The Genetic and Transcriptional Basis of Short and Long Term Adaptation across Multiple Stresses in *Escherichia coli*

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Abstract

Microbes exhibit short and long term responses when exposed to challenging environmental conditions. To what extent these responses are correlated, what their evolutionary potential is and how they translate to cross-stress fitness is still unclear. In this study, we comprehensively characterized the response of *Escherichia coli* populations to four abiotic stresses (*n*-butanol, osmotic, acidic, and oxidative) and their combinations by performing genome-scale transcriptional analysis and growth profiling. We performed an analysis of their cross-stress behavior which identified 15 cases of cross-protection and one case of cross vulnerability. To elucidate the evolutionary potential of stress responses to individual stresses and stress combinations, we re-sequenced *E. coli* populations evolved in those four environments for 500 generations. We developed and applied a network-driven method that integrates mutations and differential expression to identify core and stress-specific gene communities that are likely to have a phenotypic impact. Our results suggest that beyond what is expected from the general stress response mechanisms, cross-stress behavior arises both from common pathways, several including metal ion binding and glycolysis/gluconeogenesis, and stress-specific expression programs. The stress-specific dependences uncovered, argue that cross-stress behavior is ubiquitous and central to understanding microbial physiology under stressful conditions.

Key words: **cross-stress protection, evolutionary trade-offs, microbial evolution, stress adaptation, network analysis.**

Introduction

Regardless of their complexity, organisms have developed a rich molecular and behavioral repertoire to cope with environmental variations in order to maintain homeostasis and cellular function. The term *stress* is used to describe conditions where environmental parameters differ substantially from an organism's optimal growth conditions. Given its industrial and medical importance, stress response has been an active area of research for decades in bacteria (Storz and Hengge-Aronis 2000), usually with a focus on single stress responses such as acidic (Zhao and Houry 2010; Hong, et al. 2012; Kanjee and Houry 2013), heat (Riehle et al. 2003; Bennett and Lenski 2007; Sleight et al. 2008), oxidative (Chiang and Schellhorn 2012; Imlay 2013), and UV (Alcantara-Diaz et al. 2004; Santos et al. 2013) stresses. *Cross-stress behavior*, a phenomenon that occurs when exposure to a given stressor confers a fitness advantage or disadvantage against a second stress has also attracted considerable interest over the years (Tesone et al. 1981; Jenkins et al. 1990; Leyer and Johnson 1993; Mattimore and Battista 1996; Lou and Yousef 1997; Fletcher and Csnoka 1998; Garren et al. 1998; Canovas et al. 2001; Begley et al. 2002; Sghaier et al. 2007; Bergholz et al. 2012; Pittman et al. 2014).

Evidence of cross-stress behavior has been well documented throughout the microbial kingdom. Early work on glucose- and nitrogen-starved *Escherichia coli* cells showed increased survival rates after heat shock or hydrogen peroxide (H_2O_2)-mediated stress compared with non-stressed cells (Jenkins et al. 1988), with the alternative sigma factor *rpoH* being a crucial link for heat shock protein production during starvation stress (Jenkins et al. 1990). *E. coli* cells adapted to high ethanol concentrations had decreased growth under acidic stress (Goodarzi et al. 2010) and high temperature environments induce a similar transcriptional program to that observed under low oxygen (Tagkopoulos et al. 2008). Pre-adaptation to elevated temperature can reduce both cell death rate and mutation frequency caused by H_2O_2 in *Lactobacillus plantarum* (Machielsen et al. 2010). Osmotic stress was found to confer inducible heat tolerance in *Salmonella typhimurium* (Fletcher and Csnoka 1998), while trehalose synthesis, which is important for osmoprotection, was shown to feature a heat-inducible component that increases heat tolerance of osmo-adapted *Salmonella enterica* cells (Canovas et al. 2001). More recently, gene-deletion libraries were used to investigate H_2O_2 tolerance in yeast (Berry et al. 2011). How cross-stress behavior emerges in different environments remains unclear. In a recent study, the cross-stress fitness of *E. coli* populations in five stressors was measured after adaptation and some of the mutations implicated were identified for a single cell line (Dragosits et al. 2013).

Data from transcriptional and genetic profiling can be used to create the genetic signatures for each response and construct networks that integrate different layers of information

