Following evolution of bacterial antibiotic resistance in real time

Adam Z Rosenthal & Michael B Elowitz

A new study reports the development of the 'morbidostat', a device that allows for continuous culture of bacteria under a constant drug selection pressure using computer feedback control of antibiotic concentration. This device, together with bacterial whole-genome sequencing, allowed the authors to follow the evolution of resistance-conferring mutations in *Escherichia coli* populations in real time, providing support for deterministic evolution of resistance in some situations.

Microorganisms can and do evolve resistance to antibiotics. In recent decades, the spread of bacterial drug resistance has been accelerated by the widespread use of antibiotics in agriculture and in healthcare. This trend has raised fundamental and urgent questions about the dynamics and genetic basis of bacterial resistance. What specific mutations arise in response to low drug concentrations, and how do additional mutations strengthen resistance at higher drug concentrations? Does resistance result from mutations in just one or a few genes, or is it spread more broadly across the genome? Finally, to what extent are the specific mutations that confer antibiotic resistance and the temporal sequence in which they arise predictable? Addressing these questions requires the ability to map mutations at a genome-wide scale and to do so over time during a continuous evolutionary process. On page 101 of this issue, Roy Kishony and colleagues[1] achieve such real-time measurements by combining whole-genome sequencing with a new, powerful feedback system for continuous cultivation of bacteria under constant selection pressure. This technical advance in laboratory-based evolution is beginning to answer many of these questions.

Microbial evolution studies have been revolutionized by recent applications of whole-genome sequencing. For example, Lenski and colleagues in their groundbreaking evolution experiment identified the specific genetic changes in *E. coli* that occurred over the course of 50,000 generations in the laboratory[2,3]. This work showed the genetic effects of evolutionary adaptation to a repeated environmental regimen (glucose limitation) in parallel cultures. More recently, Collins and colleagues[4] used whole-genome sequencing to analyze the evolutionary adaptation of *E. coli* to the antibiotic norfloxacin. This study identified an altruistic mechanism for antibiotic resistance whereby resistance in a small fraction of cells could support a larger (drug-sensitive) population.

Adam Z. Rosenthal and Michael B. Elowitz are at the Howard Hughes Medical Institute, Division of Biology, California Institute of Technology, Pasadena, California, USA.
e-mail: melowitz@caltech.edu

Morbidostat

In such laboratory evolution studies, it has been technically challenging to analyze evolutionary adaptation to continuous selection pressure from antibiotics under constant conditions. The problem is that as cells become resistant to an antibiotic, its selection pressure is reduced or eliminated. To compensate, one must continually increase the antibiotic concentration in proportion to the increased resistance in the culture[4].

To address this challenge, Toprak *et al.*[1] have now developed a sophisticated computer-controlled microbial cultivation system that can maintain a constant antibiotic selection pressure. Their device, which they name the 'morbidostat', is a modern version of the turbidostat. In both devices, fresh medium enters a vessel at the same rate that culture medium containing cells is expelled, and this rate can be controlled in response to continuous measurements of cell density (**Fig. 1**)[5]. The morbidostat inserts a computer program into this feedback loop and adds additional control over antibiotic concentration. It thereby implements a simple feedback loop in which faster cell growth leads to more antibiotic in the culture, which in turn leads to slower growth, keeping cell densities constant over long periods of time despite evolutionary adaptation.

The morbidostat thus acts like a virtual treadmill whose speed (antibiotic concentration) is determined by cell growth rate. As cells develop resistance to the antibiotic and begin to grow faster (running toward the front of the treadmill), the morbidostat, like a tough coach, compensates by increasing antibiotic concentration (treadmill speed) just enough to keep cells at the original growth rate. Cells that can't keep up die, or are swept off the back of the treadmill, as the most resistant proliferate faster.

The morbidostat allows automated evolution experiments over extended timescales of weeks or longer. Furthermore, because cells are continually expelled from the device, it is possible to sample cells in a nearly continuous manner during the evolutionary experiment and to follow their genetic changes by whole-genome sequencing of cells captured at each time point. Finally, by maintaining 15 replicate cultures in parallel, Toprak *et al.*[1] were also able to explore the reproducibility of mutational trajectories during parallel evolution.

Evolution of resistance

Toprak *et al.*[1] used the morbidostat to examine the evolution of drug resistance in *E. coli* populations grown for up to 25 days under continuous challenge with one of three different antibiotics: trimethoprim, chloramphenicol or doxycycline. They performed whole-genome sequencing of strains sampled over time, identifying specific resistance-conferring mutations and reproducible temporal sequences of their appearance.

