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The very hungry bactericidal antibiotics

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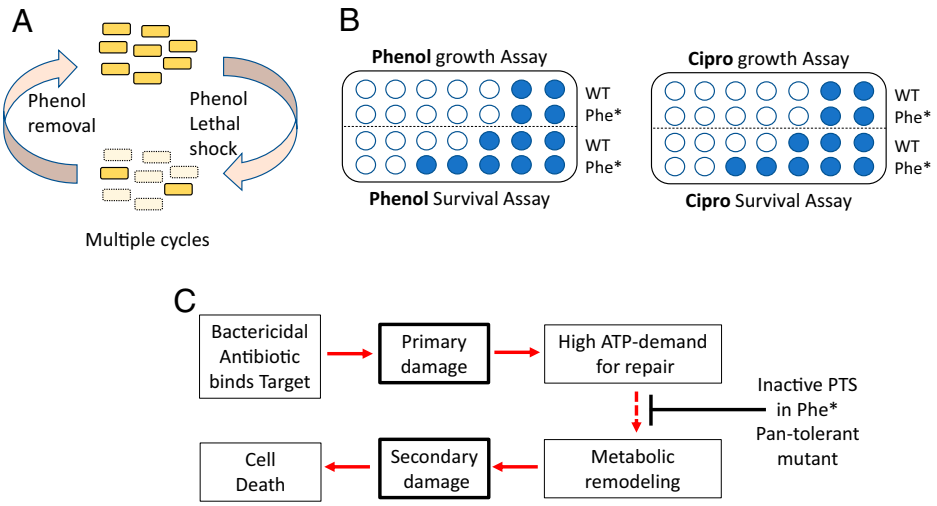

Fig. 1. Bacterial PTS orchestrates metabolic remodeling in response to bactericidal antibiotics. (*A*) Enrichment of phenol-tolerant mutants (Phe*). (*B*) Phenol-tolerant mutants are pan-tolerant to many bactericidal antibiotics. (*C*) Metabolic remodeling depends on the PTS system.

The combination of primary and secondary damages accounts for the lethality of bactericidal antibiotics. Binding of bactericidal antibiotic cripples the essential function of its target and engenders antibiotic-specific primary damage. In response, bacteria activate repair factors and replace corrupted targets, an energy-costly effort which entails a massive metabolic remodeling (1). Cells gradually exhaust available adenosine 5′-triphosphate (ATP), reducing equivalents, and increase production of toxic waste products, such as reactive oxygen species (ROS). The ensuing secondary damage, as well as the amplification of the primary damage, is a general attribute of the bacterial response to different classes of bactericidal antibiotics and lethal stressors (2). Without it, cells actually tolerate bactericidal antibiotics well, and the growth inhibition becomes reversible once the antibiotic is removed. Blocking protein synthesis with a bacteriostatic inhibitor (1), decreasing the activity of the ATP synthase complex with subinhibitory concentrations of bedaquiline (3), or disabling cellular respiration with a genetic knockout of the cytochrome complex (4) all confer a high level of tolerance to bactericidal antibiotics. However, new studies suggest that slow or no bacterial growth, per se, are neither necessary nor sufficient for antibiotic tolerance (5). Taking ATP or the nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide ratio as a proxy for a metabolic state, the lethality of bactericidal antibiotics is more strongly correlated to it than to the growth rate. The more resources bacteria can spend on a failing response, the more potent are the bactericidal antibiotics.

The understanding that tolerance precedes resistance (6, 7) spurred genome-wide mapping of genes with a potential to promote or compromise tolerance. Thus far, high-tolerance mutants with a low metabolic state were mapped to genes with a function in cellular respiration or in processes that are associated with high ATP demand (4). Less is known about regulatory genes that orchestrate the metabolic remodeling of cells to bactericidal antibiotics, and what effect they have on tolerance.

