stresses (O500B500 *vs* B1000, $DF = 1.16 \pm 0.08$; B500O500 *vs* O1000, $DF = 1.24 \pm 0.12$) (**Figure 4A, Supplementary Table S11, Supplementary File S4**). This *memory effect* is present long after the selection pressure for this particular stressor was removed (500 generations) and it can be attributed to the genetic background that has been acquired during the adaptation in the first stressor. In addition, this fitness increase is sufficiently large to overcome the additional fitness increase that is observed in the O1000/B1000 strains when compared to their O500/B500 counterparts due to their prolonged stress adaptation ($DF = 1.12 \pm 0.03$, $p\text{-value} < 7.15 \cdot 10^{-3}$).

Recent theoretical results show that the rate of evolution can be increased by guiding populations from states that are correlated and have increasing complexity⁵⁴. To challenge this notion in the case of osmotic and *n*-butanol adaptation, we competed populations evolved sequentially in both environments

to those evolved in only one stress. In both cases the population evolved for a prolonged duration in a single stressor had similar or higher fitness than populations evolved in the two stressors sequentially (O500B500 vs. B1000, $DF = 1.03 \pm 0.02$, $p\text{-value} < 0.58$; B500O500 vs. O1000, $DF = 0.87 \pm 0.01$, $p\text{-value} < 7.54 \cdot 10^{-11}$). Concomitantly, we investigated if the order in which stressors are introduced play a role on the final fitness. We found that order does not have a significant impact to the fitness of the final populations when it is measured under competition in either stress (O500B500 vs. B500O500, $DF = 0.99 \pm 0.11$, $p\text{-value} < 0.99$ in *n*-butanol stress; $DF = 0.99 \pm 0.010$, $p\text{-value} < 0.88$ in osmotic stress).

We further probed on the environment's impression on the genetic blueprint by investigating whether mutations are reversed when the stressor is removed. As such, populations that were adapted to environments for 500 generations with either *n*-butanol or osmotic stress (B500 and O500 populations, respectively) were competed to those where the initial 500 generations of stress-adaptation is followed by another 500 generations where the stressor is removed (medium-only environment; B500G500 and O500G500 cell lines). In the case of *n*-butanol, there is no significant difference in the Darwinian fitness when populations are competed under *n*-butanol stress (B500 vs. B500G500, $DF = 0.99 \pm 0.01$, $p\text{-value} < 0.34$), while in the case of osmotic stress, the difference is profound in competition under osmotic stress, as beneficial mutations that are advantageous in osmotic stress have been lost (O500 vs. O500G500, $DF = 0.79 \pm 0.01$, $p\text{-value} = 8.30 \cdot 10^{-6}$). In both cases, growth in media without the presence of stress was not affected ($DF = 1.05 \pm 0.04$, $p\text{-value} = 0.39$; $DF = 0.97 \pm 0.03$, $p\text{-value} = 0.44$).

Finally, to investigate the effect of alternating stresses to the evolutionary trajectory of each population, we evolved the O500 and B500 populations to alternating environments of osmotic and *n*-butanol stress (50 generations per environment, 10 switching events total; O500BO50 and B500OB50 cell lines). These populations have been competed with O500B500 and B500O500, respectively, in three distinct environments: *n*-butanol, osmotic stress and a varying environment with 8 hours for each stresses (**Figure 4A**). We did not observe any difference in the growth curves and competition assays between any of the populations grown in varying and static environments (B500OB50 and O500BO50 vs. B500 and O500 respectively; **Supplementary Table S11**).

Acquired genetic mutations and their effect to phenotypic fitness during short-term evolution

Previous studies on the effect of single stress adaptation to cross-stress behavior have been focused on the identification of mutations on one cell line over short timeframes³⁹. To further elucidate the genetic basis of the acquired stress resistance and cross-stress behavior, we first sequenced four cell lines for each of the four of the stressors (osmotic, *n*-butanol, acidic and oxidative; alkaline stress was excluded because of media incompatibility) and the control environment (4 biological replicates, 5 environments, 20 lines total), where we have identified a number of loci that are mutation hotspots over two or more cell lines (**Figure 4B, Supplementary Table S12**).

Across all lines, two mutations were found to be synonymous, *ynfL* (G500) and *cydD* (O500) (**Supplementary Table S13**). The predominant mutations are in the intergenic zones *rph:pyrE* (G500, P500, H500) and *fes:fepA* (O500, B500), as well as the sigma factors *rpoB* and *rpoC* (G500, O500, B500, H500). Interestingly, both *fes* and *fepA* are involved in siderophore enterobactin production that has recently been found to be linked with oxidative stress and M9 growth⁵⁵. Although its function is unknown, mutations in *rph:pyrE* have also appeared in a previous study in evolution on lactate⁵⁶. Both *rpoB* and *rpoC* encode for RNA polymerases and their mutations are very usual in evolution, more specifically mutations in *rpoB* have been linked to an increased evolvability and fitness⁵⁷ and mutations in *rpoC* to bacterial growth optimization in minimal media⁵⁸ and metabolic efficiency⁵⁹. We also found mutations in other genes already described like *pykF* in G500 which mutation is known to be beneficial for growth in M9⁵⁷, *marC* and *acrAB* for resistance in *n*-butanol^{39,60,61}, *proV* in osmotic stress^{39,46,47}, *evgS* and *rpoD* in acidic stress^{39,42,62} and *katG*, *oxyR*, *rsxD* and *ccmD* for resistance in oxidative stress^{39,63,64}. Interestingly, in oxidative stress we find mutations in the *yagA* and *yncG* genes, which is consistent with a previous finding that a deletion between *argF-lacZ* (that includes *yagA*) confers a high hydrogen peroxide resistance⁶⁵. In addition, *yncG* is homologous to Glutathione S-transferases known to help in the defense against oxidative stress⁶⁶. Other mutations with unclear importance in the stress where they appeared that were found are *ybcS*, *lon* and *prc* in G500; promoters of *yccF*, *ychF* and *pgi* and genes *rob* and *relA* in B500; *yijO* in O500; *lon* in P500; and *motB*, *yigA* and the intergenic zone *panC:panB* H500. The mutations in the *ybcS*, *prc*, *yccF* and *yigA* genes are of unknown function, while others have known, but unrelated functions. For example, *lon* is a DNA-binding protease that degrades abnormal proteins⁶⁷, *ychF* is a ribosome binding catalase⁶⁸, *pgi* is an oxidative stress induced gene⁶⁹,

270 *rob* is a transcriptional activator involved in antibiotic resistance ^{70,71}, *relA* synthesizes ppGpp, an
271 alarmone active under aminoacid starvation conditions ⁷² and *motB* is part of the flagellar motor ⁷³.

