Genetic Approaches to Facilitate Antibacterial Drug Development

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Very few chemically novel agents have been approved for antibacterial chemotherapies during the last 50 yr. Yet new antibacterial drugs are needed to reduce the impact on global health of an increasing number of drug-resistant infections, including highly drug-resistant forms of tuberculosis. This review discusses how genetic approaches can be used to study the mechanism of action of whole-cell screening hits and facilitate target-driven strategies for antimicrobial drug development.

Many classes of antibiotics in clinical use today stem from the golden age of antibiotic discovery, which had its most productive period from 1940 to 1960 (Davies 2006; Silver 2011). These drugs are one of the reasons why most bacterial infections can be cured with a few pills, generally without side effects, within a couple of weeks. It is thus easy to forget that bacterial infections have remained a major cause of preventable deaths in developing countries and continue to exert a profound impact on human health. The relative paucity of novel antibacterials discovered after 1960 coincided with the appearance and contributed to the spread of drug-resistant bacterial infections. When resistant to not only one, but multiple drugs, such infections threaten to erode a cornerstone of modern health care.

Tuberculosis (TB) is a prime example of how treatment has succeeded in developed countries while meeting frequent failure in the developing nations. Treatment failure contributed to the re-emergence of a thought-to-be-conquered dis-

ease that now constitutes as a major public health threat. First-line treatment of TB consists of drugs originally identified during the golden age of antibiotic discovery. Together with social changes, these drugs contributed to a drastic reduction of TB cases in Europe and North America while continuing to kill millions in Africa and Asia. Drug-resistant *Mycobacterium tuberculosis* (*Mtb*) appeared shortly after the introduction of TB chemotherapy and evolved into extremely drug-resistant *Mtb*, which threatens the TB control programs worldwide. Therefore, the need for new therapies for treatment of this deadly disease remains unchanged since the pre-antibiotic era. This review discusses how genetic approaches can facilitate the development of drugs for such new therapies.

GENETICS AND CLASSICAL WHOLE-CELL SCREENING

Whole-cell screening (WCS)-based antibacterial drug discovery begins with identifying small

Editors: Stefan H.E. Kaufmann, Eric J. Rubin, and Alimuddin Zumla
Additional Perspectives on Tuberculosis available at www.perspectivesinmedicine.org

Copyright © 2015 Cold Spring Harbor Laboratory Press; all rights reserved; doi: 10.1101/cshperspect.a021139
Cite this article as *Cold Spring Harb Perspect Med* 2015;5:a021139

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molecules (hits) that inhibit bacterial growth. This inhibitor-first approach provided the foundation of the golden age of antibacterial drug discovery and remains the only development strategy that has delivered antibacterial drugs to the clinic (Brotz-Oesterhelt and Sass 2010). This includes all first-in-class drug candidates currently under clinical development for the treatment of TB (Lechartier et al. 2014). The superior success rate of inhibitor-first strategies is not unique to antibacterial drug development as phenotypic screening has been more productive than target-driven approaches across a spectrum of diseases (Swinney and Anthony 2011).

Several features contribute to the success of classical WCS. These screens allow for selective investment into the chemical structures that can overcome the major challenge of antibacterial drug development: ability to penetrate the bacterial cell envelope. Furthermore, WCS provides an opportunity to discover compounds that inhibit growth by diverse mechanisms, including those that engage multiple targets. The drawback is that the mechanism of all hits remains to be determined.

Whereas it is not required to understand a drug candidate's mechanism of action (MOA) to obtain regulatory approval for use in humans, knowing how a compound exerts its biological function during development is desirable for several reasons. First, many WCS hits act in a nonspecific manner (e.g., as alkylating agents or detergents) and therefore cannot be developed into drugs (Payne et al. 2007; Roemer and Boone 2013). Second, not all hits that are toxic to the host have to be discarded as toxicity need not necessarily result from a compound's antibacterial activity. Antibacterial activity and toxicity are linked if they both stem from inhibition of the same target, for example, an enzyme that is conserved in both the pathogen and the host. Such compounds are not suited for further development. However, in cases in which the mechanism of toxicity differs from that of bacterial growth inhibition, chemical modifications can sometimes eliminate a compound's toxicity without compromising its antibacterial activity. Third, after identifying the target, the structure activity relationship (SAR)

of the compound can be explored in more detail. Fourth, and perhaps most importantly, the antibacterial MOA of a compound is a determinant of its in vivo potency. This is exemplified by recently identified pyrimidine-imidazoles, which were active against *Mtb* in vitro, had appropriate pharmacokinetic properties, but showed no potency in *Mtb*-infected mice (Pethe et al. 2013). The activity of these molecules relied on the presence of glycerol, which is used in many standard liquid media, but it is not a physiological carbon source. Therefore, lack of in vivo potency in these pyrimidine-imidazoles was mechanism-based, leaving no path to develop these compounds further.

