ACS Infectious Diseases

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Gene Expression Variability Underlies Adaptive Resistance in Phenotypically Heterogeneous Bacterial Populations

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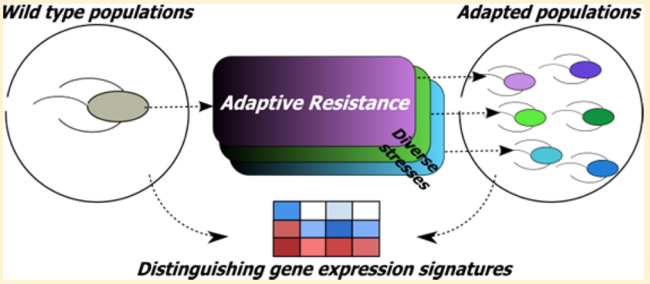
Supporting Information

ABSTRACT: The root cause of the antibiotic resistance crisis is the ability of bacteria to evolve resistance to a multitude of antibiotics and other environmental toxins. The regulation of adaptation is difficult to pinpoint due to extensive phenotypic heterogeneity arising during evolution. Here, we investigate the mechanisms underlying general bacterial adaptation by evolving wild-type *Escherichia coli* populations to dissimilar chemical toxins. We demonstrate the presence of extensive inter- and intrapopulation phenotypic heterogeneity across adapted populations in multiple traits, including minimum inhibitory concentration, growth rate, and lag time. To search for a common response across the heterogeneous adapted populations, we measured gene expression in three stress-response networks: the *mar* regulon, the general stress response, and the SOS response. While few genes were differentially expressed, clustering revealed that interpopulation gene expression variability in adapted populations was distinct from that of unadapted populations. Notably, we observed both increases and decreases in gene expression variability upon adaptation. Sequencing select genes revealed that the observed gene expression trends are not necessarily attributable to genetic changes. To further explore the connection between gene expression variability and adaptation, we propagated single-gene knockout and CRISPR (clustered regularly interspaced short palindromic repeats) interference strains and quantified impact on adaptation to antibiotics. We identified significant correlations that suggest genes with low expression variability have greater impact on adaptation. This study provides evidence that gene expression variability can be used as an indicator of bacterial adaptive resistance, even in the face of the pervasive phenotypic heterogeneity underlying adaptation.



KEYWORDS: *adaptive resistance, stress response, gene expression, antibiotic resistance*

Bacteria are able to rapidly adapt to myriad environmental pressures, a factor which has led to the widespread emergence of drug-resistant and multi-drug-resistant pathogens.[1] Initially susceptible bacterial populations are able to survive and propagate through a nongenetic response known as adaptive resistance.[2] Adaptive resistance has been observed for many conditions across a variety of bacterial species,[3−7] suggesting that adaptive resistance is controlled by an inherent, conserved regulatory response. While a conserved gene expression profile has been observed in eukaryotes in response to multiple stress conditions,[8,9] such behavior remains to be elucidated for adaptive resistance in bacteria.

The extensive phenotypic heterogeneity that underlies evolution is a major obstacle to identifying a signature for bacterial adaptive resistance. Intrapopulation heterogeneity allows adapting populations to sample multiple states, without genetic alteration, in order to maximize the probability of survival. For instance, several studies indicate that stress-response genes tend toward noisy gene expression characteristics.[9−11] Persistence is an example of a "bet-hedging" strategy[12] that leads to increased odds of continued existence. Remarkable diversity in gene expression,[13,14] lag time,[15] and sugar consumption[16] has also been observed to emerge upon exposure to selection pressure within isogenic populations. Additionally, conjugation[17] and growth in the presence of antibiotic[18] have been found to be controlled by bistable feedback regulation, thus enabling phenotypic switching when expression varies sufficiently to cross a threshold. The ubiquity of such diversity-promoting mechanisms supports heterogeneity as a necessary component of adaptation.

While adaptive resistance is generally considered to be a nongenetic response, it has also been recognized that beneficial mutations arise during adaptation and contribute to heritable resistance. While the fixation of spontaneous or stress-induced mutations over many generations obligates a genetic component to interpopulation heterogeneity,[19−21] divergence between populations can also be imposed by nongenetic changes. These include the amplification of intrapopulation stochasticity in gene expression[13] or the influence of epigenetic

Special Issue: Gram-Negative Resistance

Received: June 22, 2015
Published: September 9, 2015

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

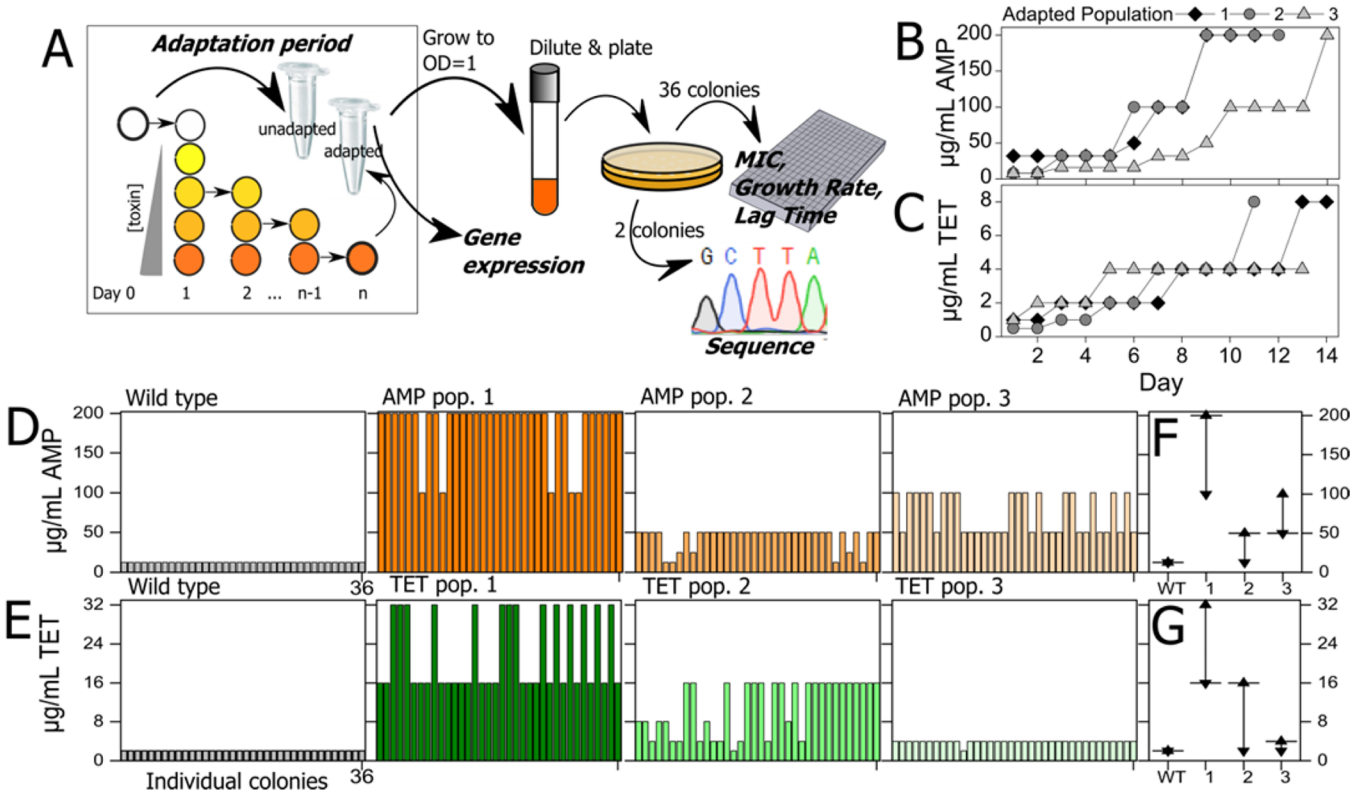


Figure 1. Adaptation protocol and inter- and intrapopulation heterogeneity in MIC. (A) *E. coli* was subjected to increasing levels of stress in the form of a chemical toxin. On day 1, the initial MIC for the toxin of interest was determined. The culture growing at the highest concentration of toxin was propagated by serial dilution for the remainder of the adaptation period. mRNA levels were quantified in samples from the final day of adaptation and day 0 wild-type strains. To measure intrapopulation variability, adapted and unadapted populations were plated and colonies selected for sequencing, MIC, and growth analysis. (B,C) *E. coli* MG1655 was adapted to ampicillin (B) and tetracycline (C). The *x*-axis starts from day 1, and *y*-axis shows the MIC of three independently adapted cultures per toxin on each day, where MIC is defined as 2*x* the toxin concentration at which a culture reaches $OD_{600} \geq 0.5$ after 24 h of growth. (D−G) MIC on LB agar of individual colonies from wild-type and adapted populations ($n = 36$ for all). MIC of ampicillin-adapted populations is shown in (D). MIC of tetracycline-adapted populations is shown in (E). Leftmost panels show wild-type colonies, and additional panels show colonies from each adapted population. (F,G) Distribution in MIC from respective panels to the left. Arrows indicate minimum and maximum values. The horizontal line is the median value.