In PNAS, Zeng et al. (8) bring to the limelight the role of a key metabolic regulatory system in the susceptibility of bacteria to the lethality of bactericidal antibiotics. Previous studies mapped high-tolerance mutants from experiments wherein cycles of antibiotic treatment, antibiotic removal, and recovery of cells enrich for tolerant mutants (9). This kind of setup is sensitive to the number of enrichment cycles, because, with time, the resistant mutants are able to take over the culture. In a different experimental design (10), high-tolerance mutants are separated from resistant mutants through cell immobilization on a filter, and a step-wise passage of the filter from plates with high antibiotic concentration to plates free of drugs. With this approach, the growth of resistant and tolerant mutants is separated in time. Zeng et al. present yet another

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approach for avoiding resistant mutants (Fig. 1*A*). For a lethal stressor, the authors decided to choose the toxic compound phenol for which bacteria are unable to evolve resistance. The authors hypothesized that, given enough enrichment cycles, the high-tolerance mutants would eventually evolve from a surviving fraction of the bacterial population. Whole-genome sequencing of multiple isolated clones mapped a missense mutation in the *ptsI* gene, which encodes the phosphorelay EI component of the "phosphoenolpyruvate (PEP): carbohydrate transferase system (PTS)." Strikingly, the phenol-tolerant *ptsI* mutant was much more tolerant than wild-type (WT) cells to bactericidal antibiotics from different classes, and to other toxic lethal stressors (Fig. 1*B*). Thus, the authors have isolated a mutant that, under conditions of their experimental system, is pan-tolerant to many lethal stressors.

The current study from Zeng et al. continues the riveting exploration of the metabolic dependence of many bactericidal antibiotics. The authors establish that the PTS system, via metabolic remodeling, contributes to the lethality of bactericidal antibiotics.

PTS is one of the most complex metabolic networks in the bacterial cell (11). It facilitates the uptake and intracellular retention of numerous fermentable sugars from the environment, prioritizes utilization of PTS-dependent sugars by a mechanism of inducer exclusion, and coordinates catabolism of sugars and amino acids via sensing of oxoglutarate. In WT, the complex of EI with Hpr (encoded by *ptsH*) transfers a phosphate group from the glycolytic intermediate PEP to the EIIA enzyme (encoded by *crr*), thereby initiating a phosphorylation cascade. When glucose is available from the environment, EIIA supports glucose import and retention through phosphorylation of the glucose transporter EIIB (encoded by *ptsG*), and dephosphorylated EIIA interacts with transporters of non-PTS sugars to inhibit their activity. When the growth environment is depleted of glucose, EIIA phosphorylates adenylate cyclase (encoded by *cyaA*) which synthesizes the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP). Binding of cAMP to CRP, a master transcription regulator, activates transcription of dozens of CRP-dependent genes, including operons dedicated for the utilization of non-PTS sugars. Therefore, *ptsI* null mutants cannot grow in the media where the fermentable sugars are the sole carbon source. Even when nutrients are plentiful, under conditions of high ATP demand, which drives an increase in glycolytic flux in WT cells, *pts* null mutant is ostensibly less fit, for it cannot utilize sugars from the media.

Zeng et al. (8) were able to reproduce the phenotype of the pan-tolerant mutant with a gene knockout of *ptsI*, suggesting that the isolated mutant from the enrichment experiments had a loss-of-function mutation in *ptsI*. Next, the authors explored the effect of chemical inhibition of EI. Interestingly, when both sugar and amino acids are available to *Escherichia coli* in a growth medium, cells preferentially catabolize low-cost amino acids (glycine, serine, threonine, and aspartate) over glucose (12). Accordingly, the growth rate of the *ptsI* knockout strain is comparable to WT in rich growth medium. Here again, the regulation is PTS dependent. Keto acids, such as oxoglutarate and oxaloacetate degradation products of low-cost amino acids, are known to inhibit EI (12). The authors cleverly leverage this knowledge to demonstrate that media supplemented with oxoglutarate increases tolerance of WT cells to bactericidal antibiotics in a dose-dependent manner. Therefore, either genetic or chemical inhibition of EI promotes pan-tolerance to lethal stressors.