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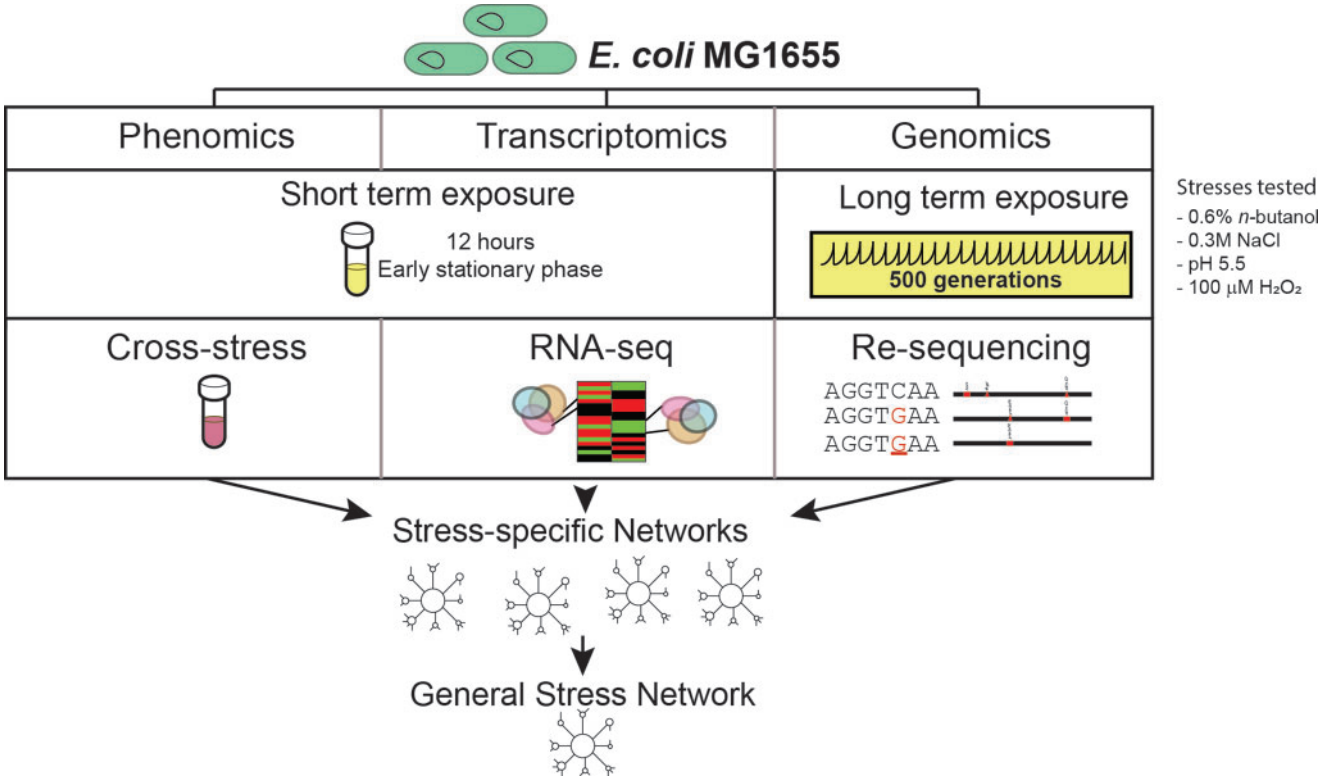


Fig. 1. Overview of the experimental approach. First, *E. coli* cells (4 biological, 2 technical replicates) were pre-conditioned in a first stressful or control environment for 12 h (early stationary phase) before competition under a second stress for other additional 12 h for cross-stress behavior assessing. Samples were taken before stress change for RNA extraction and sequencing. For long term adaptation, bacteria were evolved for 500 generations under each stress was sequenced to analyze the mutations associated to each stress. Data from all the experiments was used to produce a network.

(Imam et al. 2015). Along these lines, here we have constructed a comprehensive map of stress response in *E. coli* when exposed to four stressors and their combinations (osmotic, oxidative, acidic, and *n*-butanol), which have industrial and biological importance (Stephanopoulos 2007; Zheng et al. 2009; Imlay 2013; Kanjee and Houry 2013; Winkler et al. 2014). We profiled the genome-wide gene expression responses associated to each stress and then determined their cross-stress phenotypic behavior by measuring the population fitness in pair-wise stress combinations. We then sequenced evolved replicate populations coming from the same ancestral strain as ours and that were evolved over 500 generations. This identified 23 new mutations that are associated with long term adaptation to these stresses. We quantified the effect of acquired mutations through competition assays between the evolved strains and repaired mutants, in each respective stress. Finally, we integrated the available data to create stress-specific and cross-stress genetic networks that highlight key genes and pathways associated with the respective responses (fig. 1).

Results

Comprehensive Map of the Cross-Stress Behavior in *E. coli*

We tested the cross-stress behavior of *E. coli* MG1655 cells in all the pair combinations across four stressful environments (osmotic, oxidative, acidic, and *n*-butanol; four biological and two technical replicates) by performing competition assays against a control population that has been exposed only to M9 and calculating the Darwinian Fitness (W). Out of the 16 possible combinations, 12 pairs (75%) had significant cross-stress behavior which is in accordance with the presence of a general stress response in *E. coli* (Fig. 2A, supplementary table S1 and file S1, Supplementary Material online). Among them, the highest cross-protection was observed when bacteria were exposed to acidic stress prior to *n*-butanol stress ($W = 1.15 \pm 0.04$, $P\text{-value} < 1.46 \times 10^{-3}$). There was only one significant case of cross-vulnerability when bacteria moved from oxidative to acidic stress ($W = 0.97 \pm 0.01$, $P\text{-value} < 1.91 \times 10^{-4}$). We found a significant cross-protection effect between acidic and osmotic stresses ($W = 1.10 \pm 0.02$, $P\text{-value} < 5.17 \times 10^{-4}$), which is a cross-stress behavior with previously inconclusive results in *E. coli* cells (Garren et al. 1998; Ryu and Beuchat 1998) but it is known to occur in *S. typhimurium* (Leyer and Johnson 1993). We also detected a cross-protection similar to that described for *S. typhimurium* and *Listeria monocytogenes* (Fletcher and Csnoka 1998; Bergholz et al. 2012) between osmotic and oxidative stresses ($W = 1.14 \pm 0.02$, $P\text{-value} < 6.41 \times 10^{-3}$).