The potential for discovering other compounds with MOAs that intrinsically have no in vivo potency (here referred to as *novip* compounds) is significant because screening media can, at best, only mimic some of the environments encountered during an infection. More often, they create a physiological context that is quite different from that within the host as many standard media contain nutrients *Mtb* cannot obtain from the host in the provided form (e.g., iron) (Rodriguez and Smith 2006), cannot obtain from the host at all (e.g., biotin) (Sassetti and Rubin 2003; Woong Park et al. 2011), and lack nutrients that *Mtb* is apparently able to scavenge from the host (e.g., NAD precursors) (Boshoff et al. 2008). These differences can cause some mutations to either result in death of *Mtb* in vitro but not during infections (e.g., mutations in *nadA* [Boshoff et al. 2008]) or prevent growth during infections but not in standard media (e.g., mutations required to scavenge iron from the host [Rodriguez and Smith 2006]). One conceptually straightforward strategy to reduce the risk of identifying *novip* compounds is to screen against intracellular *Mtb* (Christophe et al. 2009). However, such screening procedures can also reveal genes that are required ex vivo but not during an infection (Munoz-Elias et al. 2006). Furthermore, *Mtb* induces a complex and dynamic pathology and can be found in multiple different intracellular and extracellular locations (Barry et al. 2009; Dartois and Barry 2013). Some of these locations permit the pathogen to replicate, whereas others may

restrict its growth and yet allow its persistence. Therefore, it seems impossible to design screening conditions that mimic the pathophysiological context of an *Mtb* infection in humans well enough to completely bias against *novips*. The best insurance against MOA-based attrition is therefore a solid understanding of a compound's MOA.

Genetic Strategies for Studying the MOA of Bioactive Small Molecules' RNA Profiling

A cell's genome-wide RNA profile is defined by the abundance of the total RNA produced at a given time. Taking snapshots of these profiles became straightforward after DNA microarrays were invented (Schena et al. 1995; Lashkari et al. 1997), and they can now be reported with even higher resolution by sequencing total RNA extracts (Nagalakshmi et al. 2008; Wilhelm et al. 2008; Arnvig and Young 2012). The foremost study to show the use of genome-wide RNA profiling for characterizing the MOA of bioactive molecules was performed with the anesthetic dyclonine (Hughes et al. 2000). Hughes et al. collected *Saccharomyces cerevisiae* RNA profiles induced by hundreds of different genetic or chemical treatments. This generated a compen-

dium of reference profiles to which the RNA profile induced by dyclonine could be compared. Pattern matching algorithms revealed that dyclonine caused changes similar to those induced by mutations in the ergosterol pathway. This suggested that dyclonine inhibited sterol biosynthesis and follow-up experiments confirmed the sterol isomerase Erg2p as a target of dyclonine. This pattern matching approach, as illustrated in Figure 1, has since been used to uncover targets and the MOA of many other antimicrobial compounds (Brazas and Hancock 2005; Smith et al. 2010; Wecke and Mascher 2011). Indeed, *Mtb* microarrays were first used to study the effect of isoniazid on the RNA profile of *Mtb* (Wilson et al. 1999). Hundreds of compound-induced RNA profiles were reported 5 yr later in a landmark study by Boshoff et al. (2004). These profiles helped to investigate a variety of antimycobacterial small molecules, including capreomycin (Fu and Shinnick 2007), isoniazid (Karakousis et al. 2008), PA-824 (Manjunatha et al. 2009), an inhibitor of menaquinone production (Dhiman et al. 2009), β -lactams (Slayden and Belisle 2009), vancomycin (Provvedi et al. 2009), the benzothiazinone, BTZ043 (Makarov et al. 2009), the natural product chelerythrine (Liang et al. 2011), thioridazine (Thorsing et al. 2013), and

Figure 1. RNA profiling. RNA molecules produced by four genes of the *Mtb* genome with and without drug treatment are represented as colored lines. The color of the squares depicted below the RNA molecules reflects their abundance, with darker colors identifying more abundant RNAs. The fictional data here would suggest that the compound of unknown MOA functions in a manner similar to RIF.

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several other WCS hits (Stanley et al. 2013; Wang et al. 2013).