272

273 We performed a similar re-sequencing analysis in the case of populations that have been evolved for a
274 total of 1000 generations in a single or multiple sequential stress combinations (sequencing of multiple
275 clones per population; **Supplementary Figure S7, Tables S14-16**). Four mutations were found to be
276 synonymous and hence neutral, *paaH* (B500O500), *flgK* (O500B500), *fixB* in (O500G500) and *ynfL*
277 (O500BO50). As shown in **Figure 4B**, the most common mutations are in the *fepA:fes* and *rph:pyrE*
278 intergenic regions (8 and 7 cell lines, respectively) and mutations in the *rpoB* and *rpoC* genes (7 and 6
279 cell lines, respectively; **Table 1**). Many of these targets are mutation hotspots (**Supplementary Table**
280 **S16**), for example in the case of *rph:pyrE* and *fepA:fes*, parallel mutations have been detected in 9
281 positions that span 140 nucleotides (**Figure S8**) and 3 positions over 22 nucleotides (**Figure S9**)
282 respectively. Populations of the B500 parental population contained several genes present in more than
283 one population, *marC*, *acrA*, *acrB*, *relA*, *glyXVY*, with *valZ:lysY*. *marC* and *acrAB* have been previously
284 described to be involved in *n*-butanol resistance ^{39,60,61} along with *nagA*. In contrast, populations evolved
285 from the O500 background have only two common mutagenized loci present: *proV* described to be
286 involved in osmotic resistance ^{39,46,47} and *nagA* present in osmo-tolerant cells ³⁵.

287

288 To decipher the genetic basis of this reversal, we compared the sequences of the B500 and O500 clones
289 (**Figure 4A**) to those from B500G500 and O500G500 populations, respectively (**Supplementary**
290 **Figure S7**). Cells that were first evolved in *n*-butanol and then adapted to stress-free media for 500
291 generations lost the *rpoB* mutation while acquired mutations in *pykF*, *gltD* and *gltB* genes. Similarly,
292 cells that were first evolved in osmotic stress and then to stress-free media lost the mutation to the
293 osmoprotectant gene *proV* and acquired mutations in the *pyrE:rph tap*, *yeiP* and *pykF* clusters. Although
294 its function is unknown, mutations in *pykF* gives an advantage for growth in M9 ⁵⁷. Both *gltD* and *gltB*
295 encode genes for glutamate synthesis ⁷⁴, *tap* produces a methyl-accepting protein ⁷⁵ and *yeiP* is an
296 elongation factor.

297

298 To associate genotype-to-phenotype, we performed growth experiments of single-knockout mutants ⁷⁶
299 in medium-only, *n*-butanol and osmotic stress (**Supplementary Table S17, Supplementary Figure**
300 **S10, Supplementary File S3**). Mutants in *pyrE* and *fes* cannot grow in any of the conditions tested,

showing that the mutations found in their promoter affect the growth in M9 and are not involved in the stress adaptation. While mutation in *fepA* affects growth in all media, it provides a higher maximum growth rate in osmotic stress (**Supplementary Table S17**, media-only $\mu_{\max} = 0.011 \pm 0.000$; *n*-butanol, $\mu_{\max} = 0.014 \pm 0.001$; osmotic, $\mu_{\max} = 0.020 \pm 0.000$). As previously described, $\Delta pykF$ enhances fitness in media-only⁵⁷, $\Delta acrA$ and $\Delta acrB$ in *n*-butanol^{39,60} and $\Delta proV$ in osmotic stress^{46,47}. Other mutations that have a positive effect are $\Delta relA$ and $\Delta sufE$ in media-only, $\Delta yjbl$ in *n*-butanol and $\Delta acrA$ in osmotic. Previous results of repaired mutations under the mutant background elucidated the effect of *acrA* mutations in osmotic stress³⁹. Cross-stress trade-offs were found in the case of $\Delta nagA$ and $\Delta sufE$ that have a negative effect under osmotic stress. In contrast, no mutation was found to have a negative effect in growth under the absence of any stressor, or in presence of *n*-butanol.

Functional and network analysis of cross-stress behavior

Re-sequencing and transcriptional profiling data were compiled in order to produce a network representation of stress resistance in *E. coli* (**Figure 5A**, **Supplementary Figure S11**). Network and functional analysis was also performed for each individual stress (**Supplementary Figures S12-S15**). Overall, enriched gene ontology clusters are related to motility, sulfur metabolic process, translation, DNA replication and cellular respiration, among others (**Figure 5A and B**). Interestingly, the glycolysis/gluconeogenesis, response to drug genes and cellular respiration are involved in *n*-butanol response, with an *pykF* mutant from this pathway being unexpectedly fit, as it has the highest growth rate in these conditions. The importance of the *fes* and *fesA* genes of the Metal Iron Binding pathway is profound in the case of osmotic and oxidative stress and similar results have been obtained in the case of *fepA* iron transporter, in *n*-butanol, osmotic and acidic stress, highlighting the importance of metal transport in stress response. In osmotic stress, several translation and transcription pathways are found to be involved, while in acidic stress regulation of transcription and cell cycle clusters are enriched. The metal binding motif also includes a differentially expressed gene, *yhjA*, that encodes for a cytochrome C peroxidase regulated by FNR and OxyR⁴⁸. This gene is been implicated in all stress responses and hence is an excellent target for further characterization. In this stress we also find motility genes involved in bacterial chemotaxis. In our analysis, a core network was identified with DEGs and mutations from every stress (33% *n*-butanol, 19% osmotic, 15% acidic and 33% oxidative). The fact that the members of the central clusters are all implicated in the acidic and osmotic stress correlates well

with the previously shown *cross-stress plots* in which we showed that exposure to acidic and osmotic stresses have the higher cross-stress protection.

Discussion

A general observation that stems from our work is that exposure of a bacterial population to one stressor is generally beneficial, as 18 out of 25 cases tested in this work showed cross-stress protection with an average Darwinian Fitness of 1.08 ± 0.01 ($p\text{-value} < 5.52 \cdot 10^{-8}$). Interestingly, we also found the first case of cross-vulnerability when bacteria adapted to oxidative stress face low pH suggesting that some stress combinations can be used for a better sterilization. By performing transcriptional profiling we found that the number of differentially expressed genes across stresses is a good predictor of the underlying cross-stress behavior. Indeed, the case of cross vulnerability has the higher number of DEGs (>750), followed by the neutral cross-stress (>100) and finally both cases of cross-protection have a small amount of DEGs (9 and 10). Although the results here support this hypothesis, cross-stress protection can be also dependent on the DEGs of the first stressor as we find several genes with unknown or unrelated function, including the putative transcriptional repressor *rpiR*, the *yfi* operon and genes *pqqL* and *ybaT* that are present in all stresses but acidic (**Fig. 3, Supplementary file S2**). These genes constitute excellent targets for further experimentation to understand the mechanism under which they affect single stress and cross-stress behavior. Additionally, we identified several genes known to be differentially expressed under anaerobic conditions, which provide a clear link between genes induced by anaerobic respiration and stress resistance⁷⁷.

