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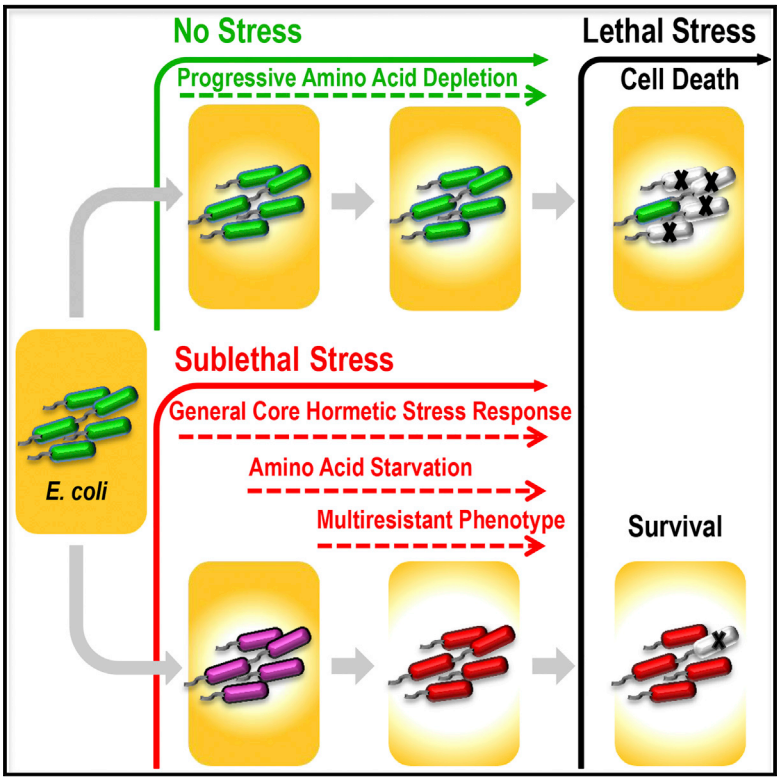
Discovery and Function of a General Core Hormetic Stress Response in *E. coli* Induced by Sublethal Concentrations of Antibiotics

Graphical Abstract



Highlights

- Sublethal stresses induce a general core hormetic stress response
- Energy metabolism and translation are increased by sublethal antibiotic treatments
- Antibiotic-treated cells deplete amino acids faster than untreated cells
- Antibiotic-treated cells induce the stringent response earlier than untreated cells

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In Brief

Mathieu et al. report that sublethal antibiotic treatments induce a general core hormetic stress response, which endows bacteria with capacity to survive different subsequent lethal stresses. It is plausible that this response plays an important role in the robustness of bacteria exposed to antibiotic treatments and constant environmental fluctuations in nature.

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Discovery and Function of a General Core Hormetic Stress Response in *E. coli* Induced by Sublethal Concentrations of Antibiotics

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SUMMARY

A better understanding of the impact of antibiotics on bacteria is required to increase the efficiency of antibiotic treatments and to slow the emergence of resistance. Using *Escherichia coli*, we examined how bacteria exposed to sublethal concentrations of ampicillin adjust gene expression patterns and metabolism to simultaneously deal with the antibiotic-induced damage and maintain rapid growth. We found that the treated cells increased energy production, as well as translation and macromolecular repair and protection. These responses are adaptive, because they confer increased survival not only to lethal ampicillin treatment but also to non-antibiotic lethal stresses. This robustness is modulated by nutrient availability. Because different antibiotics and other stressors induce the same set of responses, we propose that it constitutes a general core hormetic stress response. It is plausible that this response plays an important role in the robustness of bacteria exposed to antibiotic treatments and constant environmental fluctuations in natural environments.

INTRODUCTION

Our ability to treat infectious diseases with antibiotics is rapidly decreasing because of the massive, often inappropriate, use of antibiotics and the high capacity of bacteria to survive treatment and evolve resistance. Clearly, a better understanding of the impact of antibiotics on bacteria is required to increase the efficiency of treatments and to slow the emergence of resistance. It is generally believed that clinically relevant antibiotic resistance appears as a consequence of bacterial exposure to concentrations above the minimum inhibitory concentration (MIC) of antibiotics. For this reason, the impact of sub-MICs of antibiotics on bacterial populations has been neglected. However, growing evidence indicates that sub-MICs of antibiotics may play an important role in the emergence and spread of antibiotic resistance (Andersson and Hughes, 2014; Laureti et al., 2013). For example, concentrations of antibiotics up to several hundredfold below the MIC of susceptible bacteria can enrich the population of resistant bacteria (Gullberg et al., 2011). Sub-MICs of antibiotics can also stimulate the horizontal transfer of antibiotic resistance determinants (Laureti et al., 2013) and increase the mutation rates in different bacterial species (Baharoglu and Mazel, 2011; Gutierrez et al., 2013). Increased mutation rates are important for the de novo generation of antibiotic resistance (Denamur and Matic, 2006). Finally, sub-MICs of antibiotics can induce a state of persistence, which endows bacteria with the capacity to survive lethal doses of antibiotics (Dörr et al., 2009; Goneau et al., 2014).

The importance of studying the impact of sub-MICs of antibiotics on bacteria is emphasized because they are frequently exposed to low doses of antibiotics in their habitats. First, antibiotics are produced at low doses by bacteria and fungi in almost all environments. Second, during therapeutic treatment or antibiotic use for growth promotion, pharmacokinetic factors create gradients of antibiotic concentrations in compartmentalized human, animal, and plant bodies. Third, because the intestine poorly absorbs antibiotics, 30%–80% of ingested antibiotics are excreted unchanged in the urine and feces (Pletz et al., 2004). Consequently, active antibiotics end up in soil, lakes, and rivers, as well as in food products.

Antibiotics target essential cellular macromolecules and consequently perturb cellular physiology and induce cell death (Belenky et al., 2015). Transcriptome analysis of dying *Escherichia coli* cells treated with lethal concentrations of ampicillin or norfloxacin showed that cells induce stress responses in vain efforts to repair damage; decrease synthesis of nonessential proteins, energy metabolism, and transport of small molecules; and suppress cell division and growth (Kaldalu et al., 2004). Similar transcriptional reprogramming was observed in *E. coli* cells killed by an overproduction of restriction endonucleases (Asakura and Kobayashi, 2009) or when cells were exposed to heat (45°C) or oxidative shock (hydrogen peroxide, or H_2O_2) (Jozefczuk et al., 2010). These responses to severe stresses

are consistent with the hypothesis that a universal effect of stress on cells is the cessation of growth, which preserves energy and redirects it to maintenance and repair (Kültz, 2005). However, cells exposed to sublethal stresses, such as the sub-MIC of antibiotics, do not stop growing and are not killed. This suggests that they are able to adjust their gene expression patterns and metabolism to simultaneously respond to the antibiotic-induced stresses and maintain rapid growth. Thus far, we know little about the molecular mechanisms underlying these bacterial responses. Furthermore, an emphasis on the therapeutic potential of antibiotics has resulted in a paucity of studies related to impacts they may have on bacteria other than the evolution of antibiotic resistance. For example, some stress responses induced by sub-MICs of antibiotics, such as the *E. coli* RpoS-dependent general stress response (Gutierrez et al., 2013), provide resistance to many types of stresses that are unrelated to the inducer stress (Battesti et al., 2011).