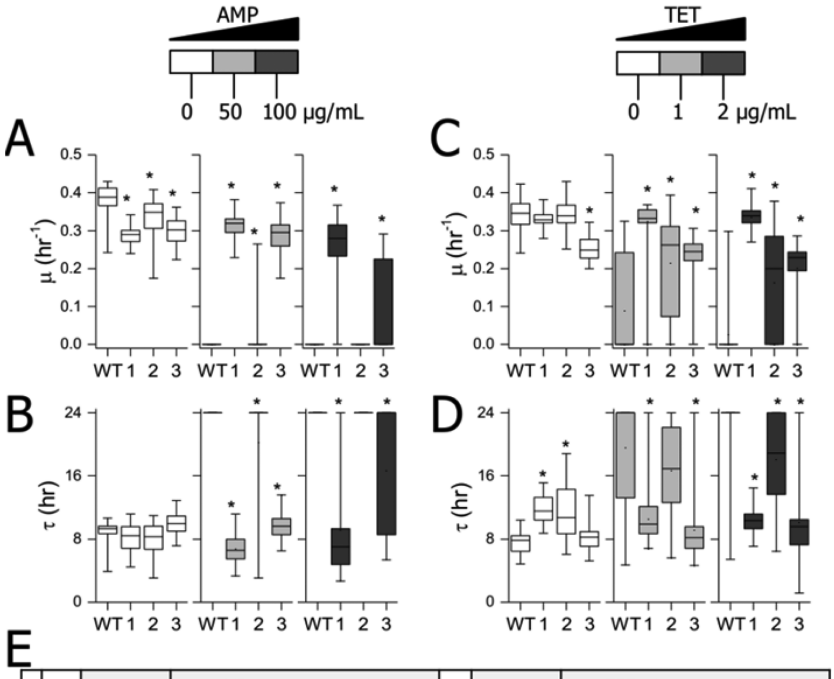
modifications.[22] The intersection of genetic and nongenetic explanations for resistance provides added complexity when attempting to identify signals of adaptation, as gene expression changes can both cause[23,24] and be caused by[25,26] mutations. Furthermore, even genetically diverse populations during long-term adaptation experiments establish the occurrence of gene expression patterns associated with response to environmental pressure.[25] Given that genetic diversity and gene expression heterogeneity can both occur simultaneously during evolution studies, distinguishing general regulators of adaptive resistance is a challenging task.

In this study, we characterize general mechanisms of adaptive resistance by searching for a conserved gene expression response in heterogeneously adapted *Escherichia coli* populations. We provide evidence for intra- and interpopulation heterogeneity at multiple levels during the de novo evolution of resistance. We observe rapid interpopulation divergence in minimum inhibitory concentration (MIC), growth rate, and lag time, and that adapted populations have increased intrapopulation variability when compared to wild-type. Probing population-level gene expression patterns in three stress-response networks (the *mar* regulon, the general stress response, and the SOS response) revealed additional interpopulation variability. We hypothesize that, despite substantial heterogeneity, population-level stress-response gene expression may provide a means by which to distinguish adapted and unadapted bacterial populations and pinpoint key genes involved in adaptive resistance. By measuring expression from a representative set of stress-response genes, we show that interpopulation gene expression variability in adapted populations is dissimilar to that in unadapted populations. Evaluating adaptive performance of strains containing clustered regularly interspaced short palindromic repeats interference (CRISPRi)[27] constructs that perturb stress-response gene expression, as well as performance of single-gene knockout mutant strains,[28] allowed for observation of distinctive relationships between the degree of a gene's expression variability and that gene's involvement in adaptation. These results suggest that interpopulation gene expression variability is a metric by which to identify adapting bacterial strains and realize genes participating in adaptive resistance.

RESULTS AND DISCUSSION

Bacterial Adaptation to Antibiotics Promotes Inter- and Intrapopulation Heterogeneity in MIC, Growth Rate, and Lag Time. To analyze the innate adaptation abilities of bacteria, wild-type *E. coli* strain MG1655 was evolved in the laboratory against two broad-spectrum antibiotics with different modes of action: ampicillin and tetracycline. Dissimilar antibiotics were selected in order to investigate a general

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567



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	Media	WT	AMP adapted populations 1	2	3	Media	WT	TET adapted populations 1	2	3
μ	0	0.38±0.04	0.29±0.02	0.33±0.06	0.30±0.03	0	0.34±0.04	0.33±0.02	0.34±0.04	0.25±0.03
	50	0±0	0.31±0.04	0.04±0.07	0.29±0.04	1	0.09±0.13	0.32±0.06	0.22±0.14	0.23±0.06
	100	0±0	0.24±0.12	0±0	0.10±0.11	2	0.03±0.08	0.34±0.03	0.16±0.13	0.20±0.08
τ	0	8.9±1.3	8.1±1.8	7.9±2.1	9.9±1.4	0	7.5±1.3	11.7±1.7	11.±3.6	8.2±1.6
	50	24±0	6.8±1.9	20±7.4	9.7±1.7	1	19.6±6.9	10.5±3.1	16.7±5.9	9.1±4.3
	100	24±0	9.2±7.0	24±0	16.7±7.9	2	22.7±4.0	10.5±1.8	18.1±5.3	10.2±4.9

Figure 2. Interpopulation and intrapopulation heterogeneity in growth characteristics of adapted populations. (A,B) Colonies from each ampicillin-adapted population were regrown in media with no toxin, 50 μg/mL, or 100 μg/mL of ampicillin. (C,D) Colonies from tetracycline-adapted populations were regrown in media with no toxin, 1 μg/mL, or 2 μg/mL of tetracycline. Growth rate, μ (A,C), and lag time, τ (B,D), are presented for adapted and wild-type populations in the same concentrations of toxin. Growth parameters significantly different ($P < 0.05$) than those of wild-type in the same condition are indicated with $*$. Box plots show the interquartile range (IQR) for $n = 36$ colonies from each population. The median is indicated with a horizontal line. Whiskers depict the minimum and maximum value. (E) Average growth rate and lag time from (A–D), with standard deviation.

adaptive response and not a response to a specific selection pressure. While ampicillin is a bactericidal β-lactam antibiotic that impairs cell wall formation,[29] tetracycline inhibits protein synthesis by blocking ribosome–tRNA interactions.[30] Three biological replicates were adapted to each toxin for a medium-term time period of 11–14 days, intended to mimic a standard antibiotic course[31] (Figure 1A; see Methods). A medium-term adaptation period also mitigates certain complications associated with short- or long-term adaptation. For instance, long-term adaptation experiments spanning months have successfully isolated resistant mutants,[7,32,33] but these mutant populations are highly enriched. On the other hand, short-term exposure (minutes to hours) leads to a larger number of gene expression changes, many of which appear to be nonspecific to an adaptive response.[34] Additionally, short-term exposure periods may point to mechanisms of cell death[35,36] rather than avenues of successful adaptive resistance.

Samples were collected at the beginning and the end of the adaptation period. Cultures isolated on the final day of the adaptation period are henceforth referred to as ampicillin- or tetracycline-adapted populations 1, 2, and 3. We observed that biological replicates exposed to the same antibiotic treatment demonstrated rapid divergence in adaptation rates, as exemplified by the MIC of the adapting populations (Figure 1B,C). Initially, the ampicillin-adapted populations had MICs of 8–32 μg/mL of ampicillin (day 1). After up to 14 days of propagation, all ampicillin-adapted populations demonstrated MICs increasing to 200 μg/mL of ampicillin. Similarly, cultures adapted to tetracycline possessed initial MICs of 0.5–1 μg/mL of tetracycline and reached a MIC of 4–8 μg/mL of tetracycline at the end of the adaptation period.