There is substantial similarity of the cross-stress behavior between *n*-butanol and osmotic stress, as well as a clear dissimilarity of these responses to that of oxidative stress with and without evolution. Analysis of the mutations found in the 4 clones sequenced (B500 and O500, **Figure 4A**) discovered two genes in the *n*-butanol evolved populations that are also involved in oxidative stress that might be related with this differential behavior, the *ychF* catalase⁶⁸ and the *pgi* gene that are induced under oxidative conditions⁶⁹. Although not directly related to oxidative stress, in these clones we also found a mutation in the *rob* gene involved in antibiotic resistance⁷⁰. Unpredictably, in the O500 clones only one mutation is not related to the media, a mutation of the gene *yijO* which is of unknown function and remains to be

investigated. Another explanation of the cross stress vulnerability is the *fepA:fes* mutation that, although it is expected to be related to the use of M9 as evolution media, it is only present in the B500 and O500 populations. The corresponding proteins belong to the enterobactin operon and have been recently implicated in growth under oxidative stress in M9 media⁵⁵ and thus its mutation might have a significant effect in M9 with oxidative stress.

If we are to predict where mutations will occur under evolution in a specific stress, it is reasonable to assume that the genes that are differentially expressed in that given stress will be likely candidates for polymorphisms or silencing. Comparison of the transcriptional profiles after short-term exposure and re-sequencing of evolved populations after 500 and 1000 generations argue that, in general, this hypothesis is not correct, as differential expression under exposure in a single stressor is not a proxy for mutation events during evolution in that stressor. Only in the case of osmotic stress we observe a mutation in the gene *proV* after evolution, which is in the same operon as the *proX* gene that was found to be over-expressed after the 12h exposure. Interestingly, after 1000 generations under *n*-butanol stress a mutation in *relA* appears and by network analysis we show that this gene is very close to several DEGs described in this stress like *gadE*, *mdtE* and *mdtF*. A similar case exists in oxidative stress where we detected a mutation in the intergenic region between *panB* and *panC*, with the network analysis showing that these genes have several interactions with other oxidative stress specific DEGs, such as *dnaJ*, *hypD* and *lolB*.

The evolved cell lines provide an interesting view of the evolutionary trajectories under the various stress combinations and it shows that their diversity is environment-specific. As shown in Figure 4B, cell populations that evolved under osmotic stress share few genetic mutations while populations evolved under *n*-butanol stress share many. Interestingly, despite the already described *n*-butanol related mutations, *acrA*, *acrB* and *marC*, we also found point mutations in several regions between tRNAs, *metW:metV*, *valZ:lysY*, *glyC:glyY* and *valT:lysW*. Mutations in four different spots argue against this being a random effect, which in turn implicates the tRNA production as a factor of *n*-butanol stress resistance. An expected result is the high abundance of mutations in the *fes:fepA* genes in all the populations as we already described this mutations in the parental population, B500 and O500. These mutations have been already described in similar scenarios and recently the involvement of the *fep* operon in growth in M9^{39,55,56}. Surprisingly, another widely present mutation, *pyrE:rph*, is only present in the population evolved for 500 generations in a stress-free M9 medium, which argues for the *fes:fepA*

mutation to be stress-related. We can track the emergence of the most common mutations by the number of polymorphisms detected in each mutation, for example the *fes:fepA* mutations appears in three different positions (**Supplementary Figure S9**) for the B500 and O500 populations. In the case of *rph:pyrE*, we have 9 distinct mutation positions (12 different mutations **Supplementary Figure S8**) arguing for the emergence of these mutations in the last 500 generations of the evolution. In addition, based on our results, we cannot conclusively dismiss or confirm the guided-evolution hypothesis although the O500B500 and B500O500 cell lines were found to have similar fitness to the 1000 generation populations, B1000 and O1000, in *n*-butanol and osmotic stresses, respectively (**Figure 4A**). This was surprising as the O500 and B500 have a significantly lower fitness with respect to their 1000 generation counterparts.

Finally, our last experiment involved fitness in a varying environment. More experimentation and in larger scale is needed to state with high confidence the magnitude and occurrence of this phenomenon. A recent work was also inconclusive regarding this phenomenon in *S. cerevisiae*³¹ with five times shorter cycles (approximately 10 generations per cycle). We also tested the hypothesis that shorter lag phases, instead of better fitness, should be observed under evolution in varying environments (New *et al*, 2014). Comparison of the $\mu_{\max}$ of the varying environments population (**Supplementary Table S10**) shows that the O500BO50 general fitness is better than the O500B500 in osmotic stress (0.064 *vs.* 0.0525) but not in *n*-butanol (0.041 *vs.* 0.0375). In the case of the B500OB50 populations, a higher $\mu_{\max}$ in osmotic stress than the B500O500 (0.0575 *vs.* 0.052) and a similar growth in *n*-butanol stress (0.039 *vs.* 0.043) was observed.

Although cross-stress behavior has been extensively studied in the past years, with many such cases related to food safety⁷⁸ and human health⁷⁹ this is one of the first studies where genome-wide profiling and a systems biology approach was used to create a comprehensive mapping of static and evolved cross-stress responses. This approach allows us to investigate the systemic response and molecular underpinnings of each condition combination, with the potential to inform new sterilization techniques and more efficient methods in clinical decision support⁸⁰.

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424

425 **Author Contributions**

426 VZ performed cross-stress experiments, growth curves, strain characterization, and library preparation
427 for genome sequencing and RNA-Seq. SQS performed the strain evolution and performed competition
428 assays for validating the fitness of the populations. MK analyzed the sequences and the DEGs. NR
429 performed the libraries for the X500 clones. VZ and IT evaluated data and prepared the manuscript. IT
430 conceived of the study and supervised all aspects of the project.

431

432 **Conflict of Interest**

433 The authors declare no competing interests.

434

435 **Materials and methods**

436 **Bacterial strains and culture conditions.** *E. coli* MG1655 and MG1655 $\Delta lacZ$ strains were used in all
 437 experiments of this study. The inclusion of $\Delta lacZ$ mutants allowed us to perform competition assays
 438 using X-Gal and IPTG staining. The neutrality of the $\Delta lacZ$ mutation was confirmed in all environments
 439 in a previous work of the laboratory³⁹. Minimal M9 salt medium with 0.4% (w/v) glucose as carbon
 440 source was used for cross-stress behavior competition assays. The stresses used are osmotic stress (0.3M
 441 NaCl), acidic stress (pH 5.5), alkaline stress (pH 9.0), oxidative stress (100 mM H₂O₂), *n*-butanol stress
 442 (0.6% *n*-butanol), and control (no-stress).