The principal limitations of RNA profiling are twofold. First, these profiles fail to identify the direct target of a compound as they only reveal the biological pathway(s) that respond to the drug. In part, this is because inhibition of different proteins can produce similar RNA profiles, such as in situations in which the proteins form a complex or are required for the same metabolic pathway. Second, the profiles (and other pattern matching approaches) are most informative for compounds, which induce a signature similar to the compounds with a known MOA. Specific predictions are difficult to make for compounds with an entirely novel MOA as appropriate reference profiles are lacking in such cases. This drawback can be overcome by enriching the reference compendium with RNA profiles from genetically defined mutants (Hughes et al. 2000; Mnaimneh et al. 2004; Freiberg et al. 2005). Unfortunately, too few such profiles are available for *Mtb* to apply this principle to facilitate the analysis of antimycobacterial compounds.

Even for relatively small bacterial genomes, genome-wide RNA profiles consist of thousands of individual measurements. This complexity offers the potential to generate unique signatures for many bioactive small molecules while making it difficult to identify the most informative RNAs. The amount of data generated by RNA profiling is not only large, but more importantly, the biological responses to growth perturbation are often complex. This foremost applies to RNA profiles collected at time points or at drug concentrations that impact the abundance of hundreds of RNAs. The nonspecific stress responses contained in such profiles can mask a compound-specific response. Reducing the treatment time and/or drug concentration often simplifies the analysis of bioactive molecules by RNA profiling. Furthermore, the ability of genome-wide RNA analyses to detect minute changes in any single gene coupled with a highly dynamic transcriptional response prevalent in bacteria increases the existing complexity by severalfold. Therefore, obtaining very similar RNA profiles in two ap-

parently identical experiments can be a challenge. Description of all experimental details, strict standardization, and avoidance of procedural changes of gene expression (as can occur, for example, by centrifugation of live *Mtb*) are therefore crucial for producing informative RNA profiles.

Selection and Identification of Resistance Mutations

The first indication that bedaquiline (BDQ, formerly known as TMC207 and originally published as R207910) prevents the growth of *Mtb* by inhibiting the adenosine triphosphate (ATP) synthase was provided by the mutants selected for their ability to grow in the presence of BDQ (Andries et al. 2005). Whole-genome sequencing of these BDQ-resistant strains revealed point mutations in *atpE*, which encodes subunit c of ATP synthase. Genetic and biochemical studies further established that BDQ inhibits ATP synthesis by binding to AtpE, thus confirming ATP synthase as a direct target (Koul et al. 2007). Selecting for resistance followed by whole-genome sequencing (Fig. 2A) has identified mutations that permit growth in the presence of several other small molecules with antimycobacterial activities. Examples include mutations in genes involved in respiration (*qcrB*) (Abrahams et al. 2012b; Pethe et al. 2013), fatty acid synthesis (*inhA*) (Hartkoorn et al. 2012), protein synthesis (*aspS*) (Ioerger et al. 2013), protein secretion (*eccB3*) (Ioerger et al. 2013), polyketide biosynthesis (*pks13*) (Ioerger et al. 2013; Wilson et al. 2013), mycolic acid transport (*mmpL3*) (Grzegorzewicz et al. 2012; La Rosa et al. 2012; Stanley et al. 2012; Tahlán et al. 2012; Ioerger et al. 2013; Remuinan et al. 2013), and arabinogalactan synthesis (*dprE1*) (Christophe et al. 2009; Makarov et al. 2009; Magnet et al. 2010; Stanley et al. 2012; Wang et al. 2013).

Resistance mutations can directly identify high-value drug targets. Selection and identification of such mutations requires no target-specific assay development, can be fast, and is comprehensive as it surveys the entire genome. However, this approach is not without limitations and fails when a resistant strain cannot be

Figure 2. Forward and reverse genetic approaches to investigate small molecule MOAs. (A) Selection of resistant mutants. Mutants that are resistant to the compound are selected on agar plates supplemented with the compound of interest. The mutations present in these resistant isolates are identified by whole-genome sequencing. (B) Under/over-expression. Reducing or increasing the expression of a candidate target (black circles) can either decrease or increase the MIC of a compound (yellow triangle) inhibiting the target. (C) Phenotyping pooled mutants. Mutants are labeled with a genetic barcode and their fitness is analyzed in a competitive growth experiment. This principle can be applied to deletion mutants, underexpressors, overexpressors, or a mix of these strains. The orange-label mutant shows increased sensitivity to the tested drug; the black-labeled mutant (which is only shown in the graph) shows decreased sensitivity.