To characterize interpopulation and intrapopulation heterogeneity, we picked 36 colonies from each adapted population for MIC (Figure 1D–G) and growth analysis (Figure 2). Average MICs of ampicillin-adapted populations 1, 2, and 3 were 186.11 ± 35.07, 43.75 ± 13.19, and 73.61 ± 25.00 μg/mL of ampicillin, which were higher and more diverse compared to a MIC of 12.5 ± 0.00 μg/mL of ampicillin for wild-type populations (Figure 1D). Similarly, average MICs of tetracycline-adapted populations 1, 2, and 3 were 22.22 ± 7.91, 16.28 ± 5.54, and 3.94 ± 0.33 μg/mL of tetracycline compared to 2.00 ± 0.00 μg/mL of tetracycline for wild-type populations (Figure 1E). One-way ANOVA with Bonferroni test was used to establish that the MICs for each set of adapting populations were significantly different from one another (significance level set at $P < 0.05$). Furthermore, we found that the wild-type

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555–567

population displayed a very consistent degree of resistance, with all colonies exhibiting the same MIC of 12.5 μg/mL of ampicillin (Figure 1D) and 2 μg/mL of tetracycline (Figure 1E). In contrast, a wide range of MIC was observed within all of the adapted populations. Colonies from ampicillin populations 1, 2, and 3 had MICs ranging between 100 and 200 μg/mL, 12.5−50 μg/mL, and 50−100 μg/mL of ampicillin, respectively (Figure 1F). Colonies from tetracycline populations 1, 2, and 3 exhibited MICs ranging between 16 and 32 μg/mL, 4−16 μg/mL, and 2−4 μg/mL of tetracycline, respectively (Figure 1G). This difference in the spread of the data shows that the MIC for the adapted populations is more heterogeneous both within as well as across populations than the wild-type population.

The divergence in MIC led us to investigate the growth rate and lag time of adapted populations as another indicator of heterogeneity. We grew the selected colonies at two sub-MIC levels of antibiotic for 24 h. Significant interpopulation and intrapopulation differences in growth rate and lag time were observed, further highlighting the phenotypic divergence in adapted populations (Figure 2A−D). One-way ANOVA with Bonferroni tests (significance at $P < 0.05$) was used to compare growth across wild-type and adapted populations. Without selection pressure, all of the ampicillin-adapted populations demonstrated an average specific growth rate and/or lag time that was significantly different than that of the wild-type (Figure 2E). Additionally, each ampicillin-adapted population exhibited an average growth rate and/or lag time in the presence and the absence of selection pressure that was significantly different from that of the other ampicillin-adapted populations in the same condition. As in MIC, we observed different ranges of growth rates and lag time in the presence of ampicillin for the adapted populations, especially in ampicillin population 3, which demonstrated the largest interquartile range both in growth rate (0−0.23 h^{-1}) and lag time (8.5−24 h) in 100 μg/mL of ampicillin. Interestingly, a unique strategy is evident in ampicillin-adapted population 2, which when taken as a whole was able to grow at 100 μg/mL of ampicillin (Figure 1B), while few (8 out of 36) of the individual colonies were able to survive even in 50 μg/mL of ampicillin (Figure 2A,B). This indicates that ampicillin population 2 could be using a mutualism strategy, one that requires the contribution of multiple players for survival. Comparable cooperative single-species populations have been observed to emerge in evolution experiments[37] and have also been created synthetically.[38]

Similar to ampicillin-adapted populations, tetracycline-adapted populations also exhibited significantly different average growth rate and/or lag time in every condition in the presence of tetracycline (Figure 2C,D). Although the average growth rates of tetracycline-adapted populations 1 and 2 were not significantly different in media without selection pressure, population 2 demonstrated intrapopulation heterogeneity higher than that of tetracycline populations 1 and 3 in both growth rate and lag time, as shown by the spread of the interquartile range (Figure 2C,D).

These results demonstrate significant divergence in MIC and in growth parameters within and across populations in clinically relevant time spans, a key issue to consider when searching for antibiotic targets. To further study heterogeneity in the adapted bacterial populations, we examined stress-response gene expression, hypothesizing that patterns in gene expression would provide insight into general mechanisms of adaptive evolution.

Interpopulation Variability in Gene Expression across Antibiotic-Adapted Strains. We measured the dominant mRNA levels for a representative set of 14 stress-response genes in each of the adapted and unadapted populations. Genes selected are associated with at least one of three potential adaptive resistance pathways: the multiple antibiotic resistance (*mar*) regulon, the general stress response, or the SOS response. We measured expression of five genes associated with the *mar* regulon: *marA*, *rob*, *soxS*, *acrA*, and *tolC*. MarA is the activator of the *mar* phenotype, which confers increased tolerance to drugs and solvents by increasing efflux activity, decreasing porin expression, and regulating metabolism, among other activities.[39] One multidrug efflux pump positively controlled by MarA is AcrAB-TolC, known for playing a role in conferring resistance to multiple toxins.[40] MarA binds to sequences of DNA called "mar-boxes", located upstream of the promoters of regulated genes. Two other regulatory proteins, Rob and SoxS, also bind to the mar-box, generating a large network of genes that can be induced in response to different stress conditions.[41]

To determine if the general stress response was active, we measured expression of *rpoS*, *mutS*, *cyoA*, and *hfq* genes. The general stress response plays a role in activating mutagenic DNA repair[42] and is regulated by the sigma factor RpoS. More than 30 proteins have been shown to act upstream to trigger RpoS activity.[43] We evaluated gene expression of two of these upstream proteins, CyoA, a subunit II of the cytochrome *bo* terminal oxidase complex,[44] and Hfq, a small RNA chaperone.[45] Recently, it was demonstrated that the MutS enzyme in the methyl-directed mismatch repair system is down-regulated via an RpoS-controlled sRNA, *sdsR*, increasing mutation rates in stressed bacteria.[24]

Additionally, we assessed expression of five genes involved in the SOS response: *lexA*, *recA*, *dinB*, *polB*, and *dam*. The SOS response is induced to repair double-stranded DNA breaks.[46] The enzyme LexA binds to SOS-box promoter regions, effectively repressing SOS response genes at a transcriptional level.[47] RecA activates the SOS response by inducing autolytic cleavage of DNA-bound LexA[47] and also participates in repairing DNA strand breaks by stabilizing single-stranded DNA and catalyzing strand exchange.[48] Some downstream constituents of the SOS response include *polB*[49] and *dinB*,[50] which code for the DNA polymerases PolII and PolIV, respectively. PolII functions as a DNA polymerase and has a proof-reading exonuclease; thus, mutants defective in exonuclease function are found to have increased mutation rates.[51] PolIV is an error-prone DNA polymerase that is also regulated by RpoS.[50,52] Loss of the DNA−adenine methyltransferase (Dam) results in activation of SOS response genes,[53] potentially due to an increase in DNA damage from random mismatch repair.

Normalized gene expression in each adapted population was calculated by comparison of fold change normalized to reference gene expression and average fold change across three wild-type populations (see eqs 4 and 5 in Methods). Consistent with the heterogeneity observed in MIC and growth, we again noted considerable differences between the dominant gene expression patterns in each antibiotic-adapted population (Figure 3A,B). Interestingly, while expression of some genes was found to be highly variable across populations, other genes demonstrated consistent up- or down-regulation across all adapted populations or across antibiotics. Both ampicillin- and tetracycline-adapted populations demonstrated