In this study, we examined how *E. coli* maintains its rapid growth rate during chronic exposure to the sub-MICs of antibiotics. We first used the β-lactam ampicillin as a model antibiotic. The β-lactams interfere with the synthesis of the peptidoglycan layer, which provides structural strength to the cell and balances osmotic pressure in gram-negative and gram-positive bacteria cells. Consequently, treatment with the β-lactam antibiotics results in a loss of cell wall integrity and ultimately in cell lysis (Yao et al., 2012). We found that exposure to the sub-MIC of ampicillin resulted in adjustments in the gene expression profiles and metabolism. We also compared the response to ampicillin treatment with the responses induced by other antibiotics. In doing so, we identified a set of cellular functions and metabolic modifications—in particular, amino acid catabolism, the translation apparatus, and energy metabolism—that are stimulated by sub-MICs of different antibiotics independent of their mechanism of action. We also demonstrated that the exposure to the sub-MIC of ampicillin has hormetic effects; i.e., treated cells acquired increased tolerance to different lethal stresses, including lethal ampicillin treatment. Therefore, given the ubiquitous presence of sublethal concentrations of antibiotics in natural environments, they may play an important role in the robustness of bacterial populations that are exposed to constant environmental fluctuations.

RESULTS

Gene Expression Profiles of Ampicillin-Treated Cells in Different Growth Phases

To evaluate how the sub-MIC of ampicillin affects gene expression, we used a library of 1,920 *E. coli* strains carrying transcriptional fusions of *gfp* to different promoters (Zaslaver et al., 2006). In contrast to DNA microarrays or RNA sequencing (RNA-seq) methods, this methodological approach allows high-temporal resolution measurement of promoter activity in living cells. Cells were treated, or not, with 1 μg/mL of ampicillin, which corresponds to 50% of the MIC for *E. coli* strain MG1655 cultivated in liquid Luria-Bertani (LB) medium. The growth curves showed two distinct phases (Figure 1A): phase 1, during which both populations grow at almost the same rate, and phase 2, representing the last two to three cell doublings, during which the growth of the treated population slows relative to the untreated control. We found that 64 promoters were significantly and differentially expressed during growth in the presence of ampicillin relative to the untreated controls (Table 1; Figure S1). We clustered the identified promoters, first as a function of the growth phase in which the specific regulation starts and second according to the regulation type observed, i.e., higher or lower than the untreated control. Most of these promoters were upregulated, i.e., 26 of 27 and 21 of 37, during the first and the second growth phases, respectively.

The functions of the genes (EcoCyc *E. coli* database, http://ecocyc.org/) whose expression was identified as being specifically altered by ampicillin provided information on the temporal changes in the physiological states of the treated cells (Table 1). During the first growth phase, the genes that were induced include the *mscS* gene, which codes for a mechanosensitive channel; the *msbB*, *fabR*, *pldB* and *yfeY* genes, which are involved in membrane synthesis; and *htpX*, which is involved in the degradation of misfolded proteins in the periplasm, suggesting that the treated cells attempt to repair the ampicillin-induced damage of the cell wall. We also observed that the *msrB* gene coding for methionine sulfoxide reductase, which may help to repair oxidized methionines in damaged proteins, was induced. In addition, we found increased reactive oxygen species (ROS) production and induction of genes known to be activated by the ROS, such as *sodA*, *soxS*, *ahpC*, and *oxyR* (Lu et al., 2005), in treated cells (Figure S2). Finally, many genes regulated during ampicillin treatment are controlled by the RpoS (σ^S or σ^{38}), RpoE (σ^{24}), and RpoH (σ^{32}) alternative sigma factors. Induction of these stress regulons was confirmed using *lacZ*-based reporter fusions (Tables 2 and S3). These results show that cells exposed to 50% MIC of ampicillin activated a variety of stress responses early during growth.

In addition, we found that the *rpsM* promoter, which drives expression of an operon encoding four ribosomal proteins and the RNA polymerase (RNAP) α subunit, was induced in treated cells (Table 1; Figure S1A). The *ptsG*, *manX*, and *mtfA* genes that encode proteins for carbohydrate uptake were upregulated. The *mlc* gene, coding for a transcriptional repressor of the *ptsG* and *manX* genes, was less expressed, while the *mtfA* gene that codes for the *mlc* titration factor was upregulated, which is consistent with an increase in carbohydrate uptake (Figure 1F; Table 1). Furthermore, the *deoC* gene, which encodes a protein involved in nucleoside degradation to carbon and energy sources, was upregulated. The *arcD* and *tnaCAB* operons encoding proteins involved in amino acid uptake and degradation were induced. The *lpd*, *acnB*, and *sdhC* genes coding for tricarboxylic acid (TCA) cycle enzymes were also induced. Finally, *adk*, *ubiX*, *erpA*, and *ugpB*, which are genes involved directly or indirectly in ATP production, were upregulated (Figure 1F). Most previously mentioned genes were induced during the first growth phase, during which there was no difference between the growth curves of the treated cultures and those of the untreated cultures (Figure 1A; Table 1). All these results suggest that treated cells react to ampicillin-induced stress by increasing translational capacity and energy production before growth is affected.

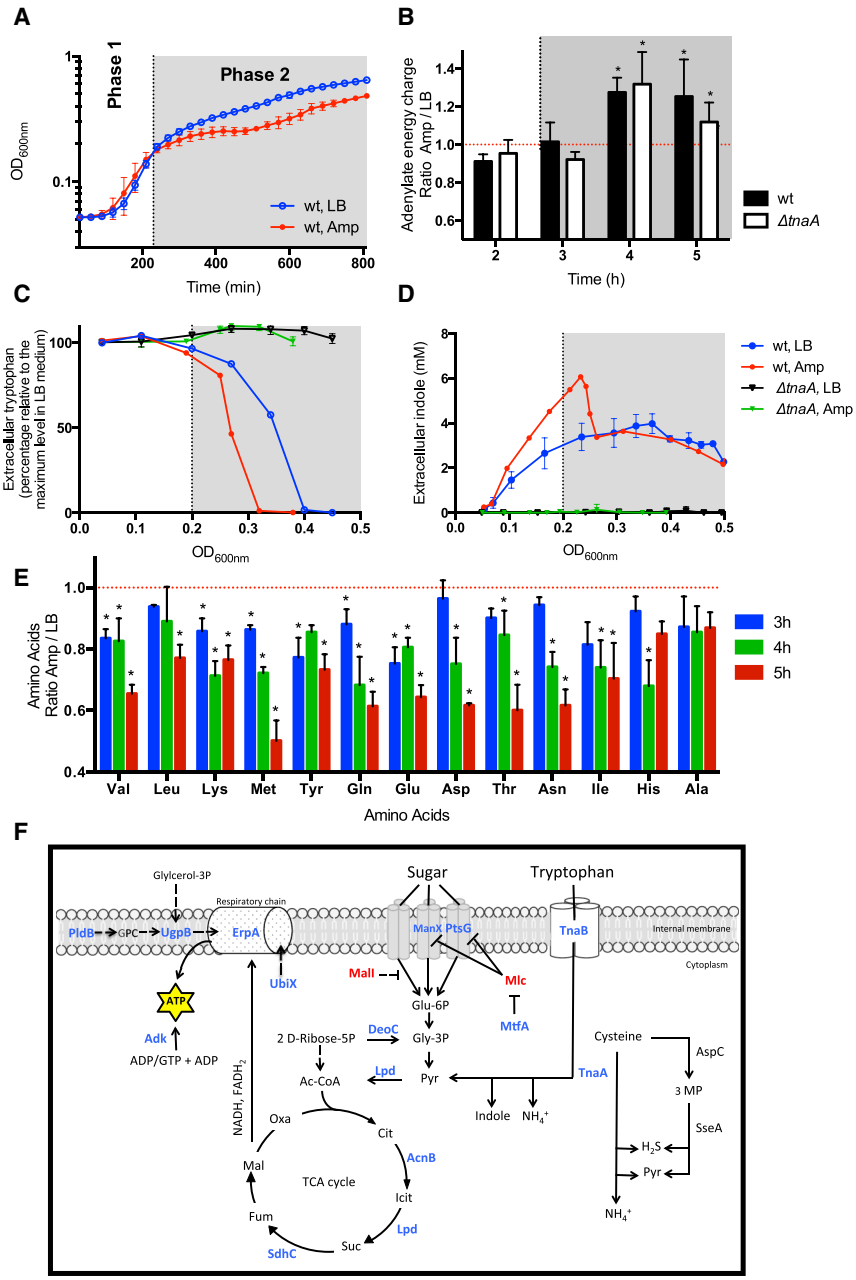

Figure 1. Growth, Energetic Metabolism, Tryptophan Catabolism, and Amino Acid Quantification of Cells Exposed to 50% MIC of Ampicillin

(A) Growth kinetics. Phase 1: period during which both populations grew at the same rate. Phase 2: period after the growth shift, during which the growth of the treated population slows relative to the untreated control.

