Functional profiling and strategic antimicrobial manipulation of a universal nutrition-sensing network to regulate microbial virulence, antibiotic tolerance, and stress protection

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KEY MESSAGES

1. Nutrient limitation can trigger time-dependent onset of antibiotic tolerance phenotypes.
2. Starvation for 24 hours produced a large population of antibiotic persisters that reverted slowly to a drug-sensitive mode.
3. Inactivation of specific tolerance mechanisms has the potential to reduce the ability of an organism to develop antibiotic resistance.
4. Supplementation of small molecules such as amino acids is highly effective in abolishing antibiotic tolerance phenotypes.

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Introduction

Bacterial stress responses have been postulated to play a role in the onset of recurrent, latent, and biofilm-associated infections, as well as the development of antibiotic resistance.[1,2] Bacteria respond to sub-inhibitory concentrations of antibiotics by modulating their metabolic and gene expression profiles.[3] Production of observable resistance phenotypes such as those attributable to mutations or acquisition of specific resistance determinants may represent the secondary effects of genetic, structural, physiological, and morphological alterations associated with stress responses. This must be taken into account when defining the molecular basis of clinical resistance. It is therefore important to determine whether bacterial stress defence mechanisms that are induced by antibiotics or other adverse factors play a role in the activation of key resistance mechanisms.

This study aimed to identify the determinants of the nutrition-dependent stress response network, their potential role in resistance formation, and the feasibility of suppressing bacterial survival fitness by manipulating the stress response.

Methods

Analyses of time-dependent starvation-induced phenotypes and recovery characteristics

The *Escherichia coli* K-12 strain BW25113 was grown in lysogeny broth to the exponential phase and re-suspended in 3-(N-morpholino)-propanesulfonic acid (MOPS) base. Induction time was set at 10, 30, and 90 minutes, and 24 hours, followed by treatment with three antibiotics (ampicillin, ofloxacin, and gentamicin at 25x minimum inhibitory concentration) for 3 and 48 hours, and assessment of the size of the surviving population as described previously.[4] A parallel persister assay was performed in which organisms were recovered from 24 hours of starvation, re-suspended in Rich Defined Medium (RDM) [Teknova, Holister, CA, USA] and incubated at 37°C for 15 minutes, followed by drug treatment for 3 hours and determination of the size of the persister subpopulation. A gene knockout study was performed to investigate the relative role of selected determinants in regulation of the sustainable tolerance and persistence phenotypes observable during prolonged starvation and the subsequent resuscitation experiments, respectively. All knockout strains tested were obtained from the Keio collection.[5]

Identification of key cellular components responsible for formation of antibiotic tolerance

The following bacterial populations were subjected to analysis using a transcriptome sequencing approach: (1) log-phase populations, (2), stationary-phase population, (3) populations subjected to spontaneous starvation, (4) populations subjected to prolonged starvation stress, (5) populations subjected to combined starvation and antibiotic stress by adding 100 μg/mL ampicillin for 30 minutes, and (6) antibiotic persisters that were produced

by re-suspending bacterial populations with 24-hour starvation in lysogeny broth, followed by treatment with ampicillin at 100 μg/mL to eradicate non-persisters. All transcriptome sequencing experiments were performed by BGI-Hong Kong (http://www.genomics.cn).

Assessment of the putative role of stress defence mechanisms in the development of antibiotic resistance

A resistance formation assay was used to determine the relative potential of selected stress response mutants to develop drug resistance upon antibiotic induction and selection. Briefly, mutation prevention concentration of the test strains was determined by spreading $1x10^9$ cells on lysogeny broth agar plates containing ciprofloxacin at a concentration of 0 to 32 μg/mL followed by incubation at 37°C for up to 72 hours. Viable counts on each plate were recorded. Mutation prevention concentration was defined as the lowest antibiotic concentration at which no colonies were observed.

Strategic development of a central approach to control microbial viability

The combined effect of nutrient limitation and deletion of key tolerance determinants on bacterial tolerance induction and survival against environmental stress was tested using a phenotype array approach (Biolog). Briefly, log-phase populations of both wild type and mutant strains were switched to a MOPS-based buffer and aliquoted into a phenotype array containing a range of compounds (P1-P8 of Biolog Phenotype Array), followed by treatment with 100 μg/mL ampicillin. Assessment of bacterial survival at 3 and 48 hours was by inoculation of 1 μL of the test population from each well of the phenotype array onto a lysogeny broth plate.

