bacterial growth, they isolated an extremely potent diarylquinoline: TMC207. Amazingly, by concentrating on chemicals with 'drug-like' structures, they found a compound that had very favorable pharmacokinetic properties and could be administered to humans without any further chemical modification.

The two other compounds, PA-824 and OPC-67863, are related nitroimidazoles. Compounds of this class act as prodrugs: they are inert until activated by cellular enzymes. Metronidazole is a nitroimidazole antibiotic that is only activated under low-oxygen conditions, and is therefore useful in treating anaerobic infections. Several other compounds of this class are activated in a similar manner and have been investigated for the imaging or treatment of tumors that create a similar hypoxic environment. In fact, the parent compound of PA-824 started as an investigational anticancer drug. What distinguishes PA-824 and OPC-67863 from other nitroimidazoles is their activity against aerobically growing *M. tuberculosis*.

Each of these three compounds was isolated using a different strategy. The parent compound of PA-824 was directly tested for activity against *M. tuberculosis*[4]. The diarylquinoline was identified in a high-throughput screen for its ability to kill the nonpathogenic, rapidly growing species *M. smegmatis*. Matsumoto *et al.* undertook the most complicated strategy[5]. They decided to focus on one of the unique properties of *Mycobacterium*, the requirement for mycolic acid synthesis to make an intact cell wall. In an amazing technical tour de force, this group spent twenty years searching for specific inhibitors of the synthesis of this lipid, ultimately identifying OPC-67683.

Clearly, the identification of an active compound is only the first step in drug development. The subsequent steps that are required to refine a lead compound into a drug critically rely on defining its mechanism of action, which is not a trivial endeavor. Consider the examples of isoniazid and *p*-aminosalicylate, two of the first drugs used to treat tuberculosis. Both were introduced in the 1950s, but the mechanisms by which they act were not elucidated until the past decade[6,7] and are still the subject of lively debate.

In the case of the new compounds, to rapidly identify their mechanism of action, two groups isolated antibiotic-resistant mutants and performed whole genome sequence analysis looking for resistance-associated polymorphisms. A mere 10 years ago, the *de novo* sequencing of a bacterial genome required hundreds of thousands of dollars and several years of work. Taking advantage of the ever increasing efficiency of sequencing technology, Andries *et al.* completely sequenced the genomes of four independent TMC207-resistant isolates and found common mutations in a single subunit of an ATP synthase. Further genetic experiments proved that an identified mutation was responsible for resistance to the drug. ATP synthase is certainly a plausible target, although further structural and biochemical work is required to prove this point.

Similarly, Manjunatha *et al.*[8] used a microarray-based resequencing method to rapidly identify polymorphisms associated with resistance to PA-824. In this case, however, the identified mutations did not define the target but, instead, identified an enzyme that is likely to be required for activation of the prodrug into the active molecule. Somewhat surprisingly, this same enzyme seems to be involved in the activation of both PA-824 and OPC-67863. It is therefore possible that both of these promising new drugs possess similar or even identical activities, although their precise targets remain elusive.

We won't know for sure if these new approaches produced better drugs until they are tested in humans, but with the existing evidence it seems likely that this will be the case. As these compounds have novel mechanisms of action, they kill MDR strains as efficiently as strains susceptible to existing drugs. Perhaps more significantly, the use of any of these three drugs in combination with standard antitubercular drugs results in significantly faster bacterial clearance in mouse models, indicating that they may be useful for shortening tuberculosis treatment by several weeks. So, if even one of these compounds succeeds in human trials, it could represent a significant breakthrough in tuberculosis treatment.