443 **Cross-stress behavior analysis.** Two competing populations (*E. coli* MG1655 and *E. coli* MG1655
 444 $\Delta lacZ$) were pre-adapted to M9 overnight and inoculated at an approximate 1:50 ratio at a starting OD₆₀₀
 445 0.004 in M9 with one stressor and only M9 for reference. After 12 h of growth, the same quantity of the
 446 strain adapted to stress and the strain grown in M9 were diluted in the second condition media (approx.
 447 1:100 dilution) and competed for additionally 12 h. Samples were taken at 12 (time 0) and 24 hours
 448 (time 1). Four independent biological replicates each with two technical replicates were performed for
 449 each individual competition. Cell counts were determined on LB agar plates containing 0.25mM IPTG
 450 (Isopropyl β -D-1- thiogalactopyranoside) and 40 mg/ml X-gal (bromo-chloro-indolylgalactopyranoside).
 451 Plates were incubated at 37°C overnight. Darwinian Fitness (DF) was calculated as described in the
 452 Supplementary Methods section.

453 **Gene expression analyses.** Cells were harvested at time 0 and 1. In all, 3ml aliquots were harvested and
 454 mixed with 1.5 ml 5% Phenol/ethanol (v/v) and stored at 80C until use. RNA was extracted using an
 455 RNeasy kit (Qiagen) and after first- and second strand cDNA synthesis, cDNA was broken using
 456 Diagenode Bioruptor NGS. End repairing, A tailing, linker ligation and PCR enrichment was made
 457 using the KAPA Library Preparation Kit (Kapa Biosystems). Size selection was performed with
 458 Agencourt AMPure XP (Beckman Coulter). After quality control, libraries were sequenced by Illumina
 459 HiSeq 2500. The low-quality raw reads were trimmed using Trimmomatic (v0.30) with default settings.
 460 Trimmed reads were aligned on most recent reference genome of *E. coli* MG1655 by using TopHat
 461 (v2.0.10) coupled with bowtie (v1.0.0)^{27,81}. The identification of the differentially expressed gene was
 462 done as explained in the Supplementary Methods.

Adaptive Laboratory Evolution. Evolutionary adaptation experiments were performed in 10 ml volume in 125 ml shake flasks at 37°C on an orbital shaker at 150 r.p.m. of an alternate of M9 with 85 mM NaCl (evolution media) media-only or with two stressors: osmotic stress (0.3M NaCl) and *n*-butanol stress (0.6% *n*-butanol). The OD₆₀₀ of each culture was measured each day before the daily transfers to ensure that the estimated nine generations per day were reached (see Supplementary Methods).

Whole-genome re-sequencing and mutation discovery of evolved strains. Selected clones were grown on LB medium overnight and genomic DNA (gDNA) was isolated using a Wizard Genomic DNA Purification Kit (Promega) and sequenced as described in the Supplementary Methods.

Competition assays. Competing populations (*E. coli* MG1655 and *E. coli* MG1655 Δ lacZ) were grown under adaptive conditions overnight and inoculated at an approximate 1:1 ratio at a starting OD₆₀₀ 0.004. Samples were taken at 0h and 24h. Two independent technical replicates were performed for each individual competition. Cell counts were determined as described before, using LB agar plates containing 0.25mM IPTG and 40 mg/ml X-. Plates were incubated at 37°C overnight. Darwinian fitness (W) was calculated as described in the Supplementary Methods section. In the case of the population adapted to a fluctuating media, the populations competed in a fluctuating media that varied from osmotic to *n*-butanol every 8 hours.

Network analysis. We used TF-DNA binding data from RegulonDB⁸² and protein-protein interaction datasets^{83,84} from the Bacterial Protein Interaction database²⁵ to construct a functional and regulatory network. We then mapped the results from our re-sequencing and transcriptional profiling analyses to build condition-specific sub-networks for each stress. Genes that connect either mutated or DE genes in paths with a path length of three or lower were also included in the network analysis. Modular organizations and community detection was performed on the resulting networks, through spin-glass model and simulated annealing techniques⁸⁵ using *igraph* R package⁸¹.