(B) Bars represent the ratio between AEC values of treated (Amp) and untreated (LB) cells.

(C) The quantity of extracellular tryptophan is presented as a percentage of the maximum level in fresh LB medium.

(D) Extracellular indole.

(E) The intracellular concentration of 13 amino acids. Bars represent the ratio between treated (Amp) and untreated (LB) cells.

(F) Schematic representation of the energy metabolism modifications of cells exposed to 50% MIC of ampicillin relative to untreated cells based on the expression profiles obtained using the promoter library (Table 1). Proteins encoded by genes whose promoters were upregulated are in blue. Proteins encoded by genes whose promoters were downregulated are in red. Sugars, amino acids, and metabolites are in black. Indirect actions are indicated by broken lines.

E. coli strains are MG1655 (WT) and the isogenic derivative Δ*tnaA*. LB, liquid LB medium; Amp, LB with 50% MIC of ampicillin. The vertical dashed lines indicate the time of the growth shift for treated relative to untreated cells. Data are presented as the mean (±SD) values from at least three independent experiments. An asterisk indicates treated versus untreated conditions, t test, *$p < 0.05$.

Energy Metabolism and Translation Are Increased in Ampicillin-Treated Cells

The gene expression analysis suggests that cells exposed to 50% MIC of ampicillin increased the energy metabolism (Figure 1F; Table 1). We quantified ATP, ADP, and AMP using high-performance liquid chromatography (HPLC) in wild-type (WT) cells during growth with or without ampicillin and calculated the adenylate energy charge (AEC) using the Atkinson equation: ([ATP] + 1/2[ADP]) / ([ATP] + [ADP] + [AMP]). AEC is a measure of the amount of metabolic energy transiently stored in the adenine nucleotide pool (Chapman et al., 1971). The AEC ratio of treated and untreated WT cells increased over time (Figure 1B; Table S1). This result is consistent with an increase in energy production for treated cells that was suggested by the gene expression data (Figure 1F; Table 1).

In growing *E. coli* cells, approximately 50% of the energy is consumed by the translational machinery (Russell and Cook, 1995). Therefore, we sought to determine whether ampicillin-treated cells, which produce more energy, have a higher ribosome content. We observed increased expression of the *rpsM*

Next, to determine whether this gene regulation modulation is specific to ampicillin, we tested whether these 64 genes were also differentially regulated during growth with 50% MIC of the aminoglycoside gentamicin or the quinolone norfloxacin (Figure 2). We found that 62.5% and 51.5% of these genes were differentially regulated during growth with gentamicin and norfloxacin, respectively, while the expression of 39% of these genes was significantly affected by all three antibiotics. Among these 39% of genes regulated by all three antibiotics, we found genes belonging to the pathways that appeared to be important in mediating the cell response to the sub-MIC of ampicillin, such as energy metabolism and translational capacity (Figure 2; Table 1).

Table 1. Gene Promoters Differentially Expressed in Cells Exposed to 50% MIC of Ampicillin Relative to Untreated Controls

Phase 1	
Gene (27)	Function
Upregulated (26)	
arcD	putative APC transporter
deoC[a,b]	2-deoxyribose-5-phosphate aldolase, NAD(P)-linked
fabR	DNA-binding transcriptional repressor
glgS[b,c]	surface composition regulator
htpX[b,d]	heat shock protein, predicted endopeptidase
manX[a,b]	mMannose PTS permease
msbB[b,c]	myristoyl-acyl carrier protein (ACP)-dependent acyltransferase
mscS[c]	mechanosensitive channel
msrB[d]	methionine sulfoxide reductase B
pldB[a,d]	lysophospholipase L(2)
ptsG[a]	glucose PTS permease
rpsM[b]	30S ribosomal subunit protein S13
rsd[b,c]	regulator of sigma D
slyD[c]	FKBP-type peptidyl prolyl cis-trans isomerase
tnaC[a]	tryptophanase leader peptide
ubiX[a,b]	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
yahM[b,e]	predicted protein
ybiH	predicted DNA-binding transcriptional regulator
ychH[b,f]	stress-induced protein
yebO[b]	conserved hypothetical protein
yfeY[b,e]	predicted outer membrane lipoprotein
ygdR[b]	conserved hypothetical protein
yggE[b,c]	conserved protein
yggM	conserved protein
yobF	small protein involved in stress responses
ytfB[b]	predicted cell envelope opacity-associated protein
Downregulated (1)	
yheO	predicted DNA-binding transcriptional regulator
Phase 2	
Gene (37)	Function
Upregulated (21)	
acnB[a]	aconitate hydratase 2
adk[a]	adenylate kinase
cysP[b]	sulfate/thiosulfate ABC transporter—periplasmic binding
entF	apo-serine activating enzyme
erpA[a]	essential respiratory protein A
lpd[a,c,f]	lipoamide dehydrogenase
mdoB[b]	phosphoglycerol transferase I
mtfA[a,e]	mlc titration factor
phoP	response regulator
prfB[b,e]	peptide chain release factor RF-2
pyrG[b]	CTP synthetase
sdhC[a,b]	succinate dehydrogenase, cytochrome b556
ssb	ssDNA-binding protein controls activity of RecBCD nuclease

Table 1. *Continued*

Phase 2	
Gene (37)	Function
Upregulated (21)	
ugpB[a,b,c]	glycerol-3-phosphate ABC transporter—periplasmic binding
ybdL	putative PLP-dependent aminotransferase (first module)
ycjX[d,f]	conserved protein
ydfH[b]	DNA-binding transcriptional repressor
ydjN[b]	L-cystine/L-cysteine transporter
yedP[b]	predicted phosphatase
ygaZ[b]	L-valine efflux transporter
yhhQ[b]	conserved inner membrane protein
Downregulated (16)	
clpX[d,e]	ATP-dependent protease specificity component and chaperone
evgA[b,c]	transcriptional regulator
kdtA	KDO transferase
mall[a]	DNA-binding transcriptional repressor
mazE	antitoxin of the MazF-MazE toxin-antitoxin system
mlc[a,d]	transcriptional repressor for glucose uptake and glycolysis
nlpI[b]	lipoprotein involved in cell division
pheL	leader peptide of chorismate mutase-P-prephenate dehydratase
pyrB[b]	aspartate carbamoyl transferase
tig[b]	peptidyl-prolyl cis/trans isomerase (trigger factor)
ybaP[e]	conserved protein
ybiA[d]	conserved protein
ydiV[b,c,e]	anti-FlhDC factor
yeeX[b]	conserved protein
yfiF	putative tRNA/rRNA methyltransferase
yniA[b,e]	predicted phosphotransferase/kinase

Growth phase 1, during which both populations grow at the same rate, and growth phase 2, during which the growth of the treated population slows relative to the untreated control (Figure 1A). See also Figures S1 and S2 and Table S5.
[a]Genes presented in Figure 1F.
[b]Regulated by ppGpp.
[c]Regulated by RpoS.
[d]Regulated by RpoH.
[e]Regulated by RpoE.
[f]Genes first upregulated and then downregulated.

promoter in treated cells compared to untreated cells (Figure S1A). Moreover, treated cells had a higher L19 ribosomal protein content than untreated cells (Figure 3A). These results indicate that treated cells have more ribosomes early during growth. The number of ribosomes is tightly regulated, and it is the primary determinant of the level of translation (Nomura, 1984); therefore, we tested whether treated cells have a higher translational capacity. For that, we measured the capacity of treated and untreated cells to produce β-galactosidase when the *lac* operon is derepressed by isopropyl β-D-1-thiogalactopyranoside (IPTG).