Cross-protection was observed in bacteria adapted to either high salt concentration or *n*-butanol and then exposed to oxidative stress ($W = 1.14 \pm 0.02$, $P\text{-value} < 6.41 \times 10^{-3}$ and

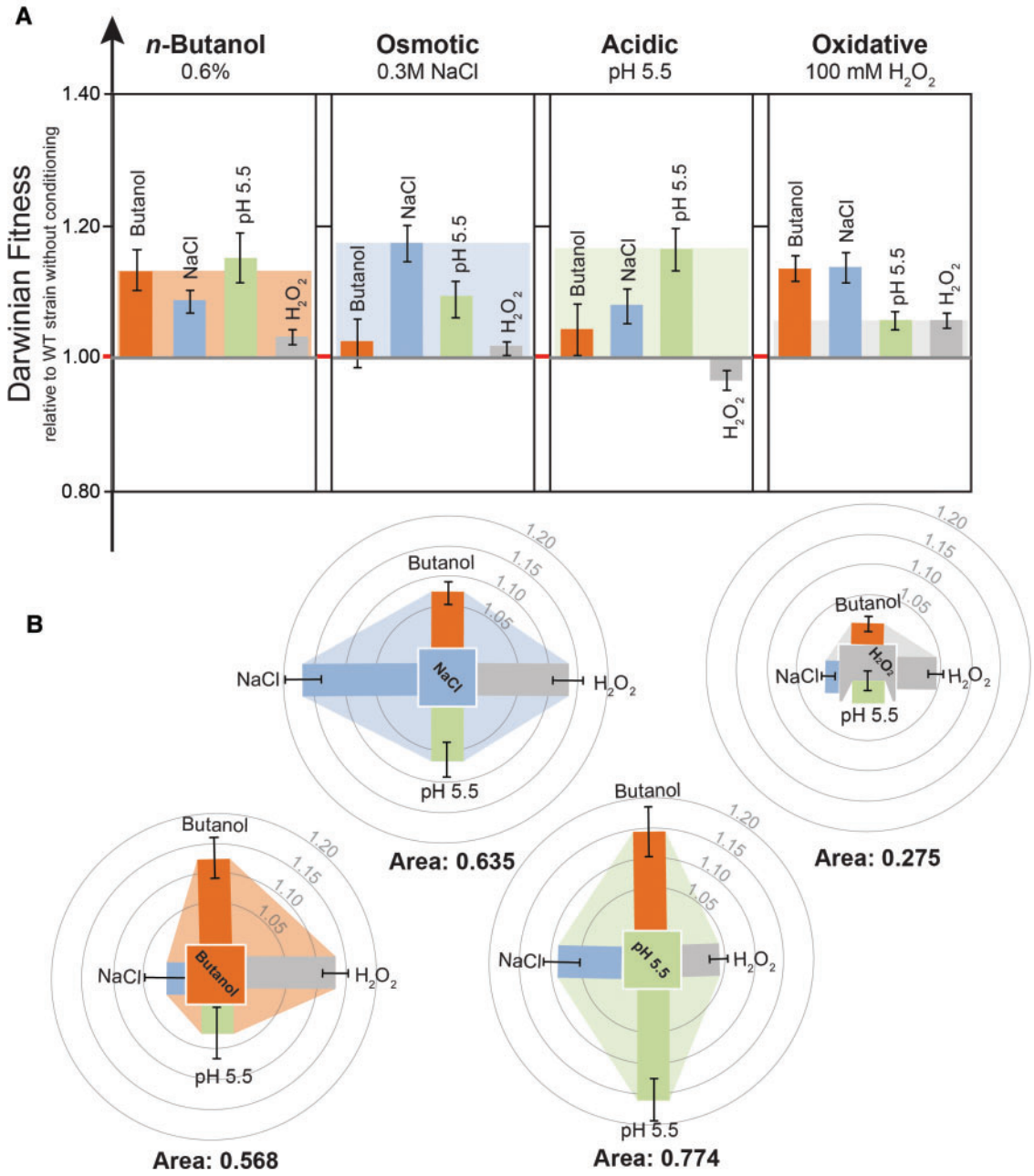


FIG. 2. Hard-wired cross-stress behavior in *E. coli*. (*A*) Change in Darwinian fitness (W) of preconditioned *E. coli* populations (12 h exposure) relative to the WT un-conditioned strain (W = 1). Each sub-plot represents a different stressful environment where fitness was assessed through competition assays. Cross-stress protection 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 pre-conditioned to oxidative stress before exposed to acidic stress (0.97 ± 0.01, *P*-value = 1.91×10^{-4}). Shaded area depicts the fitness advantage when the population has been conditioned in the same stress. (*B*) Cross-stress area plots demonstrate the level of cross-stress protection for each conditioning environment.

W = 1.14 ± 0.02, *P*-value < 0.02, respectively). A recent study has identified cross-stress vulnerability for these two combinations (Dragosits *et al.* 2013), however in that case, *E. coli* was evolved for 500 generations in the first stress, hence accumulating mutations that can alter its cross-stress profile with respect to the ancestral line. Indeed, we found that evolved and un-evolved cell lines had significantly different behaviors in both control media and under oxidative stress (supplementary figs. S1 and S2, Supplementary Material online).

In the presence of *n*-butanol or oxidative stresses, a first exposure to three out of the four stresses gave a selective advantage to the bacteria. Hence, pre-adaptation to other stresses except acidic provides a fitness advantage in the oxidative environment, partially analogous to what has been described for *L. monocytogenes* (Lou and Yousef 1997). Surprisingly, in acidic stress we observed the least cross-stress protection from all the other conditioned strains and we also observed the sole case of cross-stress vulnerability in the oxidative-adapted strains (fig. 2*A*). On the other hand,