491 References

- 492 1 Storz G, H.-A. R. *Bacterial stress responses*. (American Society for Microbiology, 2000).
- 493 2 Hong, W., Wu, Y. E., Fu, X. & Chang, Z. Chaperone-dependent mechanisms for acid resistance in enteric
494 bacteria. *Trends in microbiology* **20**, 328-335, doi:10.1016/j.tim.2012.03.001 (2012).
- 495 3 Kanjee, U. & Houry, W. A. Mechanisms of acid resistance in Escherichia coli. *Annual review of*
496 *microbiology* **67**, 65-81, doi:10.1146/annurev-micro-092412-155708 (2013).
- 497 4 Zhao, B. & Houry, W. A. Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for
498 survival. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **88**, 301-314, doi:10.1139/o09-
499 182 (2010).
- 500 5 Bennett, A. F. & Lenski, R. E. An experimental test of evolutionary trade-offs during temperature
501 adaptation. *Proceedings of the National Academy of Sciences of the United States of America* **104 Suppl**
502 **1**, 8649-8654, doi:10.1073/pnas.0702117104 (2007).
- 503 6 Riehle, M. M., Bennett, A. F., Lenski, R. E. & Long, A. D. Evolutionary changes in heat-inducible gene
504 expression in lines of Escherichia coli adapted to high temperature. *Physiological genomics* **14**, 47-58,
505 doi:10.1152/physiolgenomics.00034.2002 (2003).
- 506 7 Sleight, S. C., Orlic, C., Schneider, D. & Lenski, R. E. Genetic basis of evolutionary adaptation by
507 Escherichia coli to stressful cycles of freezing, thawing and growth. *Genetics* **180**, 431-443,
508 doi:10.1534/genetics.108.091330 (2008).
- 509 8 Chiang, S. M. & Schellhorn, H. E. Regulators of oxidative stress response genes in Escherichia coli and
510 their functional conservation in bacteria. *Archives of biochemistry and biophysics* **525**, 161-169,
511 doi:10.1016/j.abb.2012.02.007 (2012).
- 512 9 Imlay, J. A. The molecular mechanisms and physiological consequences of oxidative stress: lessons from
513 a model bacterium. *Nature reviews. Microbiology* **11**, 443-454, doi:10.1038/nrmicro3032 (2013).
- 514 10 Alcantara-Diaz, D., Brena-Valle, M. & Serment-Guerrero, J. Divergent adaptation of Escherichia coli to
515 cyclic ultraviolet light exposures. *Mutagenesis* **19**, 349-354, doi:10.1093/mutage/geh039 (2004).
- 516 11 Santos, A. L. et al. Role of transition metals in UV-B-induced damage to bacteria. *Photochemistry and*
517 *photobiology* **89**, 640-648, doi:10.1111/php.12049 (2013).
- 518 12 Begley, M., Gahan, C. G. & Hill, C. Bile stress response in Listeria monocytogenes LO28: adaptation,
519 cross-protection, and identification of genetic loci involved in bile resistance. *Applied and environmental*
520 *microbiology* **68**, 6005-6012 (2002).
- 521 13 Bergholz, T. M., Bowen, B., Wiedmann, M. & Boor, K. J. Listeria monocytogenes shows temperature-
522 dependent and -independent responses to salt stress, including responses that induce cross-protection
523 against other stresses. *Applied and environmental microbiology* **78**, 2602-2612, doi:10.1128/AEM.07658-
524 11 (2012).
- 525 14 Canovas, D., Fletcher, S. A., Hayashi, M. & Csonka, L. N. Role of trehalose in growth at high temperature
526 of Salmonella enterica serovar Typhimurium. *Journal of bacteriology* **183**, 3365-3371,
527 doi:10.1128/JB.183.11.3365-3371.2001 (2001).
- 528 15 Fletcher S.A, C. L. N. Characterization of the induction of increased thermotolerance by high osmolarity
529 in Salmonella. *Food Microbiology* **15**, 307-317 (1998).
- 530 16 Garren, D. M., Harrison, M. A. & Russell, S. M. Acid tolerance and acid shock response of Escherichia coli
531 O157:H7 and non-O157:H7 isolates provide cross protection to sodium lactate and sodium chloride.
532 *Journal of food protection* **61**, 158-161 (1998).
- 533 17 Jenkins, D. E., Chaisson, S. A. & Matin, A. Starvation-induced cross protection against osmotic challenge
534 in Escherichia coli. *Journal of bacteriology* **172**, 2779-2781 (1990).
- 535 18 Leyer, G. J. & Johnson, E. A. Acid adaptation induces cross-protection against environmental stresses in
536 Salmonella typhimurium. *Applied and environmental microbiology* **59**, 1842-1847 (1993).

- 537 19 Lou, Y. & Yousef, A. E. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes*
538 against lethal preservation factors. *Applied and environmental microbiology* **63**, 1252-1255 (1997).
- 539 20 Mattimore, V. & Battista, J. R. Radioresistance of *Deinococcus radiodurans*: functions necessary to
540 survive ionizing radiation are also necessary to survive prolonged desiccation. *Journal of bacteriology*
541 **178**, 633-637 (1996).
- 542 21 Pittman, J. R. *et al.* Proteomic Analysis of Cross Protection Provided Between Cold and Osmotic Stress in
543 *Listeria monocytogenes*. *Journal of proteome research*, doi:10.1021/pr401004a (2014).
- 544 22 Sghaier, H., Narumi, I., Satoh, K., Ohba, H. & Mitomo, H. Problems with the current deinococcal
545 hypothesis: an alternative theory. *Theory in biosciences = Theorie in den Biowissenschaften* **126**, 43-45,
546 doi:10.1007/s12064-007-0004-x (2007).
- 547 23 Tesone, S., Hughes, A. & Hurst, A. Salt extends the upper temperature limit for growth of food-poisoning
548 bacteria. *Canadian journal of microbiology* **27**, 970-972 (1981).
- 549 24 Jenkins, D. E., Schultz, J. E. & Matin, A. Starvation-induced cross protection against heat or H₂O₂
550 challenge in *Escherichia coli*. *Journal of bacteriology* **170**, 3910-3914 (1988).
- 551 25 Goll, J. *et al.* MPIDB: the microbial protein interaction database. *Bioinformatics* **24**, 1743-1744,
552 doi:10.1093/bioinformatics/btn285 (2008).
- 553 26 Mozhayskiy, V. & Tagkopoulos, I. Microbial evolution in vivo and in silico: methods and applications.
554 *Integrative biology : quantitative biosciences from nano to macro* **5**, 262-277, doi:10.1039/c2ib20095c
555 (2013).
- 556 27 Tagkopoulos, I., Liu, Y. C. & Tavazoie, S. Predictive behavior within microbial genetic networks. *Science*
557 **320**, 1313-1317, doi:10.1126/science.1154456 (2008).
- 558 28 Mozhayskiy, V. & Tagkopoulos, I. Horizontal gene transfer dynamics and distribution of fitness effects
559 during microbial in silico evolution. *BMC bioinformatics* **13 Suppl 10**, S13, doi:10.1186/1471-2105-13-
560 S10-S13 (2012).
- 561 29 Berry, D. B. & Gasch, A. P. Stress-activated genomic expression changes serve a preparative role for
562 impending stress in yeast. *Molecular biology of the cell* **19**, 4580-4587, doi:10.1091/mbc.E07-07-0680
563 (2008).
- 564 30 Dragosits, M. & Mattanovich, D. Adaptive laboratory evolution -- principles and applications for
565 biotechnology. *Microbial cell factories* **12**, 64, doi:10.1186/1475-2859-12-64 (2013).
- 566 31 Dhar, R., Sagesser, R., Weikert, C. & Wagner, A. Yeast adapts to a changing stressful environment by
567 evolving cross-protection and anticipatory gene regulation. *Molecular biology and evolution* **30**, 573-
568 588, doi:10.1093/molbev/mss253 (2013).
- 569 32 Mao, J., Blanchard, A. E. & Lu, T. Slow and Steady Wins the Race: A Bacterial Exploitative Competition
570 Strategy in Fluctuating Environments. *ACS synthetic biology*, doi:10.1021/sb4002008 (2014).
- 571 33 New, A. M. *et al.* Different levels of catabolite repression optimize growth in stable and variable
572 environments. *PLoS biology* **12**, e1001764, doi:10.1371/journal.pbio.1001764 (2014).
- 573 34 Charusanti, P. *et al.* Exploiting adaptive laboratory evolution of *Streptomyces clavuligerus* for antibiotic
574 discovery and overproduction. *PloS one* **7**, e33727, doi:10.1371/journal.pone.0033727 (2012).
- 575 35 Winkler, J. D., Garcia, C., Olson, M., Callaway, E. & Kao, K. C. Evolved *Escherichia coli* osmotolerant
576 mutants frequently exhibit defective n-acetylglucosamine catabolism and point mutations in the cell-
577 shape regulating protein MreB. *Applied and environmental microbiology*, doi:10.1128/AEM.00499-14
578 (2014).
- 579 36 Zheng, Y. N. *et al.* Problems with the microbial production of butanol. *Journal of industrial microbiology*
580 *& biotechnology* **36**, 1127-1138, doi:10.1007/s10295-009-0609-9 (2009).
- 581 37 Stephanopoulos, G. Challenges in engineering microbes for biofuels production. *Science* **315**, 801-804,
582 doi:10.1126/science.1139612 (2007).