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

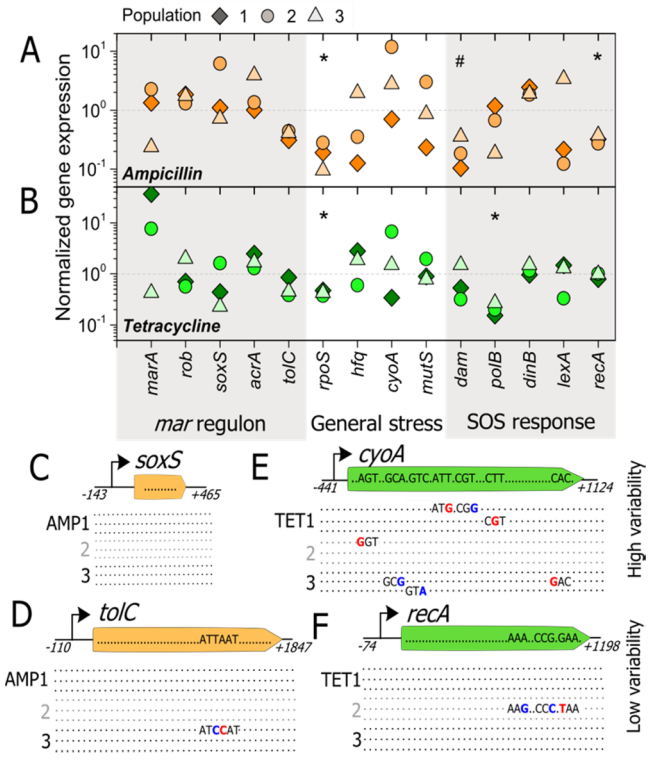

Figure 3. Variable expression of stress-response genes in ampicillin- and tetracycline-adapted populations. (A,B) Normalized expression of stress-response genes for ampicillin- (A) and tetracycline-adapted (B) populations (calculated as described in Methods). The horizontal line at 1 marks expression equal to wild-type. Significant differential expression is denoted with ∗ for $P < 0.05$ and # for $P < 0.10$. (C−F) Genes expressed with high and low interpopulation variability were sequenced. (C) *soxS* and (D) *tolC* were sequenced in three colonies from each ampicillin-adapted population (indicated by AMP1, 2, 3). (E) *cyoA* and (F) *recA* were sequenced in three colonies from each tetracycline-adapted population (indicated by TET1, 2, 3). Regions sequenced for each gene are indicated with respect to +1 of the most upstream promoter. Sequence is shown only for codons with mutations. Single-nucleotide polymorphisms are indicated in either red (nonsynonymous) or blue (synonymous). Dots indicate no change with respect to wild-type.

high interpopulation variability in expression of *marA*, *soxS*, *hfq*, *cyoA*, and *lexA* genes. *marA* transcript levels in tetracycline-adapted cultures were found to be more than 30-fold up-regulated in one population and 6-fold down-regulated in another (Figure 3B). In contrast, *rob*, *tolC*, *dinB*, and *recA* were tightly expressed across ampicillin-adapted populations, and *acrA*, *rpoS*, *polB*, *dinB*, and *recA* were tightly expressed across tetracycline-adapted populations. Within these transcripts, we found *rpoS*, *recA* ($P = 0.01$ for both), and *dam* ($P = 0.10$) to be significantly down-regulated in ampicillin-adapted populations (Figure 3A) and *rpoS* and *polB* ($P = 0.02$ for both) to be down-regulated in tetracycline-adapted populations (Figure 3B).

Genes with High and Low Expression Variability Demonstrate Interpopulation Sequence Heterogeneity. It is well-established that gene expression changes can result from either genetic[25,54] or nongenetic[4,14,55] mechanisms. To ascertain whether the interpopulation variability observed in gene expression can be attributed to mutations, we examined genes expressed with higher and lower degrees of interpopulation gene expression variability. We sequenced *soxS* (high variability) and *tolC* (low variability) genes in three colonies from each of the three ampicillin-adapted populations (Figure 3C,D) and *cyoA* (high variability) and *recA* (low variability) genes in three colonies from each of the three tetracycline-adapted populations (Figure 3E,F). We found no mutations in promoter or upstream regions from any colony (all 36 colonies) in any of the four genes sequenced, which indicates that interpopulation gene expression variability here does not stem from mutations impacting promoter binding affinity. Interpopulation gene expression variability could result from other factors, including epigenetic changes, stochasticity of transcription factor binding, or mutations in *trans* regulatory regions.

We examined the open reading frame in order to evaluate whether mutations were present that could influence protein function and enable selection for differential regulation. Though *soxS* was expressed with high interpopulation variability, there were no mutations in *soxS* in any sequenced population (Figure 3C). Ampicillin-adapted populations 1 and 2 did not have any mutations in *tolC*, though one of three colonies in population 3 was found to contain two adjacent single-nucleotide polymorphisms (SNPs), a synonymous I356I and a nonsynonymous N357H (Figure 3D). As the population-level expression of *tolC* in ampicillin population 3 was similar to that in populations 1 and 2 (Figure 3A), these SNPs did not have a measurable impact on *tolC* gene expression. In tetracycline populations, a number of SNPs were located in *cyoA*, although none of the mutations were shared between multiple colonies nor was the same codon mutated in any two colonies (Figure 3E). In *cyoA*, we did not observe a consistent trend between the number of mutations found in a population and the impact on population-level gene expression. For instance, tetracycline population 2 had only one mutation in one of three colonies but, overall, had the highest increase in gene expression with respect to wild-type (Figure 3B). For *recA*, only one of the nine colonies contained mutations: a synonymous K311K, a synonymous P336P, and a non-synonymous E351stop in one colony from tetracycline population 2 (Figure 3F). As discussed for *tolC*, the *recA* gene expression level in tetracycline population 2 was similar to that in tetracycline populations 1 and 3, so again, we see that the mutations observed in one colony did not impact population-level gene expression. Thus, in general, we do not observe a correlation between expression variability and the number of mutations in a given gene, as out of the higher variability genes, *soxS* had no mutations while *cyoA* had many mutations. We also find in *recA* and *tolC* that mutations do not prevent gene expression from being tightly controlled. These results taken together emphasize that gene expression variability is not readily explained by gene sequence and underscores the challenge of identifying general attributes of bacterial adaptation.

Interpopulation Variability in Gene Expression in the Presence of Alternate Toxins. To further probe if interpopulation variability in mRNA levels is related to adaptive resistance, we expanded the study to observe *E. coli* MG1655 populations exposed to alternate chemical toxins: biofuel compounds *n*-hexane and *n*-butanol. While it has been shown previously that *E. coli* K12 derivatives are intrinsically resistant to *n*-hexane via AcrAB-TolC efflux,[56] *n*-butanol is known to be toxic to *E. coli*, disturbing cellular respiration, metabolism, and transport, as well as activating the *mar* regulon, the oxidative stress response, the membrane stress response, and the heat shock response.[57] As expected, *E. coli* growth was not hindered

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

in 5 or 10% v/v *n*-hexane (Figure S1), while *E. coli* did not gain resistance to more than 1% v/v *n*-butanol (Figure S2). Analogous to ampicillin- and tetracycline-adapted populations, independently adapted populations in *n*-butanol demonstrated heterogeneous initial and transient MICs. However, the MIC of the *n*-butanol-adapted populations was found to be less divergent than that of the antibiotic-adapted populations, likely due to a high degree of selection as a result of the particularly disruptive nature of *n*-butanol stress.

We measured gene expression in populations adapted to *n*-butanol or grown in 10% v/v *n*-hexane (Figure 4A,B). In *n*-butanol-adapted cultures, *hfq* (P = 0.02), *lexA* (P = 0.04), and *rpoS* (P = 0.06) were differentially under-expressed, while *recA* (P = 0.01) was differentially overexpressed. Again, few genes were tightly expressed, while others were variable across the individually adapted populations. In *n*-butanol cultures, *tolC*, *rpoS*, *hfq*, and *recA* gene expression was less variable across the adapted populations, while the most variable genes were *cyoA* and *soxS* (Figure 4B). In contrast, none of the genes was significantly under-expressed in the *n*-hexane populations, though *rob* (P = 0.08) and *soxS* (P = 0.09) were overexpressed (Figure 4B). *cyoA* expression had the lowest variability across the *n*-hexane populations, a striking contrast to the high expression variability in *cyoA* across populations adapted to ampicillin, tetracycline, or *n*-butanol. Other genes with lower variability in *n*-hexane included *rob*, *soxS*, and *recA*, while *lexA*, *marA*, and *dinB* had the largest interpopulation variability.