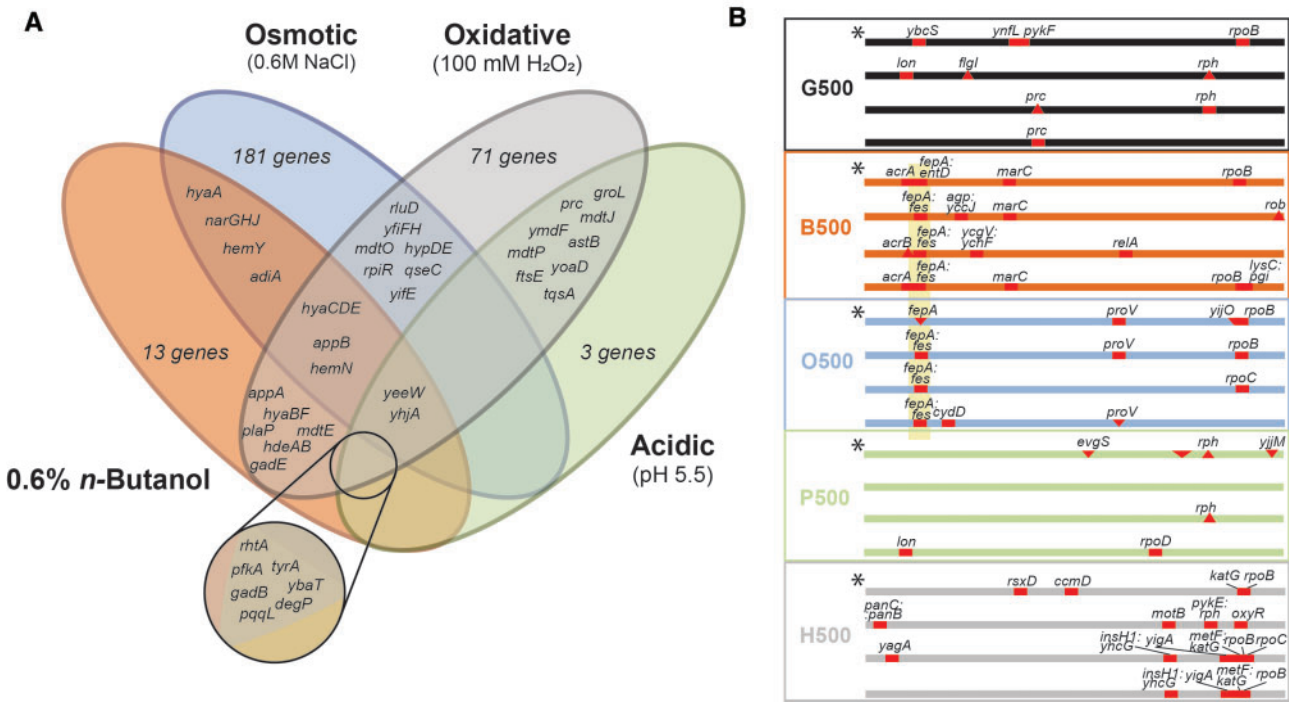


Fig. 3. Molecular profiling in single stressors: (*A*) Venn diagram showing the genes differentially regulated in presence of one stress and the commonalities of the transcription profile for the four stresses. When more of one gene of the operon, they were compiled due to space constraints. (*B*) Genomic analysis of the populations evolved for 500 generations. Map of mutations in clones of populations evolved for 500 generations under one stress. Four clones of each stress were selected by fitness assessment. Mutations are draw relative to the reference genome of MG1655 and symbolized with a line if SNP and with a triangle if it is an insertion or deletion (upside and downside respectively). Data of the first clone (depicted with an *) was provided from a previous publication (Dragosits et al. 2013). Details of the mutations can be find in supplementary table S4, Supplementary Material online.

bacteria adapted in acidic stress were well-positioned to compete in all other stresses. To quantify the degree of positive or negative cross-stress behavior, we adopted the use of *cross-stress plots* (fig. 2*B*), a visualization and quantitative tool introduced in our previous work (Dragosits *et al.* 2013). As shown in the plots, the acid-conditioned cells possess the highest area of cross-stress protection (0.774), whereas oxidative-conditioned cells have the lowest area (0.275) while also harboring the sole case of cross-stress vulnerability.

Transcriptional Response to Short-Term Stress Exposure

To analyze the underlying mechanism of the stress resistance, we performed genome-wide transcriptional profiling for each stressor (RNA-Seq; supplementary tables S2–S4, Supplementary Material online). The RNA profiles of the cells growing under one stressor were compared with bacteria grown in minimal media in order to remove the interference of the general stress response mediated by RpoS (Landini et al. 2014). We performed a rigorous selection of differentially expressed genes (DEGs) in which only genes significant in at least two out of the three computational methods used (Cuffdiff, edgeR, and DESeq) were selected. We identified 41, 203, 111, and 21 differentially expressed genes (P-value < 0.05 after Bonferroni correction) for *n*-butanol, osmotic, oxidative, and acidic stress, respectively (supplementary fig. S3 and file S2, Supplementary Material online) and depicted their overlap through a Venn diagram (fig. 3*A*).

Interestingly, under acidic stress 18 of the 21 DEGs found (86%) overlap with other stresses correlating with the fact that this specific environment has the highest cross-stress area. As shown before, pre-exposure to osmotic and *n*-butanol produces a high resistance to oxidative response and the three stresses share several DEGs. 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 (King and Przybyla 1999; Blasco et al. 2001). The *hya* operon was also down-regulated under oxidative stress when compared with its expression under control conditions. The operon encodes for the synthesis of a hydrogenase that is implicated in pH stabilizing responses (King and Przybyla 1999). The *gadE*, *gadB* genes from acidic resistance system AR2, the *adiA* gene from AR3 and the *hdeAB* acidic response (Lin et al. 1995; Gajiwala and Burley 2000; Foster 2004; Kern et al. 2007) were found to be under-expressed under the stressor of osmotic and *n*-butanol compared with their expression in the absence of a stressor.

We have identified the top ranked DEG for individual stresses (supplementary table S5, Supplementary Material online). In osmotic stress, *proX* is significantly overexpressed (fold change = 4.28, P-value < 0.0025). This gene belongs to the *pro* known for encoding osmo-protectant transportation (Perroud and Le Rudulier 1985; Lucht and Bremer 1994).