583 38 Ryu, J. H. & Beuchat, L. R. Influence of acid tolerance responses on survival, growth, and thermal cross-
584 protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *International journal of food*
585 *microbiology* **45**, 185-193 (1998).

586 39 Dragosits, M., Mozhayskiy, V., Quinones-Soto, S., Park, J. & Tagkopoulos, I. Evolutionary potential, cross-
587 stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*. *Molecular systems*
588 *biology* **9**, 643, doi:10.1038/msb.2012.76 (2013).

589 40 Blasco, F. *et al.* The coordination and function of the redox centres of the membrane-bound nitrate
590 reductases. *Cellular and molecular life sciences : CMLS* **58**, 179-193 (2001).

591 41 King, P. W. & Przybyla, A. E. Response of hya expression to external pH in *Escherichia coli*. *Journal of*
592 *bacteriology* **181**, 5250-5256 (1999).

593 42 Foster, J. W. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nature reviews.*
594 *Microbiology* **2**, 898-907, doi:10.1038/nrmicro1021 (2004).

595 43 Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L. & Foster, J. W. Comparative analysis of extreme acid survival in
596 *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *Journal of bacteriology* **177**, 4097-4104
597 (1995).

598 44 Gajiwala, K. S. & Burley, S. K. HDEA, a periplasmic protein that supports acid resistance in pathogenic
599 enteric bacteria. *Journal of molecular biology* **295**, 605-612, doi:10.1006/jmbi.1999.3347 (2000).

600 45 Kern, R., Malki, A., Abdallah, J., Tagourt, J. & Richarme, G. *Escherichia coli* HdeB is an acid stress
601 chaperone. *Journal of bacteriology* **189**, 603-610, doi:10.1128/JB.01522-06 (2007).

602 46 Lucht, J. M. & Bremer, E. Adaptation of *Escherichia coli* to high osmolarity environments:
603 osmoregulation of the high-affinity glycine betaine transport system proU. *FEMS microbiology reviews*
604 **14**, 3-20 (1994).

605 47 Perroud, B. & Le Rudulier, D. Glycine betaine transport in *Escherichia coli*: osmotic modulation. *Journal*
606 *of bacteriology* **161**, 393-401 (1985).

607 48 Partridge, J. D., Poole, R. K. & Green, J. The *Escherichia coli* yhjA gene, encoding a predicted cytochrome
608 c peroxidase, is regulated by FNR and OxyR. *Microbiology* **153**, 1499-1507,
609 doi:10.1099/mic.0.2006/004838-0 (2007).

610 49 Chavarria, M., Nikel, P. I., Perez-Pantoja, D. & de Lorenzo, V. The Entner-Doudoroff pathway empowers
611 *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. *Environmental microbiology* **15**,
612 1772-1785, doi:10.1111/1462-2920.12069 (2013).

613 50 Kannan, G. *et al.* Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. *BMC*
614 *microbiology* **8**, 37, doi:10.1186/1471-2180-8-37 (2008).

615 51 Kaur, G. *et al.* A novel MntR-independent mechanism of manganese homeostasis in *Escherichia coli* by
616 the ribosome-associated protein HflX. *Journal of bacteriology*, doi:10.1128/JB.01717-14 (2014).

617 52 Fantino, J. R., Py, B., Fontecave, M. & Barras, F. A genetic analysis of the response of *Escherichia coli* to
618 cobalt stress. *Environmental microbiology* **12**, 2846-2857, doi:10.1111/j.1462-2920.2010.02265.x (2010).

619 53 Ferianc, P., Farewell, A. & Nystrom, T. The cadmium-stress stimulon of *Escherichia coli* K-12.
620 *Microbiology* **144** (Pt 4), 1045-1050 (1998).

621 54 Mozhayskiy, V. & Tagkopoulos, I. Guided evolution of in silico microbial populations in complex
622 environments accelerates evolutionary rates through a step-wise adaptation. *BMC bioinformatics* **13**
623 **Suppl 10**, S10, doi:10.1186/1471-2105-13-S10-S10 (2012).

624 55 Adler, C. *et al.* The alternative role of enterobactin as an oxidative stress protector allows *Escherichia*
625 *coli* colony development. *PloS one* **9**, e84734, doi:10.1371/journal.pone.0084734 (2014).

626 56 Conrad, T. M. *et al.* Whole-genome resequencing of *Escherichia coli* K-12 MG1655 undergoing short-
627 term laboratory evolution in lactate minimal media reveals flexible selection of adaptive mutations.
628 *Genome biology* **10**, R118, doi:10.1186/gb-2009-10-10-r118 (2009).

629 57 Barrick, J. E., Kauth, M. R., Strelisoff, C. C. & Lenski, R. E. Escherichia coli rpoB mutants have increased
630 evolvability in proportion to their fitness defects. *Molecular biology and evolution* **27**, 1338-1347,
631 doi:10.1093/molbev/msq024 (2010).

632 58 Conrad, T. M. *et al.* RNA polymerase mutants found through adaptive evolution reprogram Escherichia
633 coli for optimal growth in minimal media. *Proceedings of the National Academy of Sciences of the United*
634 *States of America* **107**, 20500-20505, doi:10.1073/pnas.0911253107 (2010).

635 59 Cheng, K. K. *et al.* Global metabolic network reorganization by adaptive mutations allows fast growth of
636 Escherichia coli on glycerol. *Nature communications* **5**, 3233, doi:10.1038/ncomms4233 (2014).

637 60 Atsumi, S. *et al.* Evolution, genomic analysis, and reconstruction of isobutanol tolerance in Escherichia
638 coli. *Molecular systems biology* **6**, 449, doi:10.1038/msb.2010.98 (2010).

639 61 Minty, J. J. *et al.* Evolution combined with genomic study elucidates genetic bases of isobutanol
640 tolerance in Escherichia coli. *Microbial cell factories* **10**, 18, doi:10.1186/1475-2859-10-18 (2011).

641 62 Itou, J., Eguchi, Y. & Utsumi, R. Molecular mechanism of transcriptional cascade initiated by the
642 EvgS/EvgA system in Escherichia coli K-12. *Bioscience, biotechnology, and biochemistry* **73**, 870-878
643 (2009).