Table 2. ppGpp and DksA Are Required for RpoS Regulon Induction by Subinhibitory Doses of Different Stressors

		Relevant Genotype				
		WT	Δ*ada*	Δ*rpoS*	Δ*dksA*	Δ*relA* Δ*spoT*
Stressor Family	Stressor	P*adaZ* Reporter Induction (Blue Rings)				
	no stress	–	–	–	–	–
Antibiotics	ampicillin	+	+	–	–	–
	gentamicin	+	+	–	–	–
	norfloxacin	+	+	–	–	–
	streptomycin	+	+	–	–	–
	tetracycline	+	+	–	–	–
	trimethoprim	+	+	–	–	–
Alkylating agent	MMS	+	–	+	+	+
Oxidizing agent	H_2O_2	+	+	–	–	–
DNA crosslinker	MMC	+	+	–	–	–
Low pH	acetic acid	+	+	–	–	–
Metal	copper	+	+	–	–	–

Induction of the P*adaZ* reporter was monitored using a disk diffusion assay on LB plates supplemented with X-Gal. Induction was visualized as blue rings close to the growth inhibition zone. +, blue rings; –, no blue rings; copper, copper (II) chloride. See also Table S3.

We found that treated cells produced significantly more β-galactosidase than untreated cells (Figure 3B). Therefore, the increased energy production may fuel increased protein synthesis in the treated cells. Finally, increased translational capacity is not specific to ampicillin, because sub-MICs of other antibiotics also increased the expression of the *rpsM* promoter (Figure 2; Table S4) and significantly stimulated production of β-galactosidase (Figure S3A).

Tryptophan Catabolism Is Increased in Ampicillin-Treated Cells

LB medium contains very low levels of fermentable sugars, which are rapidly depleted by growing cells. Consequently, cells use amino acids as a major energy source when growing in LB (Sezonov et al., 2007). Induction of the *tnaCAB* operon early during growth in ampicillin-treated cells (Table 1; Figure S1B) suggests that antibiotic treatment may stimulate the use of amino acids as an energy source. We verified this hypothesis by inactivating the *tnaA* gene that codes for a tryptophanase. TnaA converts tryptophan and cysteine into pyruvate, which feeds into the TCA cycle (Figure 1F) (Newton and Snell, 1962; Snell, 1975). However, inactivation of *tnaA* gene did not reduce energy production in ampicillin-treated cells (Figure 1B; Table S1). Therefore, TnaA must play another role in treated cells.

Degradation of tryptophan and cysteine by TnaA also produces ammonium (NH_4^+), indole, and hydrogen sulfide (H_2S). NH_4^+ is the preferred nitrogen source for *E. coli* under aerobic conditions (Reitzer, 2003), while indole and H_2S are known to be intra- and intercellular signals that can increase tolerance to antibiotics (Han et al., 2011). We found that extracellular tryptophan was depleted more rapidly by ampicillin-treated cells (Figure 1C). To confirm that tryptophan is more rapidly degraded in treated cells, we measured indole production (Figure 1D). Ampicillin-treated cells produced more indole at the transition between the two growth phases compared to untreated cells, and indole production was abolished in the *tnaA* mutant. Finally, we monitored extracellular H_2S production. Because H_2S can also be produced by the combined action of the AspC and SseA enzymes (Figure 1F) (Shatalin et al., 2011), we also tested an *aspC* mutant. The TnaA and AspC enzymes were involved in H_2S production in ampicillin-treated and untreated cells (Figure S5D). The deletion of *tnaA* and *aspC* reduced H_2S production relative to the WT level in the absence of ampicillin to 33% and 35%, respectively. However, in the presence of ampicillin, the deletion of *tnaA* and *aspC* reduced H_2S production relative to the WT to 59% and 10%, respectively. Thus, ampicillin-treated cells increased cysteine and tryptophan catabolism.

The induction of the *tnaCAB* operon is adaptive for treated cells, because inactivation of the *tnaA* gene reduced the fitness 3.7-fold and 17-fold when cells were grown in LB and LB with 50% MIC of ampicillin, respectively, relative to the *galS* control strain (Table S5). Finally, induction of the *tnaCAB* operon was observed in cells treated with sub-MICs of other antibiotics, suggesting that it may be a part of a general response to sub-MICs of antibiotics (Figure 2; Table S4).

Ampicillin-Treated Cells Have Fewer Intracellular Amino Acids and Induce the Stringent Response Earlier Than Untreated Cells

The number of ribosomes is tuned to the available amino acid supply. When amino acids become limiting, uncharged tRNAs bind to the ribosome, signaling to the ribosome-associated RelA protein to synthesize ppGpp, whose accumulation triggers the stringent response (Dalebroux and Swanson, 2012). We quantified ppGpp levels by HPLC during growth and observed up to 2.9-fold more ppGpp in treated cells than in untreated cells during the second growth phase (Figure 3C). This result suggests that ampicillin-treated cells deplete amino acids faster than untreated cells. We quantified the intracellular concentration of amino acids by HPLC and observed that concentration of most amino acids decreased over time in treated relative to untreated cells (Figure 1E; Table S2).

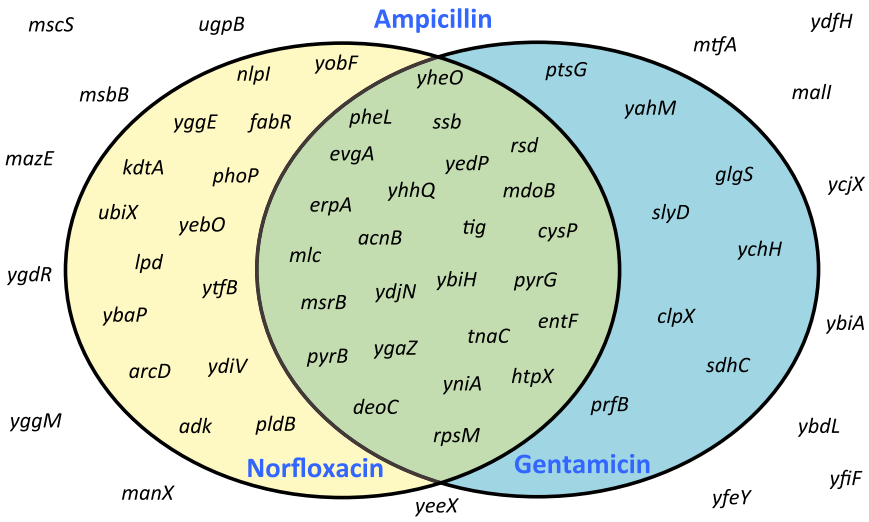

Figure 2. Genes Identified as Significantly Differentially Expressed in Cells Treated with 50% MIC of Ampicillin, Norfloxacin, or Gentamicin Relative to Untreated Controls

The 64 genes identified as differentially expressed during growth with 50% MIC of ampicillin were tested for their expression during growth with 50% MIC of norfloxacin (0.04 μg/mL) or gentamicin (0.5 μg/mL). See also Table S4.

ppGpp affects, positively or negatively, the transcription of many genes by interacting with RNA polymerase (Dalebroux and Swanson, 2012). For example, genes involved in amino acid biosynthesis are induced, while genes coding for rRNA and proteins are repressed. We observed that the *hisG* gene, which catalyzes the first step of histidine biosynthesis and is known to be induced by ppGpp (Potrykus and Cashel, 2008), showed stronger and earlier induction during the second growth phase in treated cells than in untreated cells (Figure S3B). We also measured the activity of the ribosomal promoter *rrnB* P1 fused to an unstable GFP variant ($P1_{rrnB}$-*gfp*), which is repressed by ppGpp (Figure 3D) (Maisonneuve et al., 2013). During the first growth phase, $P1_{rrnB}$-*gfp* was strongly induced in treated relative to untreated cells, similar to the *rpsM* promoter (Figure S1A). Next, as expected, the inhibition of $P1_{rrnB}$-*gfp* expression started earlier in treated cells than in untreated cells during the second growth phase, during which we observed strong ppGpp accumulation (Figure 3C). Finally, a similar $P1_{rrnB}$-*gfp* expression pattern was observed for other antibiotics, which induced the stringent response in a dose-dependent manner (Figure S3C).