In oxidative stress, we observed an overexpression of *yhjA* (fold change 5.54, *P*-value $< 5.63 \times 10^{-8}$) and an underexpression of *pfkA* (fold change −4.96, *P*-value $< 4.52 \times 10^{-7}$). The first is a *oxyR*-regulated peroxidase (Partridge et al. 2007) while the second is of unknown function and we have recently showed that it is implicated in H_2O_2 sensitivity (Chavarria et al. 2013). 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 12 h, which explains the up-regulation of the acidic-stress cluster and the resulting cross-stress protection observed in this study.

Adaptation to Long-Term Stress Exposure

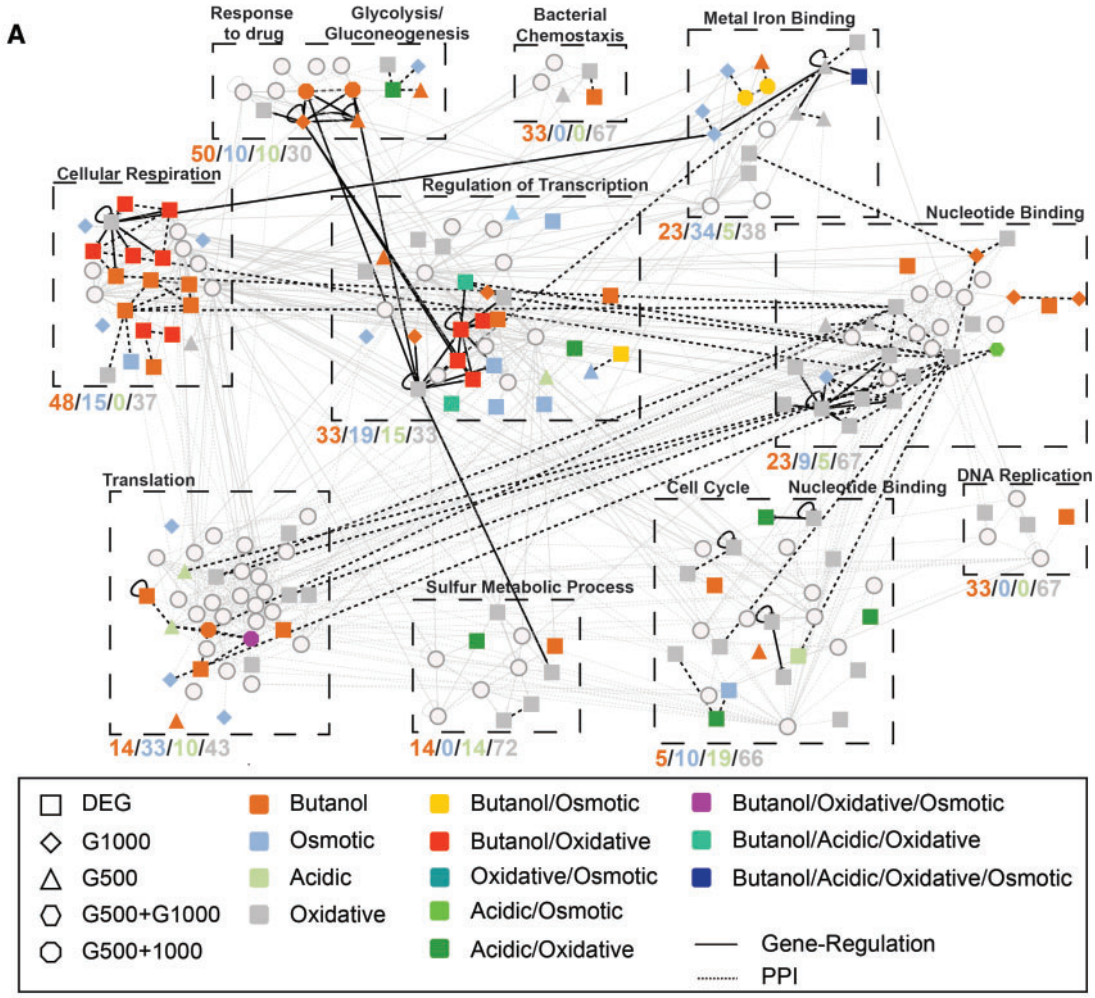
Previous studies on the effect of single stress adaptation have focused on the identification of mutations in a single cell line (Dragosits et al. 2013). To further elucidate the genetic basis of the acquired stress resistance and understand the variation during the evolutionary trajectory among clonal populations, we sequenced three additional clones selected from independent cell lines for each of the four stressors and the control environment (4 biological replicates, 5 environments, 20 lines total, supplementary table S6, Supplementary Material online) identifying a number of loci that are mutation hotspots over two or more cell lines (fig. 4). The populations were named B500, O500, P500, H500, and G500 when evolved under *n*-butanol, osmotic, acidic, oxidative, or no (control) stress, respectively.