644 63 Zheng, M. *et al.* DNA microarray-mediated transcriptional profiling of the Escherichia coli response to
645 hydrogen peroxide. *Journal of bacteriology* **183**, 4562-4570, doi:10.1128/JB.183.15.4562-4570.2001
646 (2001).

647 64 Farr, S. B. & Kogoma, T. Oxidative stress responses in Escherichia coli and Salmonella typhimurium.
648 *Microbiological reviews* **55**, 561-585 (1991).

649 65 Volkert, M. R., Loewen, P. C., Switala, J., Crowley, D. & Conley, M. The delta (argF-lacZ)205(U169)
650 deletion greatly enhances resistance to hydrogen peroxide in stationary-phase Escherichia coli. *Journal*
651 *of bacteriology* **176**, 1297-1302 (1994).

652 66 Kanai, T., Takahashi, K. & Inoue, H. Three distinct-type glutathione S-transferases from Escherichia coli
653 important for defense against oxidative stress. *Journal of biochemistry* **140**, 703-711,
654 doi:10.1093/jb/mvj199 (2006).

655 67 Van Melderren, L. & Aertsen, A. Regulation and quality control by Lon-dependent proteolysis. *Research in*
656 *microbiology* **160**, 645-651, doi:10.1016/j.resmic.2009.08.021 (2009).

657 68 Tomar, S. K., Kumar, P. & Prakash, B. Deciphering the catalytic machinery in a universally conserved
658 ribosome binding ATPase YchF. *Biochemical and biophysical research communications* **408**, 459-464,
659 doi:10.1016/j.bbrc.2011.04.052 (2011).

660 69 Niazi, J. H., Kim, B. C., Ahn, J. M. & Gu, M. B. A novel bioluminescent bacterial biosensor using the highly
661 specific oxidative stress-inducible *pgi* gene. *Biosensors & bioelectronics* **24**, 670-675,
662 doi:10.1016/j.bios.2008.06.026 (2008).

663 70 Ariza, R. R., Li, Z., Ringstad, N. & Demple, B. Activation of multiple antibiotic resistance and binding of
664 stress-inducible promoters by Escherichia coli Rob protein. *Journal of bacteriology* **177**, 1655-1661
665 (1995).

666 71 Martin, R. G. & Rosner, J. L. The AraC transcriptional activators. *Current opinion in microbiology* **4**, 132-
667 137 (2001).

668 72 Wendrich, T. M., Blaha, G., Wilson, D. N., Marahiel, M. A. & Nierhaus, K. H. Dissection of the mechanism
669 for the stringent factor RelA. *Molecular cell* **10**, 779-788 (2002).

670 73 Berg, H. C. The rotary motor of bacterial flagella. *Annual review of biochemistry* **72**, 19-54,
671 doi:10.1146/annurev.biochem.72.121801.161737 (2003).

672 74 Goss, T. J., Perez-Matos, A. & Bender, R. A. Roles of glutamate synthase, *gltBD*, and *gltF* in nitrogen
673 metabolism of Escherichia coli and Klebsiella aerogenes. *Journal of bacteriology* **183**, 6607-6619,
674 doi:10.1128/JB.183.22.6607-6619.2001 (2001).

675 75 Liu, X. & Parales, R. E. Chemotaxis of Escherichia coli to pyrimidines: a new role for the signal transducer
676 *tap*. *Journal of bacteriology* **190**, 972-979, doi:10.1128/JB.01590-07 (2008).

- 677 76 Baba, T. *et al.* Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio
678 collection. *Molecular systems biology* **2**, 2006 0008, doi:10.1038/msb4100050 (2006).
- 679 77 Du, H., Lo, T. M., Sitompul, J. & Chang, M. W. Systems-level analysis of Escherichia coli response to silver
680 nanoparticles: the roles of anaerobic respiration in microbial resistance. *Biochemical and biophysical*
681 *research communications* **424**, 657-662, doi:10.1016/j.bbrc.2012.06.134 (2012).
- 682 78 Wesche, A. M., Gurtler, J. B., Marks, B. P. & Ryser, E. T. Stress, sublethal injury, resuscitation, and
683 virulence of bacterial foodborne pathogens. *Journal of food protection* **72**, 1121-1138 (2009).
- 684 79 Tsoukalas A., A. T., Tagkopoulos I. From data to optimal decision making: A data-driven, probabilistic
685 machine learning approach to decision support for patients with Sepsis. *JMIR Medical Informatics in*
686 *press* (2014).
- 687 80 Gultepe, E. *et al.* From vital signs to clinical outcomes for patients with sepsis: a machine learning basis
688 for a clinical decision support system. *Journal of the American Medical Informatics Association : JAMIA*
689 **21**, 315-325, doi:10.1136/amiajnl-2013-001815 (2014).
- 690 81 Csardi, G. a. N., T. The igraph software package for complex network research. *InterJournal Complex*
691 *Systems* **1695**, 1695 (2006).
- 692 82 Gama-Castro, S. *et al.* RegulonDB (version 6.0): gene regulation model of Escherichia coli K-12 beyond
693 transcription, active (experimental) annotated promoters and Textpresso navigation. *Nucleic acids*
694 *research* **36**, D120-124, doi:10.1093/nar/gkm994 (2008).
- 695 83 Hu, P. *et al.* Global functional atlas of Escherichia coli encompassing previously uncharacterized proteins.
696 *PLoS biology* **7**, e96, doi:10.1371/journal.pbio.1000096 (2009).
- 697 84 Peregrin-Alvarez, J. M., Xiong, X., Su, C. & Parkinson, J. The Modular Organization of Protein Interactions
698 in Escherichia coli. *PLoS computational biology* **5**, e1000523, doi:10.1371/journal.pcbi.1000523 (2009).
- 699 85 Traag, V. A. & Bruggeman, J. Community detection in networks with positive and negative links. *Physical*
700 *review. E, Statistical, nonlinear, and soft matter physics* **80**, 036115 (2009).

703 TABLES

704 **Table 1:** List of mutations that appear in 3 or more populations. Final count of populations is 8.