In the case of trimethoprim, they found that the majority of mutations for resistance mapped to *DHFR*, the gene encoding the known target of this antibiotic, dihydrofolate reductase. Moreover, when the stepwise accumulation of *DHFR* mutations was tracked by sequencing whole genomes over time, mutations were found to occur in a particular temporal sequence in parallel cultures, suggesting that evolutionary adaptation can be deterministic[6]. This result is broadly consistent with previous predictions from Hartl and colleagues[7], who synthesized a set of combinatorial mutations in the gene encoding β-lactamase, which is involved in antibiotic resistance. These studies, which were limited to a small fraction of possible mutations in just one gene, suggested that some mutational paths could generate monotonically increasing fitness for the cell, although most could not. Toprak *et al.* now provide confirmation that relatively deterministic mutation sequences are indeed possible, even when evolution is

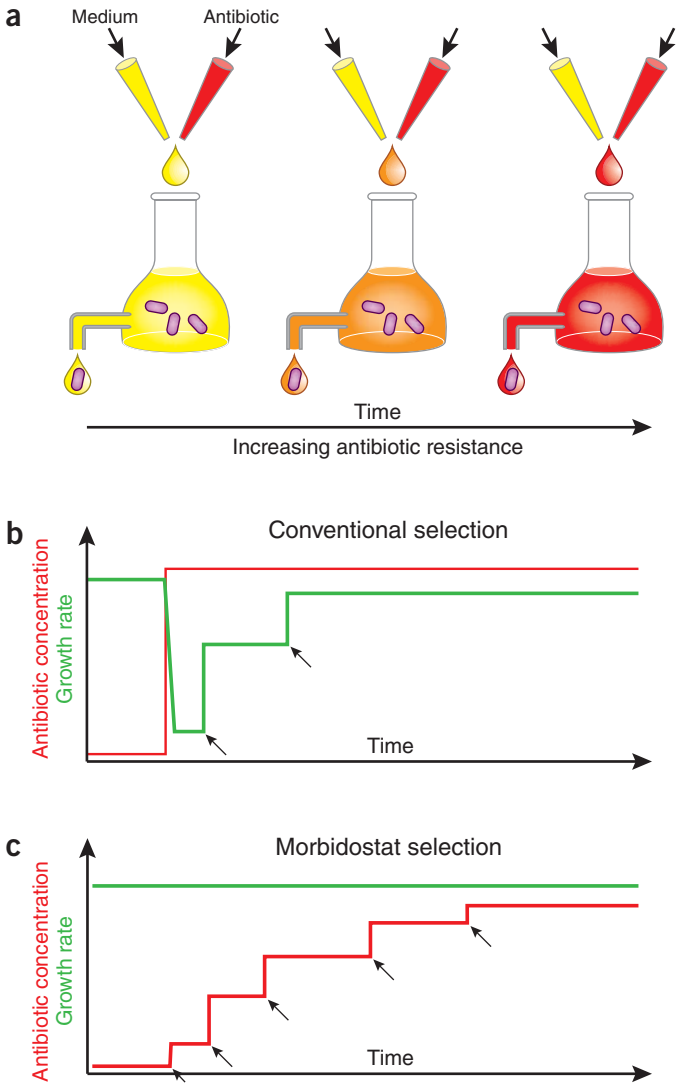

Figure 1 The morbidostat applies a continuous selective pressure to the bacterial population. (**a**) The morbidostat maintains a constant growth rate through feedback control of the mixture of medium (yellow) and medium with antibiotic (red) inflow. As bacteria acquire high levels of resistance to the antibiotic, they are able to tolerate higher antibiotic concentrations (depicted in red) while maintaining a constant growth rate. Thus, antibiotic concentrations increase progressively over time (changing color). (**b**,**c**) Evolution of antibiotic resistance in conventional and morbidostat models of antibiotic resistance. Bacterial growth rate (green line) is shown relative to increasing antibiotic concentration (red line). Black arrows indicate resistance-conferring mutations. In conventional antibiotic selection (**b**), selective pressure is applied by adding a sudden antibiotic challenge to reduce bacterial growth rate. Resistance-conferring mutations emerge and allow the population to regain growth. Under selection in the morbidostat (**c**), the bacterial growth rate is held constant as antibiotic concentrations are automatically adjusted using computer feedback to compensate for acquired mutations that affect the growth rate.

unconstrained rather than artificially limited to a single locus.