It is, however, too early to conclude that we will be closing the book on tuberculosis any time soon. Although a shorter course of therapy is desirable, only a marked shortening will have a real impact on how we provide care for those with the disease. In addition, we know that drug resistance to the new agents can be easily derived *in vitro*, and that evolution of new resistant strains is virtually inevitable once these drugs are used clinically. So any claim that tuberculosis will soon be vanquished is destined to reside alongside the famous quote that was never uttered by poor William Stewart.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

1. The Office of the Historian of the Public Health Service. <http://lhncbc.nlm.nih.gov/apdb/phsHistory/faqs.html> (2004).
2. Salomon, J.A. *et al. PLoS Med.* **3**, e273 (2006).
3. Andries, K. *et al. Science* **307**, 223–227 (2005).
4. Stover, C.K. *et al. Nature* **405**, 962–966 (2000).
5. Matsumoto, M. *et al. PLoS Med.* **3**, e466 (2006).
6. Vilcheze, C. *et al. Nat. Med.* **12**, 1027–1029 (2006).
7. Rengarajan, J. *et al. Mol. Microbiol.* **53**, 275–282 (2004).
8. Manjunatha, U.H. *et al. Proc. Natl. Acad. Sci. USA* **103**, 431–436 (2006).

Defining the 'survivasome' of *Mycobacterium tuberculosis*

Gyanu Lamichhane & William Bishai

Identification of drug targets in *M. tuberculosis* is a challenge for bench science. High-throughput mutagenesis with transposons together with microarray-based genome and transcriptome profiling has begun to meet this challenge.

The worldwide mortality and morbidity due to tuberculosis remains excessive despite the availability of drugs that can stably cure it. Multiple factors underlie this paradox. One is treatment duration: regimens of multiple drugs that need to be taken for at least six months without interruption means low rates of treatment completion, particularly in poor nations.

The authors are at the Johns Hopkins School of Medicine, 1550 Orleans Street, Baltimore, Maryland, USA.
e-mail: wbishai@jhmi.edu or lamichhane@jhu.edu

Another one is the emergence of multiply and extensively drug resistant (MDR and XDR) strains that are difficult, time-consuming and expensive to treat. The swift lethality of XDR tuberculosis reinforces the necessity of new drugs as global health priority[1].

The solution lies in the development of shortened and simplified tuberculosis drug regimens. Ideally, new drugs will inhibit new molecular targets, assuring activity against the growing burden of resistant microbes; act synergistically in multidrug cocktails; offer compatibility with highly active antiretroviral

© 2007 Nature Publishing Group http://www.nature.com/naturemedicine

therapy (tuberculosis and HIV are often comorbid) and produce effective cure in weeks rather than months. Additionally, it is highly desirable that the genes targeted by these drugs display low mutation frequencies to minimize the selection of resistant strains. Identifying essential pathways used by *M. tuberculosis* to survive during infection and disease offers a rational approach toward improved tuberculosis chemotherapy.

Sassetti *et al.*[2] addressed this challenge by combining two major advances. First, they mutagenized the genome of *M. tuberculosis* H37Rv using the *mariner*-derived transposon *Himar1*. This transposon, efficiently delivered by a mycobacteriophage, randomly inserts into TA dinucleotide sequences and therefore can potentially disrupt all but 16 of the nearly 4,000 genes of the bacterium. They generated a comprehensive collection of thousands of *Himar1* transposon insertion mutants of *M. tuberculosis*. These mutants, by virtue of their ability to survive mutagenesis and grow *in vitro*, can only harbor transposon insertion in genes that are not required for growth.

Next, they used microarrays, prepared using the recently completed[3] genome sequence of *M. tuberculosis* H37Rv, to determine what genes harbored transposon insertions. To do this, they exploited the fact that the DNA sequence across the transposon insertion junction can serve as a unique genetic tag for each mutant. The researchers prepared DNA from a pool of ~100,000 mutants along with total genomic DNA from wild-type *M. tuberculosis* H37Rv. The latter set spans the entire genome, whereas the former contains only genomic DNA adjacent to transposon insertions, which, by definition, should correspond to nonessential DNA. They labeled the two pools of DNA with different fluorescent dyes and cohybridized the DNA onto whole-genome microarrays. If a gene is essential and cannot be mutated, its corresponding microarray spot should not give a signal from the mutant pool. Using this technique—which is dubbed 'TraSH' for transposon site hybridization—Sassetti *et al.* quickly determined the full complement of 'invisible spots'. This assessment, while not a rigorous proof of essentiality, provides invaluable predictive information of the genetic complement required for growth of *M. tuberculosis*. Using TraSH, authors identified the *in vitro* 'survivasome' of the tubercle bacillus.