Ampicillin-Treated Cells Induce the RpoS Regulon Earlier Than Untreated Cells

ppGpp and the RNA polymerase-binding protein DksA were reported to participate in RpoS regulon induction upon entry into the stationary phase and during nutrient starvation (Battesti et al., 2011). We investigated whether they were also involved in the observed induction of the RpoS regulon by the sub-MIC of ampicillin (Gutierrez et al., 2013). We monitored the kinetics of RpoS regulon induction using two reporter systems induced by RpoS. The first one is based on the activity of heat-stable hydroperoxidase II (HPII) encoded by the *katE* gene (Iwase et al., 2013). The second is the P_{ada}-*lacZYA* (P*adaZ*) reporter fusion (Gutierrez et al., 2013). Both reporter systems showed stronger RpoS induction in treated cells compared to untreated cells, and their inductions were abolished in the *rpoS*, *dksA*, and *relA spoT* ($ppGpp^0$) strains (Figures 4A–4C).

RpoS is finely regulated, and this process involves many regulators. To determine whether other regulators are also activated in ampicillin-treated cells, we examined the induction of the P*adaZ* reporter in deletion mutants for genes encoding proteins involved in RpoS regulation (Figure S4; Table S6) (Battesti et al., 2011). As expected, inactivation of the *rpoS* and *hfq* genes abolished P*adaZ* induction. Weaker induction of P*adaZ*, i.e., <0.5 relative to the WT strain, was observed for three mutants: *iraP*, *iraM*, and *rsd*. The *iraP* and *iraM* genes code for two antiadaptors involved in RpoS stabilization. The Rsd protein sequesters the vegetative RpoD (σ^{70}) sigma factor, rendering the RNAP core enzyme accessible to the alternative sigma factors, including RpoS. Finally, inactivation of the Rcs phosphorelay, which stimulates *rpoS* translation via the Hfq-dependent small RNA (sRNA) RprA, also resulted in a weak P*adaZ* induction relative to the WT strain. The β-lactams trigger the Rcs phosphorelay because they damage peptidoglycan (Laubacher and Ades, 2008). Therefore, the RpoS regulon induction is principally due to the ppGpp signal, but other signals, such as peptidoglycan damage, also play an important role.

Amino Acid Starvation Induces the General Stress Response Earlier in Ampicillin-Treated Cells

Because amino acid depletion is known to induce the stringent response, we investigated whether amino acid depletion is involved in the RpoS regulon induction by ampicillin. We show that the addition of amino acids abolished the induction of P*adaZ* by ampicillin treatment (Figure 4E). Amino acids can be depleted from LB media by catabolism and/or by protein synthesis. We tested whether amino acid catabolism is responsible for the RpoS regulon induction by using *tnaA* and *aspC* mutants, which cannot degrade tryptophan and/or cysteine. We observed that P*adaZ* induction was reduced by approximately 80% and 70% in *tnaA* and *aspC* mutants, respectively, relative to the WT (Figure 4D; Table S6). The RpoS regulon can be induced by amino acid degradation or by the products of amino acid degradation. We showed that the reduction in P*adaZ* induction was not due to the absence of the products of degradation for the following reasons. First, it is not due to reduced pyruvate production, because WT and the *tnaA* mutant had similar AEC ratios between treated and untreated cells (Figure 1B; Table S1). Second, it is not the consequence of pH modification by the products of tryptophan and cysteine degradation, because WT and the *tnaA* strains

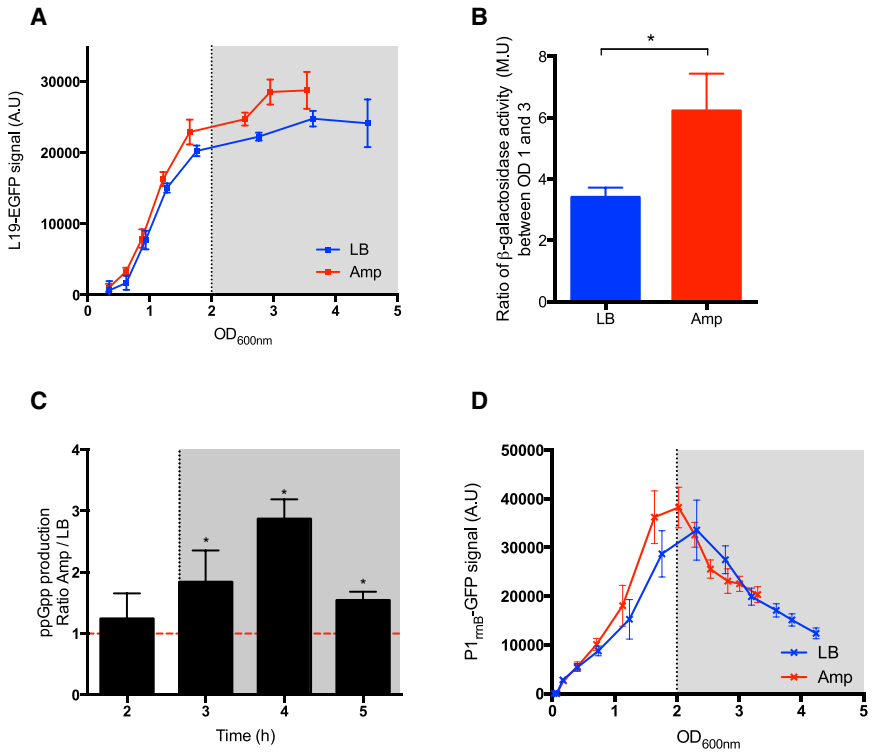

Figure 3. Ribosomal Protein Synthesis, β-Galactosidase Synthesis, ppGpp Accumulation, and Stringent Response Induction in Populations Exposed to 50% MIC of Ampicillin

(A) Intracellular levels of the ribosomal L19-EGFP fusion protein.

(B) An increased capacity of cells to perform protein synthesis was evaluated by measuring the β-galactosidase activity, expressed in MUs, during growth in LB medium supplemented with IPTG. The ratio between OD_3 and OD_1 is presented.

(C) The ratio of the ppGpp concentrations between treated (Amp) and untreated (LB) cells.

(D) Expression profiles of the *rrnB* P1 promoter-*gfp* fusion evaluated by measuring cell fluorescence (A.U.).

LB, liquid LB medium; Amp, LB with 50% MIC of ampicillin. The vertical dashed lines indicate the time of the growth shift for treated cells. Data are presented as the mean (±SD) values from at least three independent experiments. An asterisk indicates treated versus untreated conditions, t test, *$p < 0.05$. See also Figure S3 and Movie S1.

had similar pH values during growth with or without ampicillin (Figure S5A). Third, it is not due to the absence of indole or H_2S production, because the addition of sodium hydrosulfide (NaHS) (an H_2S donor) and indole did not restore P*adaZ* induction to the WT level (Figure 4E).

Therefore, RpoS regulon induction is likely affected by *tnaA* gene inactivation due to decreased amino acid degradation. This hypothesis is supported by following observations. The addition of pyruvate restored P*adaZ* induction in the *tnaA* mutant to the WT level (Figure 4E). Because pyruvate stimulates growth (Figures S5B and S5C), it should accelerate amino acid depletion. We confirmed this hypothesis by adding amino acids and pyruvate to the LB medium (Figure 4E; Table S6). As expected, the P*adaZ* induction by ampicillin was at intermediate levels between its expression after adding pyruvate and after adding amino acids in WT and *tnaA* mutants. These results show that the RpoS regulon induction by ampicillin results primarily from amino acid depletion, which is consistent with the involvement of ppGpp in this induction.