Results

Phenotypic and molecular characteristics of starvation-induced antibiotic tolerance

The time-dependent induction effects of nutrient limitation on phenotypic antibiotic tolerance were studied by reconstituting RDM-grown log phase cells in MOPS base, followed by exposure of the test organisms to starvation stress for up to 24 hours and assessment of the relationship between the exposure time and drug tolerance phenotypes. The size of the emerging tolerant population increased proportionately with the length of starvation period (Fig 1). Induction for as short as 10 minutes (the shortest induction time testable due to the requirement for medium switching) produced antibiotic tolerance that generally could not be sustained for 48 hours. In comparison, a significant level of sustainable tolerance was consistently produced if the organisms were starved for 24 hours.

Upon reconstitution in RDM, each of the test populations that had been subjected to starvation for ≥30 minutes was found to contain subpopulation persisters that exhibited at least 3-hours tolerance to the three test drugs (Fig 1). Nonetheless, no persisters survived 48 hours of drug treatment. The population size of gentamicin persisters was consistently smaller than that for ampicillin and ofloxacin in all cases. Resembling the tolerance induction characteristics, the potential to develop persisters was also time-dependent, with the level inducible by brief starvation considerably smaller than that following an extended starvation period (90 minutes and 24 hours).

A gene knockout study was performed to investigate the relative role of selected stress response genes in regulation of the sustainable tolerance and persistence phenotypes. The genetic determinants tested were found to play common, differential, or drug-specific roles in starvation-mediated responses, producing a variety of defects (Fig 2). Of particular interest, the *ubiF* and *sucB* loci, both energy production genes implicated in persister formation, were important for such a process. Nonetheless, sustainable tolerance to the test drugs was not affected in the Δ*ubiF* mutant, indicating that the product of this gene was highly specific in mediating persister formation. On the contrary, deletion of *sucB* resulted in defective production of all phenotypes except sustainable tolerance to ofloxacin, suggesting that energy production is required for maintaining the tolerance mode upon nutrient replenishment but not necessary for sustaining such phenotype during starvation.

Among the mutants that displayed an altered phenotypic pattern in the tolerance and persister assay, a drastic difference in the levels of sustainable tolerance and subpopulation persisters was often observed. This indicates that cellular mechanisms responsible for prolonged bacterial survival against antibiotic stress during nutrition starvation were not identical to those required for maintaining the tolerance phenotypes beyond the starvation phase. The fact that prolonged starvation stress enabled various mutants to develop sustainable tolerance to gentamicin, a phenotype not inducible during brief starvation, also confirmed that progressive physiological changes occurred during prolonged starvation.

Identification of key tolerance determinants by transcriptome sequencing analyses

Transcriptome sequencing was performed to probe the molecular basis of physiological changes observed under short- and long-term starvation. A significant discrepancy was observed between populations recovered under different