In a related set of papers, Rubin and colleagues used TraSH to investigate the genetic requirements of *M. tuberculosis* for growth *in vivo* (**Fig. 1**). In two separate studies, they established infections in mouse spleen and in cultured mouse bone marrow–derived macrophages[4,5] with a pool containing thousands of transposon insertion mutants. These analyses identified genes of *M. tuberculosis* that are necessary for growth and survival of the bacterium in these niches *in vivo*. These genes may offer rational targets for drugs that would inhibit pathways essential for infection (see related News and Views by Warner and Mizrahi)[6].

The subtractive TraSH approach has spawned related applications and refinements. In its original form TraSH relied upon large undefined mutant pools, but the technique can also be applied to collections of archived mutants that are deliberately pooled and then assessed simultaneously under a particular stress condition. This approach, called designer arrays for defined mutant analysis (DeADMAn), allows the concurrent analysis of pools containing fewer than 100 defined mutants and therefore permits lower inoculation titers—an important consideration when using the aerosol infection route. With a pool of 530 mutants, the DeADMAn technique has been used to determine genes that are selectively required for survival in the lungs of both mice and guinea pigs after aerosol infection[7,8].

Similarly, applying mathematical modeling to defined *Himar1* transposon collections can be useful because the eligible targets (the 74,403 TA dinucleotide sequences in the *M. tuberculosis* genome) are known *a priori*. For example, armed with the identity of 1,425 existing *mariner* insertions interrupting 770 specific genes, one such algorithm predicted that 35% of *M. tuberculosis* genes would be essential and assigned likelihoods of essentiality to each of the remaining 3,356 eligible genes that were not hit[9].

Another genetic system developed by Graham *et al.*[10] can be used to select RNAs that are synthesized in response to a stimulus. This method, called selective capture of transcribed sequences (SCOTS), was used to isolate mRNAs differentially produced by *M. tuberculosis* during infection in human macrophages. In SCOTS, cDNA is prepared from total RNA extracted from a complex mixture, such as *M. tuberculosis* residing in macrophages. cDNA derived from the bacterial mRNA is selectively captured by hybridization to immobilized bacterial genomic DNA. The desired cDNA is eluted and the processes repeated to enrich the bacterial cDNA. Although microarrays can be used for the same purpose, this tool provides an alternate way of studying transcriptional profiles in situations where microarrays may not be appropriate or available. In addition, SCOTS has proven useful in enriching desired cDNA from a complex infection mixture and improving the sensitivity of microarrays. In a study of infection of human macrophages with *Salmonella*, enrichment of bacterial cDNA using SCOTS greatly improved detection of the bacterial transcriptome by microarray[11]. SCOTS and microarrays make a powerful combination for profiling RNA from pathogens in a complex mixture.

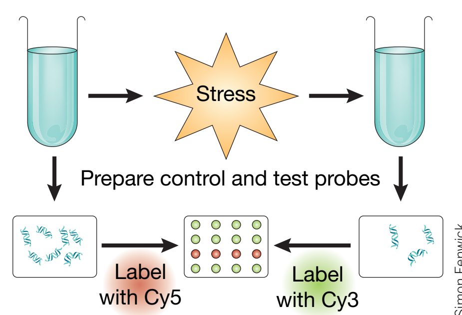

Figure 1 Transposon site hybridization (TraSH). A collection of *M. tuberculosis* transposon insertion mutants are subjected to stress. DNA addresses of input and output samples are prepared by amplifying transposon junction sequences, then labeled with fluorescent dyes and cohybridized onto a microarray. Those mutants that do not survive the stress can be readily identified (red spots).