Such deterministic genetic trajectories are not universal, however. When the authors used the morbidostat to analyze evolutionary responses to two other drugs—chloramphenicol and doxycycline—they observed qualitatively different behaviors. In response to these antibiotics, they identified resistance-conferring mutations in a larger number of gene targets, many of which are associated with a more general form of multidrug resistance. Nevertheless, most of the mutations clustered into a relatively small number of operons, suggesting that there may be a limited number of ways for the cell to circumvent a given antibiotic. However, in any particular culture, the cells did not exhaust the full evolutionary potential provided by the complete set of advantageous mutations, despite the increase of antibiotic concentration by over three orders of magnitude.

In a complementary approach to that taken by Toprak *et al.*, another group recently developed a microfluidic culture device that addresses issues of spatial heterogeneity in bacterial growth[8]. Natural environments may have strong spatial gradients of antibiotics, which could affect the evolution of resistance[9]. To mimic such conditions, Zhang *et al.*[8] created a hexagonal two-dimensional array of chambers connected by narrow channels and applied a gradient of the antibiotic ciprofloxacin to the device. They followed the growth and movement of a population of cells that were initially sensitive and sequenced the whole genomes of resistant mutants.

Beyond the laboratory

To what extent does this laboratory example of parallel evolution apply to real-life situations? The spread of an epidemic provides an opportunity to track parallel bacterial evolution, as the same strain infects multiple individuals in parallel. Lieberman *et al.*[10] provide an example of this using whole-genome sequencing of frozen clinical *Burkholderia dolosa* isolates taken from patients with cystic fibrosis followed in a single hospital over a 16-year period. Strikingly, similar bacterial mutations were seen to arise independently in different patients, as the pathogen evolved through within-host evolution and transmission between patients. A complementary study by Sebastien Gagneux and colleagues[11], on page 106 of this issue, analyzed the occurrence of rifampicin resistance during tuberculosis infections. This work identified a two-step process in which initial mutations in *rpoB*, the β subunit of the RNA polymerase, conferred resistance and were followed by the acquisition of compensatory mutations in other polymerase subunits that allowed for increased fitness.

Together, these laboratory microbial evolution and epidemiological studies offer key insights into the evolution of bacterial drug resistance. These studies have identified not only new resistance-conferring mutations, but sometimes even the order in which they occur. Furthermore, these results support the view that microbial evolution can be deterministic and, therefore, potentially predictable in some scenarios. The interface between bacterial laboratory and natural evolution studies will provide fertile ground for developing new concepts to understand the processes of resistance and evolution and new technologies to apply to increasingly urgent real-life problems.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Toprak, E. *et al. Nat. Genet.* **44**, 101–105 (2012).
2. Barrick, J.E. *et al. Nature* **461**, 1243–1247 (2009).
3 Woods, R.J. *et al. Science* **331**, 1433–1436 (2011).
4. Lee, H.H., Molla, M.N., Cantor, C.R. & Collins, J.J. *Nature* **467**, 82–85 (2010).
5. Bryson, V. & Szybalski, W. *Science* **116**, 45–51 (1952).
6. Gould, S.J. *Wonderful Life: The Burgess Shale and the Nature of History* 1st edn. (W.W. Norton, 1989).
7. Weinreich, D.M., Delaney, N.F., Depressor, M.A. & Hartl, D.L. *Science* **312**, 111–114 (2006).
8. Zhang, Q. *et al. Science* **333**, 1764–1767 (2011)
9. Hermsen, R. & Hwa, T. *Phys. Rev. Lett.* **105**, 248104 (2010).
10. Lieberman, T.D. *et al. Nat. Genet.* **43**, 1275–1280 (2011).
11. Comas, I. *et al. Nat. Genet.* **44**, 106–110 (2012).

Dnmt3a silences hematopoietic stem cell self-renewal

Jennifer J Trowbridge & Stuart H Orkin

DNA methylation is an epigenetic mark stably directing gene expression throughout development. A new study uncovers a role for the DNA methyltransferase Dnmt3a in silencing self-renewal genes in hematopoietic stem cells (HSCs) to permit efficient hematopoietic differentiation.

Proper orchestration of epigenetic patterns and transcription factors is critical to ensure hematopoietic homeostasis. The recent discovery of mutations in the DNA methyltransferase gene *DNMT3A* in human acute myeloid leukemia (AML)[1] and myelodysplastic syndrome (MDS)[2] highlights the importance of DNA methylation in hematopoietic malignancies. However, the underlying mechanisms by which Dnmt3a regulates hematopoiesis and HSC function remained unclear. On page 23 of this issue[3], Margaret Goodell, Wei Li and colleagues show that Dnmt3a is critical for silencing HSC self-renewal genes, thereby enabling efficient hematopoietic differentiation.