Across all lines, only two mutations were found to be synonymous, *ynfL* (G500) and *cydD* (O500) (supplementary table S7, Supplementary Material online). Common mutations among the stresses are the intergenic zones *rph*:*pyrE* and *fes*:*fep*, and the RNA polymerase subunits *rpoB* and *rpoC* (table 1). The first three mutations can also be found in the population that evolved in media without stress. On the one hand, both *fes* and *fepA* are involved in siderophore enterobactin production, which has recently been linked with oxidative stress and M9 growth (Adler *et al.* 2014) and mutations in *rph*:*pyrE* have also appeared in a previous study in evolution on lactate (Conrad et al. 2009). Both *rpoB* and *rpoC* encode for RNA polymerase subunits and their mutations are very common in evolution, more specifically mutations in *rpoB* have been linked to an increased evolvability and fitness (Barrick et al. 2010) and mutations in *rpoC* to bacterial growth optimization in minimal media (Conrad et al. 2010) and metabolic efficiency (Cheng et al. 2014). Specific mutations under each stress already described are *pykF* in the control population (no stress; G500), this mutation is known to be beneficial for growth in M9 (Barrick et al. 2010), *marC* and *acrAB* for resistance in *n*-butanol (Atsumi et al. 2010; Minty et al. 2011; Dragosits et al. 2013), *proV* in osmotic stress (Perroud and Le Rudulier 1985; Lucht and Bremer 1994; Dragosits et al. 2013), *evgS* and *rpoD* in acidic stress (Foster 2004; Itou et al. 2009; Dragosits et al. 2013) and *katG*, *oxyR*, *rsxD*, and *ccmD* for resistance in oxidative stress (Farr and Kogoma 1991; Zheng et al. 2001; Dragosits et al. 2013). Interestingly, in oxidative stress we found mutations in the *yagA* and *yncG* genes, consistent with a previous finding that a deletion between *argF-lacZ* (that includes *yagA*) confers a high H_2O_2 resistance (Volkert et al. 1994). In addition, *yncG* is homologous to Glutathione S-transferases which are known to help in defense against oxidative stress (Kanai et al. 2006). Other mutations with unclear importance in the stress where they appeared are *ybcS*, *lon*, and *prc* in G500; promoters of *yccF*, *ychF*, and *pgi* and genes *rob* and *relA* in B500; *yijO* in 0500; *lon* in P500; and *motB*, *yigA*, and the intergenic zone *panC*:*panB* H500. *ybcS*, *prc*, *yccF*, and *yigA* genes have an unknown function. Other genes functions have been described but seem to be unrelated to stress resistance. For example, *lon* is a DNA-binding protease that degrades abnormal proteins (Van Melderen and Aertsen 2009), *ychF* is a ribosome binding catalase (Tomar et al. 2011), *pgi* is an oxidative stress induced gene (Niazi et al. 2008), *rob* is a transcriptional activator involved in antibiotic resistance (Ariza et al. 1995; Martin and Rosner 2001), *relA* synthesizes ppGpp, an alarmone active under amino acid starvation conditions (Wendrich et al. 2002) and *motB* is part of the flagellar motor (Berg 2003).

The gene with the highest number of mutations is the RNA polymerase subunit *rpoB* (table 1). Specifically, we found seven unique mutations in seven different sequenced clones (supplementary fig. S4, Supplementary Material online), out of which six were found in the fork region (residues 500–690) involved in the conformational change during the switch from initiation to elongation mode of the RNA polymerase (Vassylyev et al. 2007). Only one mutation, found in the control population G500 #1, was out of this region and proximal to the hydrophobic pocket switch 3 loop (Vassylyev et al. 2007). The other RNA polymerase β subunit was present in two different conditions, *rpoC* (supplementary fig. S5, Supplementary Material online). In this case, the two mutations were found in two independent regions of the protein.

Functional and Network Analysis of the Stress Response

Re-sequencing and transcriptional profiling data were compiled in order to produce a network representation of stress resistance in *E. coli* (fig. 4A). Overall, enriched gene ontology clusters are related to motility, sulfur metabolic process, translation, DNA replication and cellular respiration, among others (fig. 4A and B**)**. Interestingly, the glycolysis/gluconeogenesis, response to drug genes and cellular respiration are involved in *n*-butanol response, with a *pykF* mutant from this pathway being unexpectedly fit, as it has the highest growth rate in these conditions. The importance of the *fes* and *fepA* 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 *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 fumarate and nitrate reductases (FNR) and OxyR (Partridge et al. 2007). This gene has been



B

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

Fig. 4. 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. Genomic data from cells evolved for 1000 generations was added to the network (G1000, unpublished data). 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.

Table 1. List of Mutations that Appear in Two or More Populations

Gene	Frequency	Populations
rpoB	4	G500, B500, O500, H500
rph	3	G500, P500, H500
fepA:fes	2	B500, O500
rpoC	2	O500, H500

NOTE.—Final count of populations is 5.

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 higher cross-stress protection.

Network and functional analysis was also performed for each individual stress (supplementary fig. S6–S9, Supplementary Material online). Although, we have not identified genes that are both a DEG and harbor a selected mutation, all networks are strongly connected. In *n*-butanol mutated genes (*acrA*, *acrB*, *marA*, and *rob*) multiple DEGs are regulated, such as the *hde* and *gad* operons (supplementary fig. S6, Supplementary Material online). In osmotic, mutations in *proV* and *rpoB* are related with the DEGs *proX* and *yifE*, respectively (supplementary fig. S7, Supplementary Material online). Acidic stress has several cases of mutations that are involved in downstream differential expression, such as mutations in *evgS* that regulate the DEGs *degP* and *gadP*, mutations in *lon* and *ropD* that regulate the DEG *ftsE* and mutations in *rph* with *purT* and *pfkA* (supplementary fig. S8, Supplementary Material online). In the oxidative network there are multiple connections between DEGs and mutations in the *rpoC*, *panB*, *panC*, and *katG* genes (supplementary fig. S9, Supplementary Material online).

Can the mutation–expression network help us identify mutations with larger phenotypic impact? To test this hypothesis, we selected four mutations per condition to reverse in the mutant background. For this selection, we first ranked all mutations using their number of connections to the various DEGs (table 2). We then chose the two mutations corresponding to the highest number of connecting nodes, avoiding the selection of RNA polymerase subunits as they were already described to be involved in the general stress response (table 1). As a control, we chose two random genes with mutations but no connections to the DEG network. Those 16 genes were repaired in the mutant cell lines and then their fitness was assessed by direct competition assays. Interestingly, the hypothesis holds as cell lines with repaired mutations within the DEG network have a significant fitness decrease of the repair mutant compared with the evolved clone (P-value $< 2.17 \times 10^{-5}$), while this was not the case for the controlled group (**P-value $< 0.79^3$**; supplementary fig. S10, Supplementary Material online**)**. Finally, we explored whether this method can be used in evolved cell lines, so we focused

Table 2. Ranking of Genes According to the Nodes that Connect the Mutation with the DEGs in Each Stress.