Hormesis: Sub-MIC of Ampicillin Induces Stress Responses that Confer Multi-resistant Phenotype

Using a promoter library, we observed that ampicillin modulates the expression of genes controlled by the RpoS, RpoE, and RpoH alternative sigma factors (Table 1). Using a *lac*-based disk diffusion assay, we showed that genes regulated by these sigma factors are also induced by sub-MICs of other antibiotics (Tables 2 and S3). Next, we found that the stringent response regulators ppGpp and DksA are required for RpoS regulon induction with different antibiotics (Table 2).

Induction of different stress response regulons by sub-MICs of antibiotics is expected to increase resistance to various stresses. We tested this hypothesis by exposing cells treated with the sub-MIC of ampicillin to severe heat shock and oxidative stress (Figure 5). Cells were harvested before the end of the first growth phase (Figure 1A). We observed that although there was no difference between growth curves at this stage, ampicillin-treated cells showed improved survival in 55°C and H_2O_2, with a 10-fold increase compared to untreated cells (Figures 5A and 5B). Because the capacity to resist both stresses depends on the DksA and RpoS proteins, and because the induction of the stringent and RpoS regulons depends on amino acid availability, we investigated whether addition of amino acids in the LB medium could have an impact on survival (Figures 5C and 5D). We found that this increase in cell survival depends on amino acid depletion.

Core Hormetic Stress Response Common to Multiple Stressors

Our study showed that a set of 25 genes that was significantly affected by 50% MIC of ampicillin was also regulated by 50% MIC of gentamicin and norfloxacin (Figure 2). We sought to determine whether the expression of the same set of genes would be affected when cells were exposed to other sublethal stresses. We observed that 68%, 68%, and 60% of these genes were also modulated by sublethal doses of the bacteriostatic antibiotic chloramphenicol, acid stress, and methyl methanesulfonate (MMS), respectively (Table S4). All of these stresses induced the TCA cycle gene *acnB*, the ribosomal proteins encoded by the *rpsM* operon, and the *rsd* gene coding for the anti-RpoD protein. Moreover, all of these stresses induced the RpoS regulon as determined by the P*adaZ* reporter (Table 2). The induction of this reporter was ppGpp and DksA dependent.

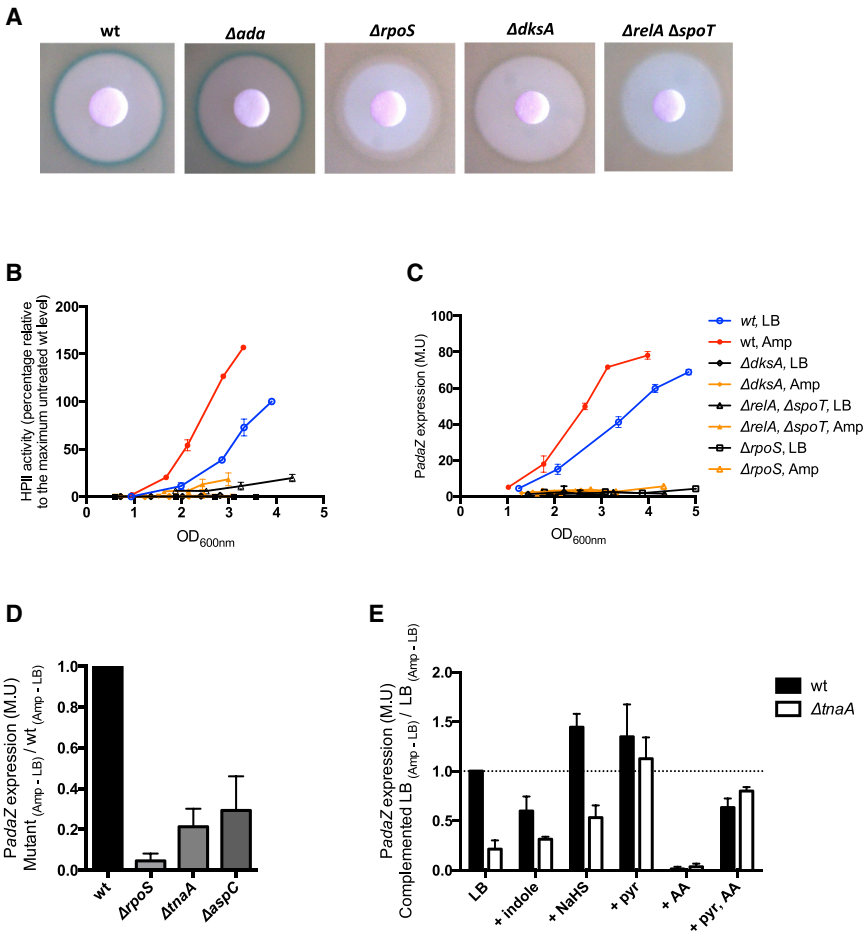

Figure 4. RpoS Regulon Induction in Populations Exposed to 50% MIC of Ampicillin in Different Growth Conditions

(A) Monitoring induction of the P*adaZ* reporter by ampicillin using a disk diffusion assay on LB plates supplemented with X-Gal. Induction was visualized as blue rings.

(B) The HPII activity was determined by measuring the amount of oxygen bubbling produced by cells. Percentages were calculated relative to the maximal level for untreated WT.

(C–E) Expression of the P*adaZ* fusion was evaluated by measuring the β-galactosidase activity, which is expressed in MUs, during growth (C) and at OD_3 (D and E). Each bar represents β-galactosidase activity calculated as follows: (D) $mutant_{(Amp\text{-}LB)}/WT_{(Amp\text{-}LB)}$ and (E) complemented $LB_{(Amp\text{-}LB)}/LB_{(Amp\text{-}LB)}$, in which LB medium was complemented with indole, NaHS, pyruvate (pyr), and/or casamino acids complemented with several amino acids (AA).

E. coli strains are P*adaZ* (WT) and isogenic derivatives. LB, liquid LB medium; Amp, LB with 50% MIC of ampicillin. Data are presented as the mean (±SD) values from at least three independent experiments. See also Figures S4 and S5 and Table S6.

In addition, ppGpp and DksA regulated the induction of the P*adaZ* reporter by the DNA crosslinker mitomycin C (MMC), H_2O_2, and copper-induced oxidative stress (Table 2). Finally, using a *lacZ*-based fusion reporter, we found that all antibiotic and non-antibiotic sublethal stresses induced the RpoE and RpoH regulons but their induction was independent of DksA (Table S3). Altogether, these data suggest that a sublethal stress core response may exist that is not specific only for antibiotics.

DISCUSSION

By studying the impact of sublethal concentrations of antibiotics on *E. coli*, we identified a general core hormetic stress response that is independent of the cellular target of a stressor and is not specific only for antibiotics. This response, which consists of increased energy production, translation, and macromolecular repair and protection capacity, allows growth when resources are available despite the constant presence of a stressor. When resources are limited, cells switch to the energy conservation mode, stop growing, and enter a multiresistant state. Therefore, given the ubiquitous presence of sublethal concentrations of antibiotics in natural environments, it is plausible that this response plays an important role in the robustness of bacteria exposed to antibiotic treatments and constant environmental fluctuations.

As a model drug, we used the β-lactam antibiotic ampicillin and assessed the most important findings with other antibiotics. Cells growing in LB medium with 50% MIC of ampicillin induced cell wall, membrane synthesis, and maintenance functions to repair ampicillin-induced cell wall damage (Table 1). Induction of the genes controlled by the alternative sigma factors RpoS, RpoE, and RpoH, which control induction of the general stress, envelope stress, and heat shock regulons, respectively, confirmed that the treated cells are under stress (Table 1). However, cells in treated and untreated batch cultures had identical growth rates until almost the end of growth, which suggests that treated cells manage to maintain cellular homeostasis (Figure 1A). The transcriptional and metabolic modifications observed in cells growing in the presence of ampicillin are undoubtedly adaptive because ampicillin pre-treated cells showed 100-fold improved survival following cytotoxic treatment with 100× MIC of ampicillin compared to untreated cells, i.e., 10^{-3}% and 10^{-1}% survival for untreated and treated cells, respectively (Wilcoxon test, $p < 0.01$).