New role for Dnmt3a

DNA methylation is established and maintained by three DNA methyltransferase enzymes: Dnmt1, Dnmt3a and Dnmt3b. During hematopoiesis, Dnmt1 is required for both HSC self-renewal and differentiation[4,5], whereas Dnmt3a has been reported to be dispensable in HSCs[6]. The recent discovery of *DNMT3A* mutations in AML[1] and MDS[2] led Challen *et al.*[3] to re-evaluate the role of Dnmt3a in HSCs by using a conditional knockout mouse model coupled with serial transplantation. In primary transplant recipients, *Dnmt3a*-null HSCs contributed normally to hematopoiesis, as reported in the previous study[6]. Challen *et al.*[3] then went a step further by isolating *Dnmt3a*-null HSCs from the primary transplant recipients and transplanting them into secondary recipient mice to force stem cell turnover. In the secondary transplant recipients, there was a striking 200-fold expansion in phenotypic HSCs, which was sustained through four rounds of serial transplantation. These HSCs did not exhibit increased proliferation or resistance to apoptosis, suggesting that an intrinsic self-renewal program was activated. Consistent with this hypothesis, *Dnmt3a*-null HSCs showed increased expression of genes implicated in HSC self-renewal and multipotency, including *Runx1* and *Gata3* (**Fig. 1a**). Despite the remarkable expansion of *Dnmt3a*-null HSCs, their contribution to overall blood production did not proportionally increase, suggesting a differentiation defect in these cells. *Dnmt3a*-null HSCs generated the full complement of hematopoietic cells, with a bias toward B-cell differentiation, and their multi-lineage differentiation capacity declined with successive rounds of transplantation. The authors conclude that conditional knockout of *Dnmt3a* impedes hematopoietic differentiation while causing the HSC population to expand in the bone marrow.

To uncover the molecular mechanism underlying the phenotype of *Dnmt3a*-null HSCs, Challen *et al.*[3] profiled changes in global gene expression and DNA methylation. Counterintuitively, abundant DNA hypermethylation was observed along with regions of DNA hypomethylation in the *Dnmt3a*-null HSCs, and the correlation between changes in DNA methylation and gene expression was weak. In contrast, a net reduction in global DNA methylation was associated with sustained expression of stem cell–specific genes in *Dnmt3a*-null B cells. These results highlight the context-dependent consequences of *Dnmt3a* loss. The impaired differentiation capacity of *Dnmt3a*-null HSCs was partially restored in rescue experiments, indicating that many of the functional changes are reversible. Taken together, these findings reveal a major role for Dnmt3a in repression of the stem cell program, which it carries out by silencing HSC genes and permitting differentiation, presumably via regulation of DNA methylation.

Direct and indirect mechanisms

These provocative findings raise intriguing questions about the role of Dnmt3a in hematopoiesis. The *Dnmt3a*-null HSC phenotype, revealed by serial transplantation, was likely overlooked in the previous report[6] or perhaps was a result of the different *Cre* drivers used in these studies. The capacity of *Dnmt3a*-null HSCs to function normally in primary transplants calls into question the mechanism underlying the dramatic expansion of HSCs seen in subsequent transplants. Because the number of HSCs residing in the bone marrow is limited by available niche space within the microenvironment[7], the expansion of *Dnmt3a*-null HSCs is surprising, and residency of HSCs outside the bone marrow niche warrants further examination. With respect to hematopoietic differentiation, the bias toward B-cell differentiation calls for elucidation of the precise phenotype of *Dnmt3a*-null lymphoid cells. As Dnmt1 is necessary to promote differentiation into lymphoid progeny[4], such elucidation will provide better insight into the cell context–dependent role of Dnmt3a and the distinct functions of DNA methyltransferases in hematopoietic differentiation.

The poor correlation between changes in DNA methylation patterns and differential gene expression in *Dnmt3a*-null HSCs is surprising. Because the profiling experiments were performed at the population level, the findings may be confounded by the heterogeneity of HSCs. The exact relationship between Dnmt3a-induced methylation and transcription will only be revealed with future technology allowing evaluation of such changes at the single-cell level. The authors attribute the poor correlation

Jennifer J. Trowbridge and Stuart H. Orkin are at the Department of Pediatric Oncology, Dana-Farber Cancer Institute and Division of Hematology/Oncology, Children's Hospital Boston, Boston, Massachusetts, USA.
e-mail: trowbridge@bloodgroup.tch.harvard.edu or stuart_orkin@dfci.harvard.edu