Stress							
Butanol		Osmotic		Oxidative		Acidic	
Gene	Nodes	Gene	Nodes	Gene	Nodes	Gene	Nodes
rob	5	*rpoC*	2	*oxyR*	6	*lon*	6
acrB	3	*rpoB*	2	*rpoC*	3	*rpoD*	5
acrA	3	*fes*	2	*rpoB*	2	*rph*	2
fepA	2	*proV*	1	*katG*	2	*evgS*	1
entD	1	*fepA*	1	*panC*	2		
				metF	1		
				panB	1		
agp	0	*yigO*	0	*rsxD*	0	*evgS*	0
relA	0	*cydD*	0	*motB*	0	*yjjM*	0

NOTE.—Underlined genes were selected for mutant repair and phenotypic analysis. Under acidic stress, indirect connections where count as no direct could be found.

on the O500 populations that evolved under osmotic stress (Dragosits et al. 2013). Similarly, the top hits in the core network were two mutations that were also DEGs, *proV* and *rpoB*, which have also been verified through mutation repair as having a profound effect in fitness under that environment (supplementary fig. S11, Supplementary Material online). As such, the reconstructed mutation–expression network presents an additional tool to highlight what we should expect the magnitude and effect of mutations to be in the respective response.

Discussion

Here, we integrated the phenotypic, transcriptomic, and genomic data of four important stressors in *E. coli*. A general observation that stems from our work is that exposure of a bacterial population to one stressor is generally beneficial, as 15 out of 16 cases tested in this work showed cross-stress protection with an average Darwinian Fitness of 1.08 ± 0.01 (P-value $< 1.51 \times 10^{-5}$). 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 to produce more effective sterilization practices. Acidic stress also has the largest cross stress area showing that its preadaptation triggers a general response in the cell. The fact that *E. coli* is an enteric bacteria that during its lifecycle has to face the digestive tub explains this phenomenon as exposure to this stress might serve as an intracellular signal for the cell to prepare for other stresses found in the gut. It is interesting to compare our findings regarding WT exposure to a sequential combination of stresses (without evolution) to our previous work, where cells evolved for 500 generations in one stress and were then exposed to another (Dragosits et al. 2013). One of the differences was in cross-stress behavior where cells preadapted in both osmotic and butanol stresses showed a high cross-protection when moved into oxidative meanwhile evolved bacteria in these conditions showed no significant differences. Adapted bacteria to both conditions showed a mutation in the enterobactin (*fes*) operon. This operon has been already described to have a protective

function under oxidative stress (Adler et al. 2014) and this mutation might lead to a lower resistance under that stress.

By connecting the transcriptional profiles of different stresses we found expression patterns are good predictors of the underlying cross-stress behavior. Indeed, exposure to acidic media produces the highest cross stress effect (fig. 2B) and the DEGs in this condition are mostly shared with the other stressors (fig. 3A). By supporting the hypothesis that cross-stress protection can be dependent on specific DEGs of the first stressor, 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. 3A). These genes constitute excellent targets for further experimentation to understand the mechanism under which they affect single stress and cross-stress behavior. In addition, we also compared the expression profiles before and after evolution under osmotic stress finding one gene to be differentially expressed in both, *proX* (supplementary file S3, Supplementary Material online). *proX* is overexpressed in the non-evolved cells (fold change 4.65, *P*-value $< 3.04 \times 10^{-5}$) and under expressed after evolution (fold change -2.13, *P*-value $< 2.17 \times 10^{-60}$). The evolved cells show a mutation in *proV*, another gene belonging to the same operon. 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 resistance (Du et al. 2012).

There is substantial similarity in the cross-stress behavior of *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 four clones sequenced (B500 and O500, fig. 3B) discovered two genes in the *n*-butanol evolved populations that are also involved in oxidative stress and are related to this behavior, the *ychF* catalase (Tomar et al. 2011) and the *pgi* gene that are induced under oxidative conditions (Niazi et al. 2008). Although not directly related to oxidative stress, in these clones we also found a mutation in the *rob* gene involved in antibiotic resistance (Ariza et al. 1995). Unpredictably, in the O500 clones only one mutation was 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 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 media (Adler et al. 2014) and thus its mutation might have a significant effect in M9 with oxidative stress. The evolved cell lines provide an interesting view of the evolutionary trajectories under the various stresses and it shows that their diversity is environment-specific. As shown in figure 3B, cell populations that evolved under acidic stress share few genetic mutations while populations evolved under the other stresses share many (table 1). Interestingly, some mutations can be found not only along several lines but also along several conditions. One example is the high abundance of mutations in the *fes:fepA* genes found in eight lines belonging to two different stresses. This mutation has been previously described in similar scenarios and recently the involvement of the *fep* operon in growth in M9 (Conrad et al. 2009; Dragosits et al. 2013; Adler et al. 2014) although, unexpectedly, in our experiment, it was not detected in the only media line showing the *fes:fepA* mutation to be stress-related. Another widely present mutation in the literature, *rph*, is present in the population evolved in acidic and oxidative stress as well as in the only-media control. It is hypothesized that mutations appear randomly with the media functions as a selector. Here, by integrating genomic data with transcriptional data in each stressor we show that both mutations are closely related as can be seen in the networks produced (supplementary figs. S6–S9, Supplementary Material online) and we verified this by repairing the respective mutations (supplementary fig. S10, Supplementary Material online).