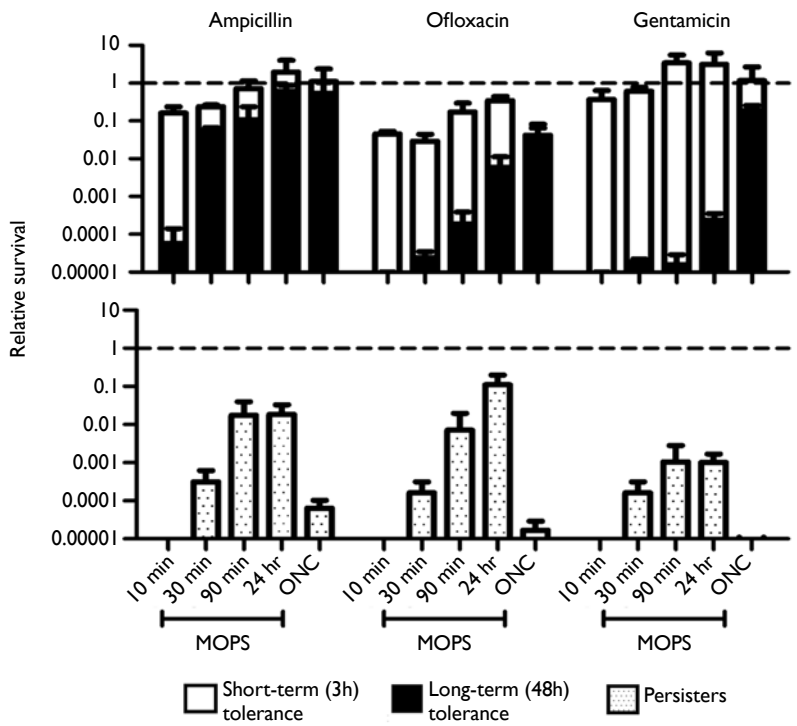

FIG 1. Progressive development of antibiotic persisters within starvation-induced antibiotic-tolerant populations. Exponentially growing *Escherichia coli* BW25113 populations (at 10^7/mL) in Rich Defined Medium (RDM) were washed and reconstituted in 3-(N-morpholino)-propanesulfonic acid (MOPS) base. At indicated times, aliquots were assayed for tolerance to ampicillin, ofloxacin, and gentamicin as described previously.[4] The relative abundance of persisters at each time point was also determined by reconstituting the MOPS-starved cells in RDM for 15 minutes, followed by 3-hour antibiotic challenge and assessment of the survival rate. An overnight culture (ONC) was included in both assays as control. Abundance of persisters in ONC was determined by 100-fold dilution in RDM, followed by 15 minutes incubation and antibiotic challenge.

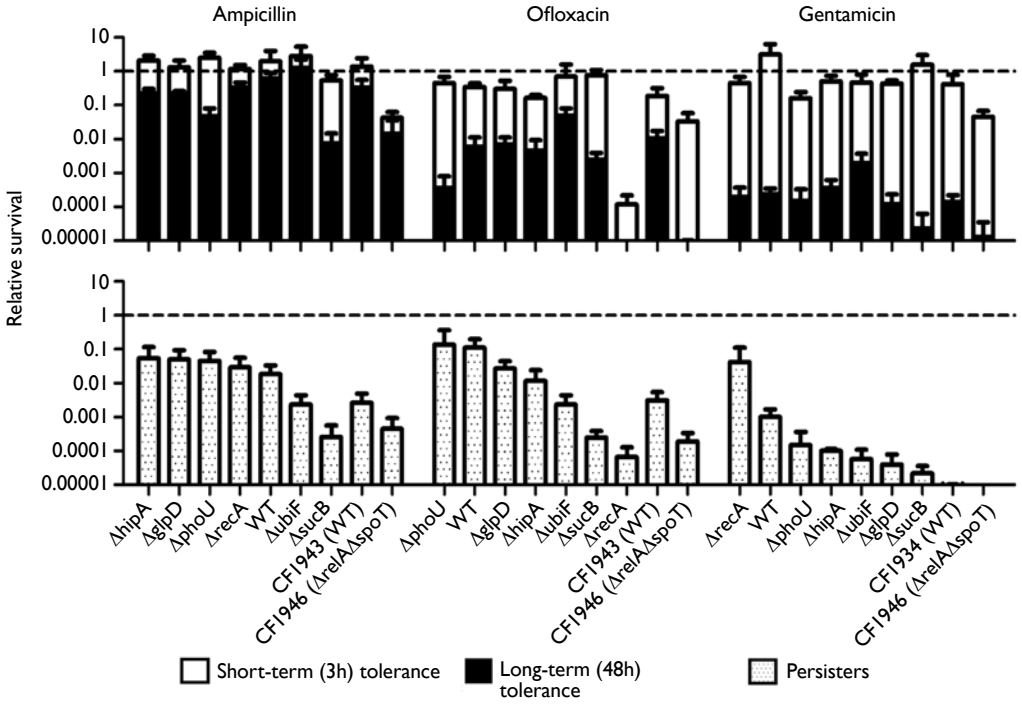

FIG 2. Gene knockout analysis of the relative role of putative persister genes in starvation-induced persister formation. Rich Defined Medium–grown log-phase populations with indicated isogenic gene deletions (arranged in descending order by their relative persister abundance except for the ppGpp+ strain and its corresponding ppGpp0 mutant) were subjected to starvation in 3-(N-morpholino)-propanesulfonic acid base for 24 hours and subsequently analysed for tolerance (to ampicillin, ofloxacin, and gentamicin) and persister development.