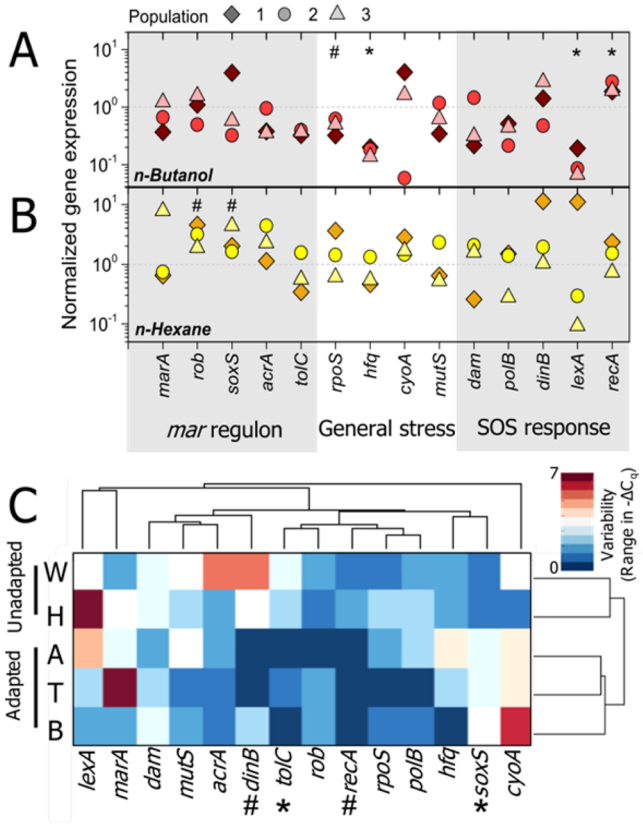

Figure 4. Gene expression variability is an indicator of adaptation. Normalized expression of 14 stress-response genes is shown for *n*-butanol-adapted populations (A) and populations grown with *n*-hexane (B). Normalized gene expression was calculated as described in the Methods. The horizontal line at 1 marks expression equal to wild-type. Significant differential expression is denoted with ∗ for $P < 0.05$ and # for $P < 0.10$. (C) Heatmap values indicate the interpopulation range of gene expression ($-\Delta C_{q,avg}$) from each of the five growth conditions (shown on vertical axis): wild-type (W), adapted to ampicillin (A), tetracycline (T), or *n*-butanol (B), or grown with *n*-hexane (H). Hierarchical clustering is based on Euclidean distance. Significantly different expression variability between unadapted and adapted populations is indicated with ∗ for $P < 0.05$ and # for $P < 0.10$.

Differential Gene Expression Variability Is an Indicator of Adaptation. We postulated that the degree of interpopulation transcript variability in adapted (ampicillin-, tetracycline-, and *n*-butanol-adapted populations) when compared to unadapted populations (wild-type and *n*-hexane populations) might signify a response associated with adaptive resistance. To compare expression variability across adapted populations, we employed hierarchical clustering as well as principal component analysis (PCA). The interpopulation range of gene expression (range of $-\Delta C_{q,avg}$) was used as the variability metric (eq 4). Notably, the unadapted and adapted populations were divided into separate clusters using hierarchical clustering (Figure 4C). Among the adapted populations, the tetracycline- and ampicillin-adapted populations were closest together. Separation of unadapted and adapted populations according to gene expression variability was also observed using PCA (Figure S3).

Hierarchical clustering highlights patterns in gene expression variability that separate adapted and unadapted populations. Importantly, we find that in adapting populations while few genes exhibit increased interpopulation gene expression variability, few other genes demonstrate decreased interpopulation gene expression variability with respect to wild-type (Figure S4) or unadapted hexane population (Figure 4C). As a result, the overall expression variability pattern across the set of genes is fundamentally different for adapted versus unadapted populations. Interestingly, several genes were significantly differentially variable in adapted conditions versus unadapted conditions, perhaps signifying a stress-specific adaptive resistance mechanism. Genes undergoing significant reductions in gene expression variability upon adaptation suggest that a specific, small range of expression levels is selected for even in divergent-adapted populations under different stress conditions. One of the most variable genes in the wild-type samples was *dinB*, for which variability significantly decreased in adapted cultures (P = 0.07). Similarly, for *tolC* and *recA*, the adapted populations displayed tighter expression than the unadapted populations (P = 0.02 and 0.08, respectively). In contrast, *soxS* expression variability was significantly higher across adapted populations than across wild-type and hexane-treated strains (P = 0.01), signifying that a high range of expression levels is tolerable for adaptation.

Clustering also allowed identification of groups of genes exhibiting similar trends in variability. For example, *lexA* and *marA* were grouped separately from the bulk of the genes and were relatively more variable in *n*-hexane-, tetracycline-, and ampicillin-adapted samples. Another noteworthy grouping contained *tolC*, *rob*, *recA*, *rpoS*, and *polB*, which were generally expressed with lower interpopulation variability. Previous studies have observed that several of these low-variability genes play a direct role in survival to diverse antibiotics, for instance, *tolC* in the presence of ciprofloxacin, sulfamethoxazole, metronidazole, and ampicillin[58] and *recA* in the presence of ciprofloxacin, sulfamethoxazole, metronidazole norfloxacin, ampicillin, and kanamycin.[35,58] Additionally, in *E. coli*, *Pseudomonas aeruginosa*, and *Vibrio cholera*, inhibition of *rpoS*

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

was shown to decrease the frequency of resistant colonies upon exposure to ampicillin.[24] Finally, identifying differences between the adapted populations from each condition may provide insight into stress-specific mechanisms of adaptive resistance. When we compare the tetracycline- and ampicillin-adapted populations, *marA* expression was relatively more variable across tetracycline-adapting strains than ampicillin-adapting strains, while *lexA*, *polB*, and *mutS* were more variable across ampicillin cultures.

Gene Expression Variability Impacts Adaptation Ability. To further scrutinize the relationship between expression variability and adaptation, we applied the synthetic CRISPRi system[27] to manipulate expression of seven genes: *marA*, *acrA*, *tolC*, *dinB*, *soxS*, *recA*, and *mutS*. CRISPRi blocks transcription via interference from the deactivated RNA-guided DNA endonuclease, dCas9, which binds to DNA in a region specified by the 20 nt sequence of a single-guide RNA (sgRNA) (Tables S1 and S4 and Figure S5). The sgRNA sequences were designed to guide dCas9 to an NGG PAM sequence in the open reading frame for each target gene. Plasmids expressing the sgRNA and dCas9 protein were co-transformed into *E. coli* MG1655. A sgRNA plasmid-targeting red fluorescent protein (RFP), which is not present in *E. coli* MG1655, was also transformed as a control strain. The CRISPRi strains were grown in media without or with 1 μg/mL of tetracycline for a period of 3 days to evaluate the impact of gene perturbation on adaptation.

To quantify adaptation ability, we calculated an adaptation factor ($\alpha_{m,c}$), which describes the average change in growth rate upon gene perturbation relative to the control ($r_{m,c}$) and relative to the initial growth rate (on day 1) upon gene perturbation ($s_{m,c}$) (eqs 1−3). The subscripts "m" and "c" denote the CRISPRi strain and adaptation condition (either tetracycline or no toxin), respectively. $r_{m,c}$ was calculated as the sum of relative change between the growth rate of the specific CRISPRi strain (μ_m) and control strain (μ_w) under the same selection condition as on days 2 and 3 of the adaptation experiment (eq 2). $s_{m,c}$ was calculated as the relative change between the growth rate of the CRISPRi strain on days 2 and 3 with respect to day 1 (eq 3). Positive α indicates that the CRISPRi strain adapted well; a negative α indicated that the strain adapted poorly, and α close to zero indicates that adaptation was not impacted (Figure S6). The magnitude of the adaptation factor ($|\alpha|$) is thus used as a gauge of the absolute impact of a gene perturbation on adaptation, as the perturbation could impact adaptation either positively or negatively.

$$\alpha_{m,c} = \frac{r_{m,c} + s_{m,c}}{2} \tag{1}$$

$$r_{m,c} = \frac{1}{2}\left[\left(\frac{\mu_{m,c,\text{day 2}} - \mu_{w,c,\text{day 2}}}{\mu_{w,c,\text{day 2}}}\right) + \left(\frac{\mu_{m,c,\text{day 3}} - \mu_{w,c,\text{day 3}}}{\mu_{w,c,\text{day 3}}}\right)\right] \tag{2}$$

$$s_{m,c} = \frac{1}{2}\left[\left(\frac{\mu_{m,c,\text{day 2}} - \mu_{m,c,\text{day 1}}}{\mu_{m,c,\text{day 1}}}\right) + \left(\frac{\mu_{m,c,\text{day 3}} - \mu_{m,c,\text{day 1}}}{\mu_{m,c,\text{day 1}}}\right)\right] \tag{3}$$

We compared the $|\alpha|$ for the CRISPRi strains to the interpopulation range of gene expression from adapted populations by calculating linear fits and Pearson correlation coefficients. Using an F-test, we show that the fits for unadapted and adapted conditions are statistically different (Figure 5A). When the strains are not under stress (unadapted condition), α values are all close to zero, indicating that mutant strains do not have inherently compromised fitness (Figure 5B). We also find no definite correlation between $|\alpha|$ and gene expression variability in the unadapted condition (Figure 5B, Pearson's r = −0.16 with P = 0.73). Contrastingly, CRISPRi strains adapted to tetracycline demonstrated a negative correlation (Figure 5C, Pearson's r = −0.53 with P = 0.23), which hints that genes with low expression variability during adaptation to tetracycline have greater impact on adaptation to tetracycline selection pressure. To further scrutinize the relationship between adaptation and gene expression variability, we extended the analysis to establish whether similar correlations were present in related *E. coli* strains.