Recognizing that combination therapy will be mandatory, another challenge for tuberculosis drug development is the selection of optimal targets that may be inhibited without drug-drug antagonism. Understanding drug mechanisms of action is critical for selecting drugs that inhibit a broad spectrum of essential biochemical pathways, in order for therapy to be effective and the possibility of selecting for resistant strains to be minimized. Boshoff *et al.*[12] used whole-genome microarrays to profile the transcriptional responses of *M. tuberculosis* to the inhibitory stress of drugs. Differential responses to 75 different drugs, their combinations, and exposure to altered growth conditions were determined using 430 whole-genome microarrays. The large amount of data collected from these arrays was statistically evaluated and grouped to uncover pathways affected by drugs. The analysis disclosed 150 groups of genes, with which the researchers constructed a genetic map of metabolic processes induced in response to drug treatment—an invaluable roadmap as new antimicrobials are developed.

The map constructed in this study showed many genes in a distinct cluster that defined drugs involved in iron metabolism. With this information, ascididemin was readily identified as affecting iron metabolism in *M. tuberculosis*. An increase in iron concentration in the media made bacteria less susceptible to the drug, confirming that it interfered with iron scavenging. Furthermore, with

© 2007 Nature Publishing Group http://www.nature.com/naturemedicine

microarray profiling the researchers were able to identify the active antimycobacterial agent in a complex mixture. The transcriptional profiles of *M. tuberculosis* when exposed to a crude extract prepared from an invertebrate known to produce ascididemin or to the pure compound were similar. This proof-of-principle shows the power of this system to readily provide insight into drug class and likely mechanism of action—vital information, particularly for antimicrobials that are developed from high-throughput screening[13,14].

Despite being an obligate human pathogen, *M. tuberculosis* inhabits a surprising number of environmental niches within the human host, and it implements survival programs that allow it to persist or proliferate according to changing patterns of immune containment. Post-genomic technologies have begun to define these bacterial survivasomes and offer cause for optimism in the search for shortened and simplified tuberculosis drug regimens.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

1. Gandhi, N.R. *et al. Lancet* **368**, 1575–1580 (2006).
2. Sassetti, C.M., Boyd, D.H. & Rubin, E.J. *Mol. Microbiol.* **48**, 77–84 (2003).
3. Cole, S.T. *et al. Nature* **393**, 537–544 (1998).
4. Sassetti, C.M. & Rubin, E.J. *Proc. Natl. Acad. Sci. USA* **100**, 12989–12994 (2003).
5. Rengarajan, J., Bloom, B.R. & Rubin, E.J. *Proc. Natl. Acad. Sci. USA* **102**, 8327–8332 (2005).
6. Warner, D.F. & Mizrahi, V. *Nat. Med.* **13**, 282–284 (2007).
7. Lamichhane, G., Tyagi, S. & Bishai, W.R. *Infect. Immun.* **73**, 2533–2540 (2005).
8. Jain, S.K. *et al. J. Infect. Dis.* (in the press).
9. Lamichhane, G. *et al. Proc. Natl. Acad. Sci. USA* **100**, 7213–7218 (2003).
10. Graham, J.E. & Clark-Curtiss, J.E. *Proc. Natl. Acad. Sci. USA* **96**, 11554–11559 (1999).
11. Faucher, S.P., Porwollik, S., Dozois, C.M., McClelland, M. & Daigle, F. *Proc. Natl. Acad. Sci. USA* **103**, 1906–1911 (2006).
12. Boshoff, H.I. *et al. J. Biol. Chem.* **279**, 40174–40184 (2004).
13. Andries, K. *et al. Science* **307**, 223–227 (2005).
14. Matsumoto, M. *et al. PLoS Med.* **3**, e466 (2006).

The survival kit of *Mycobacterium tuberculosis*

Digby F Warner & Valerie Mizrahi

Persistence of *Mycobacterium tuberculosis* in humans depends on its ability to survive within the host macrophage. So the bacterium must resist antimicrobial mechanisms or subvert macrophage signaling pathways to prevent its death. Recent evidence suggests that the two strategies are not mutually exclusive.