TABLE. Results of a representative phenotype array (Biolog) designed to test the effect of specific carbon or amino acid source in abolishing 3-(N-morpholino)-propanesulfonic acid (MOPS)–induced ampicillin tolerance. Ω and Δ denote positive detection of viable organisms in the wild type and Δ*acrA* strain, respectively, after 48 hours challenge by 100 μg/mL ampicillin, ie tolerance remains inducible by MOPS.

	1	2	3	4	5	6
A	Negative control: Ω	L-arabinose: Ω	N-acetyl-D- glucosamine	D-saccharic acid	Succinic acid	D-galactose
B	D-serine	D-sorbitol	Glycerol: Ω	L-fucose	D-glucuronic acid: Ω	D-gluconic acid
C	D-glucose-6-phosphate	D-galactonic acid-γ-lactone	D,L-malic acid	D-ribose	Tween 20	L-rhamnose: Ω
D	L-asparagine	D-aspartic acid: Ω Δ	D-glucosaminic acid	1,2-propanediol: Ω Δ	Tween 40	α-keto-glutaric acid
E	L-glutamine	m-tartaric acid	D-glucose-1- phosphate	D-fructose-6- phosphate	Tween 80	α-hydroxy glutaric acid-γ- lactone
F	Glycyl-L-aspartic acid	Citric acid	m-inositol: Ω	D-threonine	Fumaric acid: Ω	Bromo succinic acid
G	Glycyl-L- glutamic acid	Tricarballylic acid: Ω	L-serine	L-threonine: Ω	L-alanine	L-alanyl-glycine: Ω
H	Glycyl-L-proline	p-hydroxy phenyl acetic acid: Ω	m-hydroxy phenyl acetic acid: Ω	Tyramine	D-psicose	L-lyxose: Ω Δ

test conditions. A large number of genes was up-regulated in populations subjected to spontaneous and prolonged starvation, when a log-phase population was used as control. The discrepancy between the expression profile of stationary phase cells and those subjected to starvation stress revealed that nutrition limitation was not the only factor that induced development of phenotypic tolerance in stationary-phase populations. Antibiotics induced further stress responses in bacterial populations undergoing a starvation process, and the antibiotic tolerance phenotypes observed during starvation may be partially attributed to physiological changes triggered by the antibiotic itself. The transcription profile of persisters differed from that of the starving population, indicating that persisters comprised only a fraction of the original tolerant population detectable during starvation.

Transcriptome studies were performed to identify genes that are significantly up-regulated during prolonged starvation. When a bacterial population subjected to 24 hours starvation was compared with a log-phase population, >1700 genes were found to be up-regulated. Genes that were exclusively expressed during starvation and those for which the transcription level was most significantly up-regulated included the TetR/AcrA transcriptional activator, multiple stress resistance protein BhsA, and DNA repair protein RadC. A huge amount of transcriptome sequencing data are being analysed to shortlist genetic components for future studies to assess their potential as drug targets to reduce survival fitness of antibiotic tolerant and resistant organisms.

Assessment of the putative role of stress defence mechanisms in the development of antibiotic resistance

To determine whether strains that are compromised in their ability to develop or maintain antibiotic tolerance phenotypes are also less able to develop into resistant mutants, the mutation prevention concentration of selected gene knockout mutants was determined (Δ*sodA*, Δ*arcA*, Δ*dnaK*, Δ*dps*, Δ*proP*, Δ*rcsC*). The wild type and all test strains except Δ*arcA* exhibited a mutation prevention concentration of 0.25 μg/mL. For Δ*arcA*, no growth or emergence of mutants was detectable at any test concentration (the lowest being 0.06 μg/mL), indicating that failure to produce the *arcA* gene product not only leads to a lower tolerance level, but also undermines the ability of the organism to undergo mutational changes and develop resistance phenotypes. The Δ*dnaK* mutant was also found to produce a substantially smaller number of colonies on the plates containing 0.06 and 0.12 μg/mL, suggesting that a lack of *dnaK* gene product also affected development of both tolerance and resistance phenotypes.