An increased capacity to repair macromolecular damage requires increased energy production. For example, treatment with UV radiation, bleomycin, and DNA gyrase inhibitors induced ATP production in *E. coli* (Barbé et al., 1983; Dahan-Grobgeld et al., 1998; Guerrero et al., 1984). We also observed that the genes involved in ATP production, such as TCA cycle and respiratory chain genes, were upregulated, and the AEC ratio between treated and untreated cells increased over time (Figure 1B; Table 1). These results suggest that increased energy production

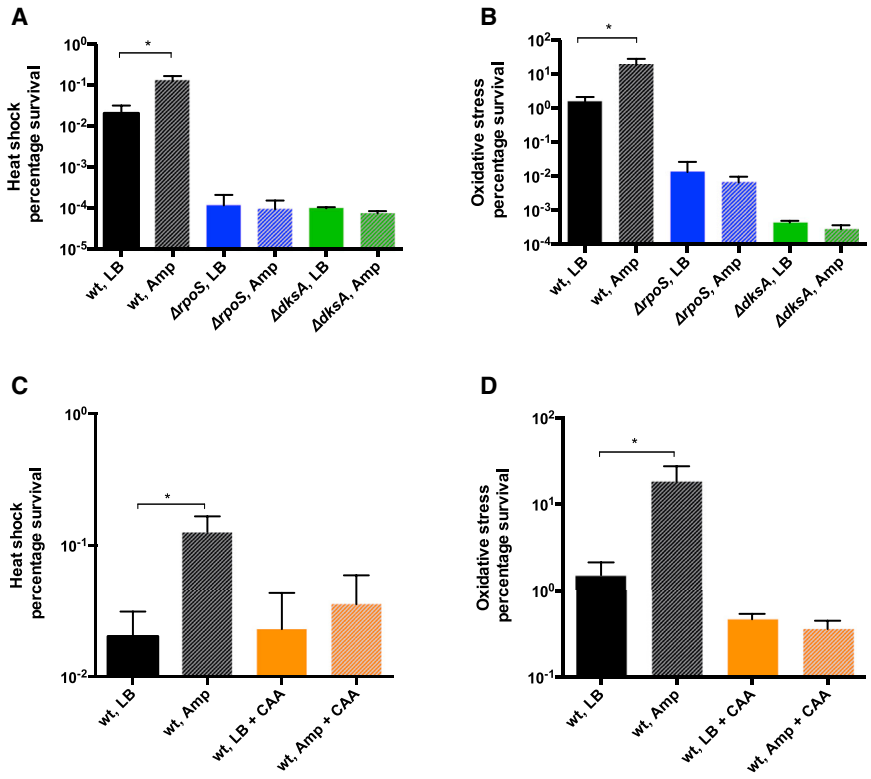

Figure 5. Resistance to Heat and Oxidative Shocks of Cells Exposed to 50% MIC of Ampicillin

(A–D) Cells were grown for 3 hr and exposed to 55°C (A and C) or to 25 mM H_2O_2 (B and D) for 30 min. The survival ratio between the number of CFUs before and after the stress was calculated for each strain in each condition.

E. coli strains are P*adaZ* (WT) and the isogenic derivatives Δ*rpoS* and Δ*dksA*. LB, liquid LB medium; Amp, LB with 50% MIC of ampicillin; CAA, supplemented with casamino acids. Data are presented as the mean (±SD) values from at least three independent experiments, t test, *$p < 0.05$.

in treated cells could be necessary for the management of the ampicillin-induced perturbations. This hypothesis was confirmed by the observation that inactivation of the TCA cycle gene *acnB* reduced fitness by 12-fold during growth with a sub-MIC of ampicillin relative to the *galS* control strain (Table S5).

In growing *E. coli* cells, approximately 50% of the energy provided by ATP is consumed by the translational machinery (Russell and Cook, 1995). Because ampicillin-treated cells maintain maximal growth rates while repairing damage, they may produce more energy to increase the translational capacity. Treated cells overexpressed genes coding for ribosomal proteins and increased the number of ribosomes and the translational capacity (Figure 3). This confirms that treated cells have abundant energy and an abundant amino acid supply, because only under these conditions does *E. coli* devote its resources to ribosome biogenesis (Chubukov et al., 2014). However, because in the LB medium amino acids are used as an energy source and as building blocks for protein synthesis, both of which are increased in treated cells, amino acid depletion is faster in treated cells than in untreated cells.

Amino acid depletion is expected to result in the appearance of uncharged tRNAs, increased amounts of the ppGpp alarmone, and induction of the stringent response (Potrykus and Cashel, 2008). We observed higher amounts of ppGpp, induction of *hisG* gene expression, and repression of *rrnB* gene expression from the P1 promoter in ampicillin-treated cells relative to the untreated cells (Figure 3). This latter result was also observed during sublethal treatments with streptomycin, gentamicin, norfloxacin, and trimethoprim (Figure S3). Our study adds antibiotics to the list of known stringent response inducers, i.e., amino acid, fatty acid, phosphate, iron, and carbon-source starvation (Dalebroux and Swanson, 2012).

Induction of the stringent response causes repression of the genes required for rapid growth, such as rRNA and tRNA genes; inhibition of DNA replication initiation; and activation of the genes involved in amino acid biosynthesis (Dalebroux and Swanson, 2012; Potrykus and Cashel, 2008; Potrykus et al., 2011). This could explain why ampicillin-treated cells in batch cultures enter the stationary phase earlier than untreated cells (Figure 1A). We are confident that amino acid depletion, not antibiotic-induced damage accumulation, is responsible for the growth rate deceleration for the following reasons. In microfluidic devices for which fresh LB medium with 50% MIC of ampicillin was constantly supplied, (1) cells maintained the same division rates as untreated cells even after 10 hr of growth (Figure S6) and (2) cells carrying a $P1_{rrnB}$::$gfp^{unstable}$ reporter fusion were constantly bright, which indicates that this ribosomal promoter is highly active (Movie S1). Subsequently, we replaced the fresh LB media with or without ampicillin by conditioned media from the batch cultures in which cells were growing with or without ampicillin. We observed that the growth slowed and the fluorescence disappeared in both cases but both occurred more rapidly in conditioned media with ampicillin (Movie S1). This result confirms that cells growing in batch cultures with ampicillin deplete amino acids more rapidly than untreated cells.

One of the consequences of the induction of the stringent response by antibiotic-induced amino acid depletion is induction of the RpoS-regulated general stress response. The stringent response was reported to induce the RpoS regulon by stimulating expression of the genes coding for the antiadaptor proteins IraP and IraD, which bind RssB and block RpoS protein degradation by ClpXP (Battesti et al., 2011). In our study, IraP is the more likely candidate, because inactivation of *iraP* reduced ampicillin-induced expression of the P*adaZ* reporter, while this was not the case for *iraD* gene inactivation (Figure S4B). ppGpp is also known to stimulate expression of the *rpoS* gene and of the

RpoS-regulated genes by interacting directly with RNAP or co-operating with the transcription factor DksA (Dalebroux and Swanson, 2012). We observed that ppGpp and DksA were required for RpoS regulon induction by all tested antibiotics but also by other stressors, such as low pH, MMC, H_2O_2, and copper (Table 2).