It has been documented that the transcription machinery is closely involved in stress response highlighting the Sigma factor *rpoS* as the most important protein involved in this phenomena (Battesti et al. 2011; Landini et al. 2014). RpoS functions as a major expression regulator when cells go into stationary phase. When harvesting cells for RNA-seq as well as during evolution, we kept the cultures out of the stationary phase skipping the *rpoS*-dependent general stress involvement. However, we found the two beta subunits of the RNA polymerase, *rpoB* and *rpoC*. These subunits form the active center where the fork region is an interface between both subunits (Vassylyev et al. 2007). Six out of the seven mutations sequenced in *rpoB* were found in the cited fork region (residues 500–690). A recent study performed a systematic analysis of this region suggesting that mutations on it debilitates the DNA clamp of the elongation complex facilitating a spontaneous melting of the RNA–DNA hybrid that triggers slippage (Zhou et al. 2013). This slippage triggers resistance to several stresses in the presence of antibiotics (Jin and Gross 1988) and acid stress (Harden et al. 2015) but the exact mechanism is still unknown. We also found two mutations in the other subunit, *rpoC*, but in this case both mutations were in different regions. Mutations in *rpoC* are rarer in evolutionary studies and have not been studied in detail. These mutations might have the same effect in the DNA clamp and their individual effect will be studied in detail in the future.

The mutation–expression network that we presented here can be used for selecting the most promising mutations for further validation, a key component to any evolution study as the phenotypic assessment of the mutations is expensive and laborious as it can lead to thousands of putative mutations (Tenaillon et al. 2012). This is particularly useful to studies with multiple single stressors and their combinations, like the one presented here. In turn, these studies are important to understand the complex interaction between short and long-term adaptation in environments with multiple stressors, which better resemble what bacterial populations tackle in everyday natural, clinical and industrial environments. Of particular interest would be to expand this study in organisms with food safety relevance, such as *Salmonella enterica* and *Staphylococcus aureus*. In addition, this work paves the way for models that can probabilistically predict mutations based

on the environmental or experimental setting where populations grow.

Materials and Methods

Bacterial Strains and Culture Conditions

E. coli MG1655 and MG1655 Δ*lacZ* strains were used in all experiments conducted. The inclusion of Δ*lacZ* mutants allowed us to perform competition assays using X-Gal and IPTG staining. The neutrality of the Δ*lacZ* mutation was confirmed in all environments in previous work from our laboratory (Dragosits et al. 2013). Minimal M9 salt medium with 0.4% (w/v) glucose as the carbon source was used for cross-stress behavior competition assays. The stresses used were osmotic stress (0.3 M NaCl), acidic stress (pH 5.5), oxidative stress (100 μM H_2O_2), *n*-butanol stress (0.6% *n*-butanol), and control (no-stress).

Competition Assays

Two competing populations (*E. coli* MG1655 and *E. coli* MG1655 Δ*lacZ*) were pre-adapted to M9 overnight and inoculated at an approximate 1:50 ratio at a starting OD_{600} 0.004 in M9 with one stressor and only M9 for reference. After 12 h of growth (early stationary phase), the same quantity of the strain adapted to stress and the strain grown in M9 was diluted in the second condition media (approx. 1:100 dilution) and competed for an additional 12 h. Samples were taken at 12 (time 0) and 24 h (time 1). Four independent biological replicates each with two technical replicates were performed for each individual competition. Cell counts were determined on LB agar plates containing 0.25 mM IPTG (Isopropyl-β-D-1-thiogalactopyranoside) and 40 mg/ml X-gal (bromo-chloro-indolylgalactopyranoside). Plates were incubated overnight at 37 °C. Darwinian Fitness (W) was calculated as described in the supplementary methods, Supplementary Material online, section.

Gene Expression Analyses

Cells were harvested at time 0 and 1. In all, 3ml aliquots were harvested and mixed with 1.5 ml 5% Phenol/ethanol (v/v) and stored at −80 °C until use. RNA was extracted using a RNeasy kit (Qiagen) and after first- and second strand cDNA synthesis, cDNA was broken using Diagenode Bioruptor NGS. End repairing, A tailing, linker ligation and PCR enrichment was made using the KAPA Library Preparation Kit (Kapa Biosystems). Size selection was performed with Agencourt AMPure XP (Beckman Coulter). After quality control, libraries were sequenced by Illumina HiSeq 2500. The low-quality raw reads were trimmed using Trimmomatic (v0.30) with default settings. Trimmed reads were aligned on most recent reference genome of *E. coli* MG1655 by using TopHat (v2.0.10) coupled with bowtie (v1.0.0) (Csardi and Nepusz 2006; Tagkopoulos et al. 2008). The identification of the differentially expressed gene was done as explained in the supplementary methods, Supplementary Material online.

Whole-Genome Re-Sequencing and Mutation Discovery of Evolved Strains

The selected clones from three independent evolutions for each condition (Dragosits et al. 2013) 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, Supplementary Material online.

Mutant Repair

Mutant repair was made as previously described (Fridman et al. 2014). Mutations were repaired by transducing mutations of adjacent genes from the Keio collection (Baba et al. 2006) using phage P1 (Lennox 1955). For a complete list of the genes used see supplementary table S8, Supplementary Material online.

Network Analysis

We used TF-DNA binding data from RegulonDB (Gama-Castro et al. 2008) and protein–protein interaction datasets (Hu et al. 2009; Peregrin-Alvarez et al. 2009) from the Bacterial Protein Interaction database (Goll et al. 2008) 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 the spin-glass model and simulated annealing techniques (Traag and Bruggeman 2009) using *igraph* R package (Csardi and Nepusz 2006).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Author Contributions

V.Z. performed cross-stress experiments, strain characterization, library preparation for genome sequencing, and RNA-Seq and repaired the mutations. M.K. performed the computational analysis. N.R. performed the libraries for the evolved clones. V.Z. and I.T. evaluated data and prepared the manuscript. I.T. conceived of the study and supervised all aspects of the project.

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