Gene	Frequency	Populations
<i>fepA:fes</i>	8	B1000, B500O500, B500G500, B500OB50, O1000, O500B500, O500G500, O500BO50
<i>rph:pyrE</i>	7	B1000, B500G500, B500OB50, O1000, O500B500, O500G500, O500BO50
<i>rpoB</i>	6	B1000, B500OB50, O1000, O500B500, O500G500, O500BO50
<i>rpoC</i>	6	B1000, B500OB50, O1000, O500B500, O500G500, O500BO50
<i>marC</i>	5	B1000, 500O500, B500G500, , O500B500, O500BO50
<i>glyXVY</i>	4	B1000, B500O500, B500G500, O1000
<i>acrB</i>	4	B1000, B500O500, B500G500, O500B500
<i>acrA</i>	3	B1000, B500G500, O500B500
<i>nagA</i>	3	B1000, O500B500, O500BO50
<i>valZ:lysY</i>	3	B500O500, B500G500, B500OB50
<i>relA</i>	3	B1000, B500O500, B500G500
<i>proV</i>	3	B500OB50, O1000, O500B500
<i>pykF</i>	3	O1000, O500G500, B500G500
<i>sufE</i>	2	O1000, B500OB50
<i>yjbI</i>	2	O1000, B500OB50

705

706 Figure Legends

707

708 **Figure 1. Overview of the experimental approach.** (A) *E. coli* strains (4 biological, 2 technical replicates) were
 709 pre-conditioned in a first stressful (condition 1) or control (condition 2) environment for 12h before they were
 710 exposed to a second stress (condition 3) for an additional 12h. Competition assays, growth curves, genome-scale
 711 transcriptional profiling and functional network analysis was performed to assess the cross-stress behavior of *E.*
 712 *coli* for combinations of five stresses (n-butanol, osmotic, acidic, alkaline and oxidative stress). (B) Sequential
 713 evolution in stressful environments and hypotheses testing related to the adaptive potential of cross-stress
 714 behavior. *E. coli* populations were adapted over 1000 generations under various stresses to assess hypotheses
 715 related to co-protection, guided evolution, order, environmental reversal and varying environments. Genome-wide
 716 re-sequencing was used to map the genotypic landscape and phenotypic characterization was performed through
 717 competition assays and growth curves. Functional and pathway analysis was performed to identify the biological
 718 processes and network neighborhoods that are implicated in the acquired stress resistance

719 **Figure 2: Hard-wired cross-stress behavior in *E. coli*.** (A) Change in Darwinian fitness (*W*) of preconditioned
 720 *E. coli* populations (12h exposure) relative to the WT un-conditioned strain (*W*=1). Each sub-plot represents a
 721 different stressful environment where fitness was assessed through competition assays. Cross-stress protection
 722 ranges from 1.15 ± 0.04 to 1.02 ± 0.01 and cross-stress vulnerability is observed only in the case of *E. coli* strains
 723 pre-conditioned to oxidative stress before exposed to acidic stress (0.97 ± 0.01 , $p\text{-value} = 1.91 \cdot 10^{-4}$). Shaded
 724 area depicts the fitness advantage when the population has been conditioned in the same stress. (B) Cross-stress
 725 area plots demonstrate the level of cross-stress protection for each conditioning environment.

726 **Figure 3: Transcriptional profiling in single stressors and cross-stress pairs.** (A) genes differentially
 727 regulated in presence of one stress. (B) expression profiles of the cross-stress behavior. 4 analyses were perform,
 728 from n-butanol to osmotic, from osmotic to n-butanol, from oxidative to acidic and from acidic to oxidative. The
 729 expression profile was compare to a sample grown for 12 hours in M9 and then to the second stress (first column)
 730 and 24 hours in the second stress. Important implicated pathways are highlighted.

731 **Figure 4: Phenotypic and genomic analysis of the populations evolved for 500 and 1000 generations.** (A)
 732 Map of mutations in clones of populations evolved for 500 generations under one stress. Four clones of each
 733 stress were selected by fitness assessment. Mutations are draw relative to the reference genome of MG1655 and
 734 symbolized with a line if SNP and with a triangle if it is an insertion or deletion (upside and downside
 735 respectively). Details of the mutations can be find in Table S4. (B) Venn diagram illustrating the presence of the
 736 mutations in all the populations (Fig S6 and Table S10). (C) Results of the direct competition assays for each of

the five hypothesis tested in this study. In all cases two biological replicates were performed (black and grey dots) but in the case of order in *n*-butanol and reversal of the *n*-butanol mutation in M9 were 6 replicates were performed.

Figure 5. Network analysis and implicated pathways in stress resistance. (A) A functional network was constructed from PPI and TF-DNA data, superimposed with the re-sequencing and transcriptional profiling results of our analyses. Modularity-based algorithms were used to identify communities within the network, which were further analyzed for enriched clusters. The name of the most statistically significant cluster and the profile of the mutants/DEGs in terms of the corresponding stress (Butanol, Osmotic, Acidic, Oxidative, in that order) for each community is shown. For example, in the case of the first community, the Glycolysis GO term is the most over-represented and 50% of the observed mutations/DEGs in the community were identified in cell lines exposed/evolved in *n*-butanol. Light pink nodes are genes that are not mutated or DEGs, but connect two or more mutated/DEG genes in a path with a length shorter than three. (B) Highly enriched pathways and their members that are implicated in each stress.

Figure 1. Overview of the experimental approach

Figure 2: Hard-wired cross-stress behavior in *E. coli*

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759 **Figure 3: Transcriptional profiling in single stressors and cross-stress pairs**

Figure 4: Phenotypic and genomic analysis of the populations evolved for 500 and 1000 generations.

B

Stress	Pathway	Interactors
<i>n</i> -butanol	Response to Drug	<i>marA, acrB, acrA, acrD</i>
	Cellular Respiration	<i>ubiC, fnr, hyaD, hyaB, hyaE, hyaF, hyaA, narL, hyaC, narG, narH, narJ</i>
	Glycolysis/Gluconeogenesis	<i>eno, pykF, pfkA, pgi</i>
Osmotic	Metal Iron Binding	<i>entD, katG, pyrC, fes, fur, fepA, yhjA</i>
	Translation	<i>rpsM, spsD, rplA, rplM, rplB, rplV, rpmB, rplC, pth, rplC</i>
	Regulation of Transcription	<i>crp, hns, flhC, gadX, evgA, evgS, gadE, phoP, yjM, uxuR</i>
Acidic	Regulation of Transcription	<i>crp, hns, flhC, gadX, evgA, evgS, gadE, phoP, yjM, uxuR</i>
	Cell cycle	<i>groL, ftsE, ftsA, ftsN, ftsY, murB</i>
Oxidative	Cellular Respiration	<i>ubiC, fnr, hyaD, hyaB, hyaE, hyaF, hyaA, narL, hyaC, narG, narH, narJ</i>
	Metal Iron Binding	<i>entD, katG, pyrC, fes, fur, fepA, yhjA</i>
	Bacterial Chemotaxis	<i>cheY, cheR, motB, tsr, trg</i>
	Nucleotide Binding	<i>groL, atpA, ftsE, ftsA, lpdA, tyrR, murB, ydiA</i>
	Sulfur Metabolic Process	<i>clb, cysB, cysU, cysW, tauB, tauC, cysP</i>

Figure 5. Network analysis and implicated pathways in stress resistance.