Therefore, our study shows that provided resources are not limited, bacteria treated with sublethal doses of different stressors can maintain high growth rates while successfully dealing with macromolecular damage and metabolic perturbations (Figure S6). However, when resources are limited, which is probably a norm in natural environments, bacteria exposed to sublethal stresses accelerate resource depletion and starvation. Under starvation conditions, there is a trade-off between functions dedicated to reproduction and those dedicated to cellular maintenance and repair (King et al., 2004; Nyström, 2004). This conflict is resolved by resource reallocation from reproduction toward increased cellular repair and maintenance. We showed that resource reallocation in cells experiencing sublethal stresses is regulated by the ppGpp alarmone, independent of the nature of a stressor (Table 2). The observation that different antibiotics induce a starvation-related stress response suggests that it could be a central hub to which all others are connected.

Stringent response-mediated transcriptional reprogramming, and in particular RpoS regulon induction, results in increased production of genetic diversity (Gutierrez et al., 2013) and phenotypic diversity, which increases the likelihood that some cells within the population will be able to endure different environmental assaults. We observed that cells treated with the sub-MIC of ampicillin survived lethal ampicillin treatment, severe heat shock, and oxidative stress better than untreated cells (Figure 5). This robustness depended on the RpoS and DksA proteins. We also showed that addition of amino acids abolished the capacity of bacteria exposed to sublethal concentrations of ampicillin to resist lethal stresses. This observation emphasizes the potential of manipulating the metabolic state of cells for the increased efficiency of antimicrobial treatments, as was previously observed for persister cell eradication by adding amino acids (Lebeaux et al., 2014; Shan et al., 2015).

EXPERIMENTAL PROCEDURES

Strains, Media, Culture Conditions, and Chemicals

All *E. coli* strains used in this study were derivatives of MG1655 (Table S7). All experiments were performed using LB medium (Difco) supplemented, or not, with 2 mM indole, 0.2 mM NaHS, 20 mM pyruvate, or 0.2% casamino acids complemented with 2 mM tryptophan, 2 mM cysteine, 0.2 mM glutamine, and 0.2 mM asparagine. Cells were grown in flasks or in 96-well plates at 37°C. All chemicals and antibiotics were purchased from Sigma. Antibiotics for the sub-MIC experiments were always freshly prepared (see also Supplemental Experimental Procedures).

Gene Expression Profiles

We used a library of 1,920 *E. coli* strains (Zaslaver et al., 2006). Each strain of this library carries a low-copy plasmid, in which a particular *E. coli* promoter region was inserted upstream of a gene coding for a fast-folding GFP. Strains were inoculated from frozen stocks and grown overnight in LB medium complemented with 25 μg/mL of kanamycin (kan) in 96-well plates. Then, cultures were diluted 1/1,000 into LB medium + kan, with or without 1 μg/mL of ampicillin, in a final volume of 160 μL for each well. On top of each well, 20 μL of mineral oil were added. Using a programmable robotic system (Freedom Evo, Tecan), plates were incubated at 37°C, and optical density of 600 nm (OD_{600}) and GFP fluorescence (excitation, 480 nm; emission, 535 nm) were measured (Infinite M200 PRO, Tecan) every 37 min over 15 hr of growth. Orbital shaking was applied before every measurement. For data analysis and analysis of the expression profiles of various reporters in cells treated with sub-MICs of gentamicin, norfloxacin, chloramphenicol, acetic acid, and MMS, refer to Supplemental Experimental Procedures.

β-Galactosidase Assays

Overnight cultures of reporter strains and MG1655 were diluted 1/300 in 50 mL of LB medium with or without 50% MIC of antibiotic. The β-galactosidase activity was measured as previously described (Miller, 1972). The β-galactosidase activity is expressed in Miller units (MUs). To induce the MG1655 *lac* operon, we added 0.1 M IPTG. We confirmed that the P*adaZ* reporter is functional in all genetic backgrounds by inducing it with MMS, which is an inducer of the Ada-regulated adaptive response (Table 2) (Sedgwick, 1983). The disc diffusion assay using reporter strains was performed as previously described (Gutierrez et al., 2013).

Fluorescence Measurement with a Microplate Reader

Overnight cultures were diluted 1/300 in 50 mL of fresh LB medium with or without 1 μg/mL of ampicillin and incubated at 37°C. During growth, OD_{600} was measured using a spectrophotometer, and fluorescence was measured using a Tecan Infinite M200 PRO. (For the L19-EGFP and $P1_{rrnB}$-GFP strains: excitation, 480 nm; emission, 510 nm. For the P_{hisG}-mKate strain: excitation, 580 nm; emission, 610 nm.)

To obtain high-throughput measurements for other antibiotics, we assayed the expression of the $P1_{rrnB}$-GFP fusion strain using 96-well plates. Overnight cultures were diluted 1/1,000 and dispensed in 96-well plates. The volume of the cultures in each well was 160 μL of LB medium with different concentrations of antibiotics and 20 μL of mineral oil. The OD_{600} and GFP fluorescence were measured every 15 min over 10 hr of growth. The obtained values were background corrected.

Quantification of HPII Activity

HPII activity was measured every hour during growth, as previously described (Iwase et al., 2013).

Tryptophan and Indole Quantification

For each time point, 1-mL aliquots of the cell suspension were centrifuged, and the supernatant was filtered. For tryptophan quantification, the supernatant was diluted 1/10 in sterile water, and the fluorescence was measured (excitation, 270 nm; emission, 342 nm). After background correction (with 6% perchloric acid [PCA] in LB medium to destroy tryptophan), the percentage of tryptophan was calculated relative to the maximum level in fresh LB medium. For indole quantification, 400 μL of Kovac's reagent was added to the supernatant, which was then incubated for 2 min. A 100-μL aliquot was removed and added to 900 μL of an HCl-amyl alcohol solution. The absorbance at 540 nm was measured. The indole concentration in the supernatant was calculated using a calibration curve.

ATP, ADP, AMP, ppGpp, and Amino Acid Quantification

Overnight cultures were diluted 1/300 in fresh LB medium with or without 1 μg/mL of ampicillin and incubated at 37°C. At different time points, approximately 10^{10} cells were harvested. For ATP, ADP, AMP, and ppGpp quantification, the extraction was performed as previously described (Huang et al., 2003), except that trichloroacetic acid (TCA) was replaced by PCA to deproteinize cells. For reversed-phase HPLC (Shimadzu HPLC system interfaced with LabSolution software), samples were injected onto a 250 × 4.1 mm PRP-1 column (Hamilton) at 50°C. The mobile phase composition for the gradient system was 25 mM KH_2PO_4, 25 mM K_2HPO_4, and 10 mM tetrabutylammonium hydroxide (pH 7.0) for mobile phase A and 100% MeOH for mobile phase B. The gradient program was 0–8 min 0% phase B, 8–62 min 0%–30% phase B, and 62–70 min 30% phase B, and the initial conditions (100% phase A) were then maintained for 30 min. A photodiode array (PDA) detector was used for detection. The products were spectrophotometrically monitored

at OD_{254} and quantified by the integration of the peak absorbance area, employing a calibration curve generated with various known concentrations of molecules. Amino acid quantification was performed as previously described (Ramond et al., 2014).

Resistance to Heat and Oxidative Stresses

Overnight cultures were diluted 1/300 in LB medium and grown with or without 1 μg/mL of ampicillin and/or complemented with casamino acids for 3 hr (until $OD_{600} \approx 1$). For heat stress, 1 mL of cells was placed in a water bath at 55°C for 30 min. For oxidative stress, cells were incubated with 25 mM H_2O_2 for 30 min. Then cultures were diluted in LB medium supplemented with 10 U/mL of bovine liver catalase to ensure the removal of H_2O_2. For both stresses, the number of colony-forming units (CFUs) was quantified before and after the stress by plating a serial dilution of cells on LB plates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, seven tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.001.

AUTHOR CONTRIBUTIONS

I.M., A.M., S.F., and J.D. conceived and designed the experiments. A.M., S.F., A.F., M-F.B., and P.S.-V. performed the experiments. I.M., A.M., S.F., A.F., and X.S. analyzed the data. I.M., A.M., and S.F. wrote the manuscript.

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