A

Strain	Condition	Slope	Pearson's r	Prob > F
CRISPRi	Unadapted	-0.005±0.012	-0.164	$P = 7\text{x}10^{-4}$
	Adapted	-0.112±0.081	-0.526	
KEIO	Unadapted	-0.008±0.021	-0.110	$P = 0.080$
	Adapted	-0.092±0.040	-0.420	

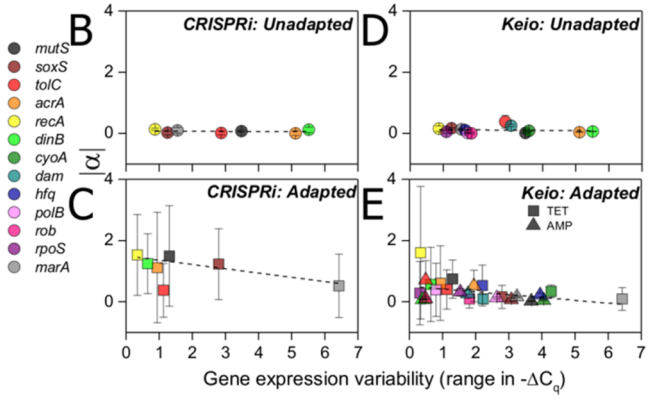

Figure 5. Genes with low expression variability have a greater impact on adaptation. (A) Parameters from linear fits shown as dashed lines in B−E, including slope with standard error, Pearson's r, and the probability value from an F-test comparing the unadapted and adapted fits. (B−E) Comparison of gene expression variability (interpopulation range in $-\Delta C_{q,\text{avg}}$) to the magnitude of the adaptation factor ($|\alpha|$) of corresponding CRISPRi (B,C) or single-gene knockout (D,E) strains. Y-error bars are the SD of n = 3 (CRISPRi) or n = 4 (knockout) biological replicates grown either without (B,D) or with (C,E) antibiotic selection pressure. Square and triangle symbols denote selection pressure of tetracycline and ampicillin, respectively. Each point represents one gene/strain pair.

Mutant single-gene knockout strains derived from *E. coli* BW25113[28] strains were grown in minimal medium with (4 μg/mL of ampicillin or 2 μg/mL of tetracycline) or without antibiotics to evaluate the improvement in growth rate for four replicate populations over 3 days of propagation. We calculated α for the mutant strains using eqs 1−3, with *E. coli* BW25113 as the control strain. Similar to the CRISPRi strains, the linear fits for unadapted and adapted strains were significantly different (Figure 5A). Again, when strains are grown without stress, α values are close to zero, and there is no clear relationship between adaptation and gene expression variability (Figure 5D, Pearson's r = 0.11 with P = 0.72). Notably, the magnitude of the adaptation factor and amount of gene expression variability are significantly negatively correlated for adapted knockout strains (Figure 5E, Pearson's r = −0.42 with P = 0.03), once

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

more, indicating that genes with a smaller range of expression have a greater impact on adaptive capability. The significance of negative correlation holds even if either of the points at the extreme end of the distribution is removed: Δ*marA* in tetracycline ($r = -0.42$ with $P = 0.04$) or Δ*recA* in tetracycline ($r = -0.40$ with $P = 0.05$). Moreover, we observe that the replicate populations with genes lower in expression variability knocked out tended to demonstrate greater heterogeneity in growth, resulting in greater variability (SD) in $|\alpha|$ (Figure 5E). This is consistent with our observation that greater heterogeneity in growth is observed during adaptation (Figure 2). Indeed, gene expression variability was also found to be significantly negatively correlated with the interpopulation variability (SD) in $|\alpha|$ for adapted knockout strains (Pearson's $r = -0.46$, $P = 0.02$) but not in unadapted strains (Pearson's $r = -0.16$, $P = 0.60$).

Summary and Conclusions. In the face of pervasive heterogeneity, it is challenging to discern key genes that enable the progression of adaptive resistance. In this report, we describe the magnitude of intra- and interpopulation heterogeneity at multiple levels, including MIC, growth rate, lag time, gene expression, and gene sequence. Our findings support previous observations that resistance has both genetic[19,25,54] and nongenetic[2,14,55] components, further obfuscating general signatures of adaptive resistance. Here, we sought to ascertain whether distinguishing gene expression patterns would emerge from the heterogeneous adapted populations and found that stress-response genes demonstrated varying degrees of interpopulation gene expression variability. The degree of heterogeneity is highlighted by the lack of synergy between expression of upstream regulators and downstream target gene expression. For instance, the transcription factor MarA promotes transcription of *acrA* and *tolC*, but we observed that in some adapted populations (e.g., ampicillin population 3, tetracycline populations 2 and 3) *marA* and either *acrA* or *tolC* mRNA registered opposite changes in expression. The lack of a clear relationship in expression levels can possibly be attributed to redundancy in these networks, as *tolC* is also regulated by SoxS, Rob, and RpoS in addition to MarA and other factors not assessed in this study.[59,60]

The multiple intersections of regulation in stress-response networks render their corresponding gene expression patterns difficult to interpret. However, while high expression variability was observed for some genes, several genes were expressed at a consistent level across adapted populations. Furthermore, clustering based on interpopulation gene expression variability enabled us to separate adapted populations from unadapted populations, indicating that the degree of variability has biological relevance. Others have shown a correlation between the number of mutations in a coding region and the magnitude of gene expression changes,[61] but here we found that mutations were not predictive of interpopulation gene expression variability, which suggests that both genetic and nongenetic changes underlie the progression of resistance. The implication of interpopulation gene expression variability on adaptive resistance was further analyzed by adapting CRISPRi strains and single-gene knockout mutants to antibiotics. Significantly different trends were found to relate adaptation and gene expression variability in adapted versus unadapted populations. Especially, in single-gene knockout mutants, we noted a negative correlation, whereby knocking out stress-response genes with low interpopulation variability in expression (e.g., *tolC*, *recA*, *dinB*) was more likely to impact adaptation (either positively or negatively), while knocking out genes with high interpopulation variability in expression (e.g., *marA*, *soxS*, and *cyoA*) had less impact on adaptation.

The value of using gene expression variability to identify key players in adaptive resistance is evident when attempting to specifically attribute a transcriptional response to any one regulatory hub. For instance, *rpoS*, the sigma factor responsible for activating the general stress response, had consistently decreased expression in all ampicillin-, tetracycline-, and *n*-butanol-adapted populations (Figure 3A,B and Figure 4A). Others have shown that knocking out *rpoS* increases persistence,[62] which can promote temporary tolerance gains. Hence, it appears that reduced expression of *rpoS* is part of a natural adaptive response. As adapting strains are growing at the same time scale as wild-type and executing increases in MIC, in this instance, persistence is not the mechanism of survival. *dinB*, encoding DNA polymerase IV, is activated by *rpoS*,[52] so conceivably, decreases in *rpoS* RNA could lead to decreases in *dinB*, though this was not evident in the gene expression data (Figure 3A,B and Figure 4A). Likewise, *rpoS* represses *mutS*,[63] but increased *mutS* expression was also not readily observed in the adapted populations. Finally, *tolC*, which is induced by RpoS,[60] did show more than 2-fold decrease in expression in 8 out of 9 adapted populations, consistent with lower *rpoS* expression levels. Three day propagation of mutant strains revealed that both *rpoS* and *tolC* impact adaptation to antibiotics (Figure 5). Δ*rpoS* strains propagated in ampicillin had compromised adaptation (average $\alpha = -0.30$, Figure S6), while the same strain in tetracycline showed increased adaptation capabilities ($\alpha = 0.27$). Δ*tolC* strains had limited adaptation in ampicillin ($\alpha = -0.70$) and tetracycline ($\alpha = -0.39$), while CRISPRi−*tolC* strains in tetracycline had increased adaptation ability ($\alpha = 0.69$). Therefore, it seems that slight reductions in *tolC* expression, mediated by *rpoS* or enacted synthetically by CRISPRi, are advantageous for adaptation to ampicillin and tetracycline, while complete knockout of *tolC* hinders adaptation. While future studies are needed to explain the advantage of decreased *tolC* expression, or why knocking out *rpoS* has variable impact on adaptation to different antibiotics, here we demonstrate that these genes can be identified as targets of interest purely by measuring their gene expression variability. Gene expression variability in both *rpoS* and *tolC* was low across adapted populations (Figure 4C), which we propose can be used as an indicator that these genes are important for adaptation.