Strategic development of a central approach to control microbial viability

A phenotype array approach was used to test the combined effects of nutrient supplementation and gene knockout on tolerance formation. A total of 760 compounds including amino acids and various carbon and nitrogen sources were tested for their ability to abolish MOPS-induced tolerance to ampicillin in a wild type strain and the *acrA* deletion mutant. A substantial number of compounds were able to abolish antibiotic tolerance. For the wild type strain, 210 (28%) of the 760 compounds tested conferred the ability of ampicillin to eradicate the entire test population in a MOPS-based environment that is supposed to confer prolonged tolerance to this drug. For the Δ*acrA* strain, as many as 363 (48%) compounds could cause abolition of ampicillin tolerance (Table), indicating that nutrient supplementation can enhance the effects of knockout of specific genes in suppressing the

7	8	9	10	11	12
L-aspartic acid	L-proline	D-alanine	D-trehalose	D-mannose	Dulcitol
D,L-α-glycerol-phosphate	D-xylose	L-lactic acid: Ω	Formic acid: Ω	D-mannitol	L-glutamic acid
D-fructose: Ω	Acetic acid	α-D-glucose	Maltose	D-melibiose	Thymidine
α-keto-butyric acid	α-methyl-D- galactoside	α-D-lactose: Ω	Lactulose	Sucrose: Ω	Uridine
α-hydroxy butyric acid	β-methyl-D- glucoside: Ω	Adonitol	Maltotriose	2-deoxy adenosine	Adenosine
Propionic acid	Mucic acid	Glycolic acid: Ω	Glyoxylic acid	D-cellobiose	Inosine
Acetoacetic acid	N-acetyl-β–D- mannosamine	Mono methyl succinate	Methyl pyruvate	D-malic acid: Ω	L-malic acid
Glucuronamide	Pyruvic acid	L-galactonic acid-γ-Lactone	D-galacturonic acid	Phenylethyl-amine	2-aminoethanol

ability of the host organism to develop and maintain antibiotic tolerance.

Discussion

This study describes a sequential developmental pattern of antibiotic tolerance in bacteria subjected to nutrient limitation, in which the physiological responses elicited are highly dependent on the duration of stress exposure. Tolerant populations generated by prolonged starvation also harboured a subpopulation of persisters that reverted only slowly into an actively growing, drug-sensitive mode upon replenishment of nutrients. The size of this persister population varies according to the length of the preceding starvation period. This indicates that starvation stress elicits long-lasting protective mechanisms that confer resistance to multiple stresses. This is consistent with our previous findings that starvation-induced stress tolerance was not solely due to physiological dormancy,[4] which would not increase strength of tolerance as starvation conditions persist. Results of our gene expression profiling and gene knockout experiments confirmed the presence of independent yet overlapping genetic pathways that regulate the drug specificity, sustainability, and reversibility of antibiotic tolerance and that active defence functions such as efflux and production of chaperone proteins constitute the key tolerance mechanisms.

Apart from being potential targets of drugs designed to eradicate tolerant or persistent bacterial populations, cellular components responsible for producing antibiotic tolerance phenotypes may also be good targets for the control of resistant organisms. The intricate relationship between tolerance and resistance needs further investigation. Our findings suggest that suppressing stress responses can reduce the chance of resistance formation. Preliminary tests have been performed to examine the feasibility of using small nutrient molecules to interfere with the ability of bacteria to withstand environmental stresses. Amino acids and various carbohydrates and nitrogen sources are highly potent in abolishing tolerance phenotypes. Thus, agents that suppress tolerance development may reduce bacterial survival fitness, thereby enhancing the effects of conventional drugs.

Conclusion

Our findings may facilitate the development of new generation antimicrobial drugs through widespread interference and suppression of bacterial stress responses. This new antimicrobial approach with both natural and specially designed agents may be used to suppress multiple cellular pathways.

Acknowledgement

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