The pan-tolerant mutant is indeed unable to meet the high ATP demand of cells treated with lethal stressors. This refractory metabolic response could account for its pan-tolerant phenotype. Under normal growth conditions, the ATP concentration was comparable between *ptsI* and WT. In a stark difference, shortly after the addition of lethal stressors, ATP increases to a far greater concentration in WT than in the *ptsI* mutant. In the context of antibiotic tolerance, the *ptsI* mutant may represent a distinct class of a low-metabolic state mutant. The mutant has no growth defect, and its ATP content is no different from WT—that is, before the antibiotic treatment. The metabolic differences manifest only after cells are treated with antibiotics.

Are there PTS mutants that emulate the pan-tolerant phenotype? The same degree of pan-tolerance was seen with a point mutation in EIIA that prevents its phosphorylation, and in gene knockouts of *cyaA* and *crp*. Moreover, either the expression of a constitutively active CRP allele or supplementing the growth medium with exogenous cAMP negated the enhanced tolerance of *ptsI* mutants to bactericidal antibiotics. Therefore, metabolic remodeling and the increased ATP production in WT cells in response to lethal stressors appears to depend on transcriptional activity of CRP. In agreement, previous studies established a strong effect for CRP and adenylate cyclase on global transcriptional reprogramming (13), as well as on survival (14, 15), in cells treated with bactericidal antibiotics.

How does the CRP regulon support metabolic remodeling? This exciting question is left somewhat open. Consider that the increased ATP demand is known to increase the glycolytic flux (16). Is it possible that the increased glycolytic flux and ATP production depend on CRP-regulated utilization of sugars? Perhaps. The authors rule out a contribution from some PTS transporters to the pan-tolerant phenotype (8). Antibiotic tolerance of the mutants with the knockouts of EIIB genes (*ptsG*, *malX*, *ascF*, and *glvC*) is indistinguishable from that of parental WT. This result indicates that a single PTS transporter is not sufficient for driving an increase in the glycolytic flux and susceptibility of WT cells to lethal stressors. Alternatively, it is possible that CRP-dependent reprogramming of gene expression achieves metabolic remodeling of cells without importing sugars from the environment. Genome-wide expression profiles indicate that many genes with a function in glycolysis, the pentose phosphate pathway, the TCA cycle, respiration, and ATP synthesis are differentially regulated in the *ptsI* mutant already, under normal growth conditions. Treatment of the cultures with bactericidal fluoroquinolone ciprofloxacin accentuates differential expression of this group of genes between the pan-tolerant mutant and WT. As expected from the lower expression of the genes with a function in the

TCA cycle and respiration, ROS do not accumulate in the pan-tolerant mutant to the same extent as in WT cells.

The torrent of studies, each presenting a novel tolerant mutant, may seem daunting for the clinical prospects of preventing or targeting antibiotic tolerance. Is that really so? There are reasons for cautious optimism. The metabolic dependence of bactericidal antibiotics is not absolute. Whereas some bactericidal antibiotics are strongly dependent on a heightened metabolic state, some are not (17). Moreover, alternating cycles of strong and weak metabolic-dependent bactericidal antibiotics protract the time for an appearance of tolerant mutants (18). A different approach to limit tolerance is to target bacterial detox systems with small-molecule inhibitors, such as those for hydrogen sulfide production (19), that impart tolerance in WT cells across various bacterial pathogens. And, in cases of tolerant mutants that are very common in clinical settings, it may be possible to target them specifically (20).

The current study from Zeng et al. (8) continues the riveting exploration of the metabolic dependence of many bactericidal antibiotics. The authors establish that the PTS system, via metabolic remodeling, contributes to the lethality of bactericidal antibiotics. The compelling data support the conclusion that the hunger of bactericidal antibiotics for ATP is left insatiable without a functional PTS system (Fig. 1*C*). The combination of readily available genome sequences of clinical isolates together with simple assays to score the tolerance level should undoubtedly encourage the efforts to test the prevalence of *pts* null mutants.

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