We postulate that a range of mRNA levels is selected for during adaptation, and as such, the observed expression variability provides a measure of a gene's participation in adaptive resistance. Genes with low and high variability in expression likely play different roles in long-term population survival. For instance, an analysis of yeast transcriptome data noted that genes serving as network hubs are more likely to exhibit low expression variability than genes with fewer interactions.[64] Similarly, our results suggest that genes with low expression variability directly promote resistance to a certain toxin, as manipulating these genes was more likely to impact adaptation. Supporting this claim, reductions in gene expression variability from negative feedback control have been previously observed in a number of biological systems, including in *E. coli* chemotaxis[65] and in synthetic circuits in yeast.[66] Additionally, differentiation in mammalian and plant cells has been linked to fine-tuning of expression by microRNAs.[67,68] In the adapted populations studied here, the

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

genes exhibiting high expression variability do not appear to be directly providing resistance to antibiotics, as repressing or knocking out these genes generally had less impact on adaptation. However, these genes may contribute to population survival by allowing cells to sample distinct physiological states and prepare for changing environmental conditions. Higher gene expression variability may provide an advantage in that cells can explore different adaptation strategies when stressed without the need for mutations. For example, variation in bistable or multistable feedback structures can promote bet-hedging by generating heterogeneous subpopulations[12,55] or can enable cells to cycle through multiple expression states.[69,70] Especially considering that we observe intrapopulation heterogeneity in MIC and growth, we acknowledge that individual cells within each population may have obtained different solutions. Single-cell gene expression studies will provide further insight into intrapopulation distributions of stress-response gene expression and enable correlations between interpopulation and intrapopulation gene expression variability.

Biological relevance of differential gene expression variability has been proposed by others. Studies in complex organisms including *Homo sapiens* report similar variability or "dynamism" at the tissue level that contributes to various disease states.[71,72] Recently, differential gene expression variability was observed during adaptive resistance to antisense therapeutics.[73] In this report, differential gene expression variability may indicate the presence of a regulatory structure that is active during adaptive resistance, though each gene's range of expression could be altered by a multitude of phenomena, including pulsatile dynamics,[70] multistability,[74,75] or divergent adaptation at the genomic level.[19,76] Thus, gene expression variability is a powerful metric to signify the occurrence of adaptive resistance, as it provides valuable information without the need to deconvolute intersecting stress-response network regulation, pinpoint specific mutations, or eradicate pervasive phenotypic divergence. Additional examination of the range of gene expression within and across adapted populations will help to illuminate the basis and rationale for differential variability in stress-response and other networks.

METHODS

Bacterial Strains, Media, and Culture Conditions. *E. coli* K-12 strain MG1655 (ATCC 700926) was used as the wild-type in adaptation experiments and the host strain for harboring CRISPRi[27] synthetic constructs. Keio collection mutants and parent strain (*E. coli* BW25113, the wild-type for knockout mutant studies) were purchased from Yale's Coli Genetic Stock Center (http://cgsc.biology.yale.edu/index.php). All strains and plasmids used are listed in Table S1. All adaptation cultures were grown in M9 minimal media with 0.4% glucose and toxin (ampicillin, tetracycline, *n*-butanol, or *n*-hexane) as indicated. Keio collection mutants were also grown in M9 minimal media (consisting of 5X M9 minimal media salts solution from MP Biomedicals, 2.0 mM $MgSO_4$, and 0.1 mM $CaCl_2$ in sterile water) with 0.4% glucose. Cells harboring CRISPRi constructs were grown in LB media (Sigma-Aldrich) supplemented with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) to maintain plasmid selection. Construct expression was induced via supplementation with anhydrous tetracycline (aTc) (10 ng/mL). Colonies of MG1655 wild-type strain were grown on LB agar plates. Colonies of Keio mutants were grown on LB agar plates supplemented with kanamycin (50 μg/mL). Colonies harboring CRISPRi constructs were grown on LB agar plates supplemented with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL).

Adaptation of *E. coli* MG1655 Strains, Culture Conditions, and Sample Collection. Wild-type and *n*-hexane samples were obtained by growing *E. coli* MG1655 to $0.5 \leq OD_{600} \leq 1.0$ in M9 minimal media with 0.4% glucose (and with 10% v/v hexane for *n*-hexane samples). OD_{600} was obtained on a Nanodrop 2000 (Thermo Scientific) using 2 μL of culture. Initially, 5 mL of overnight cultures was inoculated from three individual colonies of the wild-type MG1655 strain grown at 37 °C with 225 rpm shaking in M9 minimal media with 0.4% glucose (day −1). Portions of the triplicate wild-type cultures grown to exponential phase in minimal media were stored for transcriptome analysis (day 0). We defined MIC as 2*x* the concentration of toxin that allows culture density of $OD_{600} > 0.5$ after 24 h of growth. The initial MIC of *E. coli* MG1655 was determined by splitting the day 0 wild-type MG1655 cultures in minimal media with a range of toxin concentrations (Table S2). The culture growing to $OD_{600} > 0.5$ at the highest concentration of toxin after overnight growth was selected for continued evolution. This culture was repeatedly diluted 1:5 to 1:100 (depending on culture density) to $OD_{600} \sim 0.1$ in fresh media every 1−3 days into new cultures containing the toxin at the current and higher concentrations. Between splits and at least every 24 h, medium was refreshed by either diluting the culture 1:5−1:100 in new media (if $OD_{600} \geq 0.5$) or spinning down cells and resuspending in new media (if $OD_{600} < 0.5$) to maintain toxin pressure and nutrient supply. Evolution was continued for at least 11 days until bacterial populations were able to grow at four times the initial MIC or until the populations exhibited no improvement in tolerance for seven continuous days. Samples for qPCR were collected on the final day of adaptation when culture at the highest toxin concentration was in exponential phase, $0.5 \leq OD_{600} \leq 1.0$. To store samples for RNA extraction, 0.5 mL of culture was added to 1 mL of RNAprotect bacterial reagent (Qiagen), incubated at room temperature for 5 min, and centrifuged at 4000 rpm for 10 min. Pellets were flash frozen in ethanol and dry ice and stored at −80 °C until RNA extraction. Freezer stocks were prepared by centrifuging 0.5 mL of culture for 5 min at 4000 rpm and then suspending in LB + 50% glycerol. Three independent biological replicates were adapted to each toxin. Adaptation exposure conditions for each population are included in Table S2.

Adapted Population Growth and MIC. Adapted and wild-type populations were inoculated from glycerol stocks into M9 minimal media, grown to $OD_{600} = 1$, diluted 1:10000 in sterile water, and 10 μL was plated onto LB agar. After 16 h of growth at 37 °C, 36 colonies were picked and suspended in 20 μL of sterile water. For growth curves, 1 μL of each colony suspension was used to inoculate 50 μL cultures in a 384-well plate. Each colony from the wild-type and ampicillin-adapted populations was grown in media with no toxin, 50, or 100 μg/mL of ampicillin. Colonies from wild-type and tetracycline-adapted populations were grown in media with no toxin, 1, or 2 μg/mL of tetracycline. For MIC assessment of individual colonies, 1 μL of each colony suspension was spotted onto LB agar plates across a range of concentrations of toxin (0, 6.25, 12.5, 25, 50, 100, 200 μg/mL of ampicillin and 0, 0.25, 0.5,1, 2, 4, 8, 16, 32 μg/mL of tetracycline). The MIC was estimated as the lowest concentration of antibiotic at which no growth was observed on solid media after 24 h at 37 °C. All growth curves

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

in this study were obtained with a Tecan GENios plate reader (Tecan Group Ltd.) with Magellan software version 7.2. Absorbance was read at 562 nm and 37 °C every 20 min, with shaking between measurements. Growth curve parameters were calculated using the program GrowthRates.[77]

Sequencing of Stress-Response Genes. Three colonies from each adapted population and two from the wild-type population were picked from LB agar plates. Colony PCR was performed using Phusion high-fidelity DNA polymerase (New England Biolabs) to amplify a continuous region for sequencing, which included all annotated promoter regions and upstream transcription factor binding sites, the open reading frame, and at least 80 nt downstream of the open reading frame. PCR primers are included in Table S3. Bands were cut and purified with a Zymoclean gel DNA recovery kit (Zymo Research). Concentration was verified with a Nanodrop 2000. Sequencing primers 19−20 nt in length were designed to bind at least every 700 bp along the region of interest (Table S3). Primers were 45% GC content with a melting temperature of 50−60 °C with G or C at the 3′ end. Sequencing was performed by Quintarabio. SnapGene software version 2.7.2 (GSL Biotech LLC) was used for alignment. Sequences were aligned to the wild-type sequences as well as the *E. coli* K12 MG1655 NCBI reference genome NC_000913.3. No mutations were noted in wild-type sequences.