© 2007 Nature Publishing Group http://www.nature.com/naturemedicine

Macrophages are phagocytes at the frontline of host immune defense against microbial pathogens. They are also the primary habitat of *Mycobacterium tuberculosis*: unlike bacteria that dependent on the avoidance of phagocytosis to survive, *M. tuberculosis* preferentially targets macrophage vacuoles. This apparent incongruity demands that *M. tuberculosis* either tolerate the macrophage's antimicrobial effectors—low pH and reactive oxygen and nitrogen species—or actively subvert normal cellular mechanisms to avoid being killed. Another critical feature of *M. tuberculosis* pathogenesis, extended survival in the host in a state of clinical latency, also requires similar abilities. In this case, however, *M. tuberculosis* must evade or interfere with immune surveillance and signaling pathways. Alternatively, the bacillus must tolerate host defense mechanisms, either through the mobilization of repair or detoxification pathways, or through phenotypic tolerance developed as a result of metabolic adaptation or quiescence. Three recent papers have elucidated different aspects of these strategies[1–3], influencing our current thinking on the relationship between the invading pathogen and its host, and informing an emerging theme in mycobacterial pathogenesis—that *M. tuberculosis* is a well equipped adversary that has adapted exquisitely to life as an intracellular pathogen.

The authors are in the Molecular Mycobacteriology Research Unit and Centre of Excellence for Biomedical Tuberculosis Research, School of Pathology, University of the Witwatersrand and National Health Laboratory Service, de Korte Street, Johannesburg 2000, South Africa.
e-mail:mizrahiv@pathology.wits.ac.za

Macrophages are potent producers of reactive nitrogen species, including nitric oxide (NO), in response to immunostimulatory signals. Previous mouse studies have established a critical role for NO in controlling *M. tuberculosis* infections[4]; however, NO also participates in cellular signaling pathways and respiratory inhibition. Voskuil *et al.*[1] postulated that, in addition to its known antibacterial activity, NO might be involved in bacillary persistence. Using whole-genome expression profiling, the authors investigated the transcriptional response of *M. tuberculosis* to NO. High NO concentrations elicited a response indicative of general cellular stress. By contrast, exposure of the bacterium to nontoxic NO levels or to hypoxic conditions resulted in the rapid induction of a defined set of 48 genes—the 'dormancy regulon'.

In other words, the physiological and metabolic changes associated with adaptation to hypoxia overlap with those occurring in response to low-dose NO exposure. This overlap suggested that the reversibility of bacteriostasis induced by both hypoxia and NO treatment might depend on metabolic shutdown mediated by elements of the dormancy regulon. It also suggested that oxygen and NO might competitively modulate dormancy regulon expression.

Included in the dormancy regulon are genes associated with anaerobic metabolism and stabilization of cellular components, indicating that its transcriptional upregulation might function to prepare the cell for extended periods of metabolic quiescence. Consistent with this model, Voskuil *et al.*[1] observed elevated expression of five sentinel dormancy regulon genes in mouse lungs at 21 days after infection—a time-point that coincides with the onset of immunity and bacterial growth arrest. Notably, parallel studies in mouse macrophages[5] and in the mouse *in vivo*[6] established that upregulation of dormancy genes requires the activity of inducible nitric oxide synthase. Induction of dormancy genes within the mouse phagosome seems therefore to be NO dependent[5]. On the basis of these observations, Voskuil *et al.*[1] proposed a model in which NO production and hypoxia combine to inhibit aerobic respiration and growth of *M. tuberculosis*. That is, NO production might signal the bacterium to adopt a quiescent physiological state.

Subsequent studies[7,8] have shown that the dormancy regulon is controlled by a two-component response regulator system comprising the dormancy survival regulator—DosR—and its corresponding two sensor kinases, DosS and DosT. The precise molecular signals to which the sensors respond have yet to be identified. However, recent evidence[9] of strong antibody