CRISPRi Design and Plasmid Assembly. Single-guide RNA (sgRNA) plasmids (PBO.002-PBO.008) were derived from the RFP-targeting control plasmid pgRNA (Addgene plasmid 44251). Primers were designed to replace the 44251 plasmid's RFP-targeting sgRNA using a common reverse primer flanked with an ApaI restriction site and unique forward primers flanked with a SpeI restriction site (Table S4). PCR with Phusion high-fidelity DNA polymerase (New England Biolabs) was used to amplify these new target sgRNA insert DNA fragments, which were subsequently gel-purified (Zymoclean gel DNA recovery kit, Zymo Research), digested with ApaI and SpeI (FastDigest enzymes, Thermo Scientific), and PCR-purified (GeneJET PCR purification kit, Thermo Scientific). The 44251 plasmid backbone was also digested with ApaI and SpeI and gel-purified, and T4 DNA ligase (Thermo Scientific) was used to ligate the new sgRNA target inserts into the 44251 backbone. Ligations were transformed into chemically competent *E. coli* MG1655 cells harboring dCas9 (Addgene plasmid 44249). Plasmid minipreps were performed using Zyppy plasmid miniprep kit (Zymo Research). Sequencing of final sgRNA constructs was performed for validation of correct assembly (GENEWIZ). Repression was verified with qPCR (Figure S5).

Adaptation of *E. coli* MG1655 Harboring CRISPRi Constructs. Three individual colonies were picked from selective plates (LB agar + ampicillin + chloramphenicol). Colonies were used to inoculate 100 μL of LB cultures supplemented with ampicillin, chloramphenicol, and aTc and grown to stationary phase. From each of these cultures, 2 μL was used to inoculate two 50 μL LB cultures supplemented with ampicillin, chloramphenicol, and aTc (and one supplemented with 1 μg/mL of tetracycline) in a 384-well microplate. After 24 h of growth, 2 μL of these cultures was diluted into fresh media. This process was repeated for a total of 3 days of growth.

Adaptation of *E. coli* BW25113- and BW25113-Derived Single-Gene Knockout Mutants. Four individual colonies from each strain were picked from selective plates (LB agar + kanamycin). Colonies were suspended in 50 μL of M9 minimal media with 0.4% glucose, out of which 1 μL of each colony suspension was used to inoculate 20 μL cultures (in media only or media with 4 μg/mL of ampicillin or 2 μg/mL of tetracycline) in a 384-well microplate. Concentration of antibiotic was selected based on the MIC of 1 mL of *E. coli* BW25113 cultures in M9 minimal media. After 24 h of growth, cultures were diluted (1:40 to 1:10, depending on the absorbance recorded) into fresh media with antibiotic as indicated. Cultures were diluted twice for a total of 3 days of growth.

RNA Extraction and Purification. For transcript expression from adapted populations, cell pellets from the samples at the end of each adaptation period were resuspended in TE buffer supplemented with lysozyme and proteinase K. For CRISPRi verification, overnight cultures of cells harboring CRISPR inhibition constructs grown in M9 minimal media supplemented with 0.4% glucose, ampicillin, and chloramphenicol were inoculated with 10 ng/μL of aTc for 3 h, pelleted, and resuspended in TE buffer supplemented with lysozyme and proteinase K. RNA was extracted from all samples using the GeneJET RNA purification kit (Thermo Scientific). DNA contamination was removed with the TURBO DNA-free kit (Ambion). RNA concentrations and A_{260}/A_{280} ratios were obtained with a Nanodrop 2000 (Thermo Scientific). Extracted and purified RNA was stored at −80 °C in nuclease-free water.

RT-qPCR. The DyNAmo SYBR Green two-step RT-qPCR kit (Thermo Scientific) was used to synthesize cDNA and to prepare RT-qPCR reactions. In 10 μL reactions, total RNA (50−100 ng) was reverse-transcribed with 2 μL of M-MuLV RNase H+ reverse transcriptase (RT) and 300 ng of random hexamers in a Biorad T100 thermocycler. A no-RT control was included with each set of cDNA reactions. RT was carried out for 10 min at 25 °C, followed by 30 min at 37 °C, and 5 min at 85 °C. cDNA was diluted to 1 ng/μL with nuclease-free water and stored at −20 °C. Each qPCR reaction contained 1−2 ng of template cDNA, 0.5 μM forward and 0.5 μM reverse primer, 0.2 μL of 50x ROX dye, 5 μL of MasterMix containing SYBR green and modified *Tbr* DNA polymerase, and nuclease-free water to 10 μL total volume. Two technical replicates were included for each sample cDNA/primer combination. The qPCR cycling program was 15 min at 95 °C for polymerase activation followed by 40 cycles of denaturing at 94 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. No-RT controls were included to verify that DNA contamination was negligible. Melting curves were generated after cycling was completed by holding for 15 s at 95 °C, 15 s at 55 °C, then ramping to 95 °C and holding for 15 s. Data shown in this report were obtained on an Eco Illumina RT-qPCR, located in the CU Core Sequencing Facility.

RT-qPCR Primer Design. Gene-specific RT-qPCR primers were designed using Primer-BLAST.[78] Sequences of open reading frames were obtained from the *E. coli* K12 MG1655 NCBI reference genome NC_000913.3. Primers were selected to be 20−22 nt in length with a less than 200 nt amplicon for each primer pair (Table S5). Integrated DNA Technology and Invitrogen supplied the primers, with standard desalting. Primer specificity was verified by running no template controls and melting curves, as well as gel electrophoresis of RT-qPCR products. Primer amplification efficiency was found to be in the range of 96 ± 10% by running standard curves with template cDNA dilutions prepared from total RNA.

DOI: 10.1021/acsinfecdis.5b00095
ACS Infect. Dis. 2015, 1, 555−567

Gene Expression and Gene Expression Variability Analysis. C_q values were obtained from Eco Software version 4.1.2.0. The average of two technical replicates (per biological replicate) was used to calculate all ΔC_q values. Cycle numbers differed by 0.3 for typical technical replicates. Due to the divergent nature of the adaptation experiments, we calculated fold change in gene expression with respect to four reference genes: *gyrA*, *hcaT*, *rrsA*, and *cysG* (eq 4). Reference genes were selected from literature.[79−81] The average C_q for the four reference genes together exhibited a lower standard deviation than the average C_q across all genes, indicating that the reference genes were generally more stable than an average gene. The heatmap and dendrograms were generated using the clustergram function with a Euclidean distance metric, unweighted average linkage function, and optimal leaf ordering[82] in MATLAB's Bioinformatics Toolbox (The Mathworks, Inc., Natick, MA). Normalized gene expression of mRNA expression was calculated per the $2^{-\Delta\Delta C_q}$ method,[83] per eq 5. For adapted populations, gene expression for each gene of interest was normalized with respect to four reference genes and three wild-type strains (m = 3) by taking the geometric mean of the $2^{-\Delta\Delta C_q}$ calculated for each gene of interest (goi), reference gene (ref), and wild-type strain (wt) pairing,[84] as shown in eq 5, where m = 3. For verification of inhibition using CRISPRi constructs, normalized gene expression was calculated for individual biological triplicates as in eqs 4 and 5, using *rrsA* as a reference gene (Figure S5). P values were obtained using two-tailed, type two t tests.

$$\Delta C_{q,goi,avg} = \frac{1}{n}\sum_{i=1}^{n}\left(C_{q,goi} - C_{q,ref(i)}\right) \tag{4}$$

$$NRQ_{goi} = \left(\prod_{j=1}^{m} 2^{\Delta C_{q,goi,avg,wt(j)} - \Delta C_{q,goi,avg,sample}}\right)^{1/m} \tag{5}$$

Linear Fitting. Slope and Pearson correlation coefficients for gene expression variability (range in $-\Delta C_q$) versus $|\alpha|$ were calculated using linear fits with no weighting (OriginPro software, version 9.1.0).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00095.

Tables and figures referred to in the main text (PDF)

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by NIH Pharmaceutical Biotechnology training fellowship (Grant No. 5T32GM008732) to K.E.E., and William M. Keck Foundation (Grant No. OCG6145B) and University of Colorado start-up funds to A.C.

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DOI: 10.1021/acsinfecdis.5b00095
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