obtained. Furthermore, alteration of the target responsible for the growth inhibitory effect of a small molecule constitutes only one of the several mechanisms by which mutations can impart resistance. Alternatively, methods such as chemical inactivation, efflux, or failure to transform an inactive prodrug into its active derivative can form the basis of resistance. The knowledge of mutation frequencies that cause resistance by these mechanisms proves highly useful to determine the value of a compound. A high frequency of resistance owing to such mechanisms significantly devalues the compound. However, this knowledge rarely plays a role in defining a compound's MOA. Nevertheless, in scenarios in which a mutation can be mapped to a true target, resistance mutation provides one of the simplest ways to define a compound's MOA.

Linking Small Molecules to Candidate Targets by Increasing or Reducing Gene Activity

To be *directly* involved in the MOA of a bioactive small molecule, a protein has to physically interact with that small molecule. That a candidate protein has the potential for such an interaction within live bacteria can be confirmed indirectly by showing an immediate correlation between the protein's abundance and the minimal inhibitor concentration required to inhibit growth (MIC) (Fig. 2B). This type of experiment has helped to clarify the MOA of INH (isoniazid), which was debated to impair one of two enzymes involved in fatty acid biosynthesis, InhA or KasA. Strains that overexpressed these proteins were analyzed for their susceptibilities to INH and the KasA inhibitor thiolac-

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tomycin (TLM). Overexpression of InhA increased the MIC of INH but did not change the MIC of TLM; overexpression of KasA had the opposite effect (Larsen et al. 2002). These results supported the view that InhA and KasA are primary targets of INH and TLM, respectively. Similar overexpression studies have often been performed with *Mycobacterium smegmatis* in which the MOA of different inhibitors was linked to one or more targets, including D-cycloserine to alanine racemase (Alr) and D-alanine-D-alanine ligase (Ddl) (Feng and Barletta 2003), platensimycin to KasA and KasB (Brown et al. 2009), ethambutol to the arabinosyltransferases EmbB and EmbC (Goude et al. 2009), and certain diphenylurea compounds to inosine monophosphate dehydrogenase (Olaleye et al. 2010; Usha et al. 2011).

In mycobacteria, overexpression has primarily been used to evaluate the role of specific candidate target proteins for compound activity, but it can also provide a means to find candidate targets for a compound with an entirely unknown MOA. To achieve this, libraries of overexpression clones are systematically screened for those that improve growth in the presence of the inhibitor. Such a screen, which used an ordered library of overexpression clones for all essential *Escherichia coli* genes, identified the lipoprotein chaperon, LolA, as a candidate target of the novel antibacterial compound, MAC12343. Biochemical studies confirmed that MAC12343 binds LolA and perturbs lipoprotein trafficking (Pathania et al. 2009). Similar overexpression libraries are under construction for *Mtb* and will hopefully be available soon.

A complication of overexpression screens, especially when using random libraries, is the frequent isolation of efflux pumps. These pumps increase resistance by lowering the intracellular concentration of the compound under investigation, and their isolation does not reveal a compound's MOA. Difficult to interpret MIC shifts can also occur if the candidate target belongs to a protein family of which several members are encoded in the same genome. If the inhibitor binding site is conserved, overexpression of any member of such a protein

family will increase the MIC even when only one family member is the physiological target. This has been discussed for inosine-5'-monophosphate (IMP) dehydrogenases (GuaB1/2/3) (Usha et al. 2011), ketoacyl-ACP synthases (KasA/B) (Brown et al. 2009), and methionine aminopeptidases (MapA/B) (Olaleye et al. 2010). In some of these cases, the MIC may have increased because the overproduced protein sequestered the inhibitor from its physiological target.

As target overexpression should increase a compound's MIC, so should target underexpression decrease the MIC (Fig. 2B). This was indeed observed for several conditional *M. smegmatis* knockdown mutants described recently. Underexpression of gyrase subunit A (GyrA, target of quinolones [Sugino et al. 1977], Alr [Caceres et al. 1997], dihydrofolate reductase [DHFR, target of trimethoprim] [Schweitzer et al. 1990], RNA polymerase subunit B [RpoB, target of RIF] [Lancini et al. 1969]), and protein biotin ligase (BirA, target of BioAMS [Duckworth et al. 2011]) sensitized *M. smegmatis* to ciprofloxacin, D-cycloserine, trimethoprim, RIF, and BioAMS, respectively (Duckworth et al. 2011; Kim et al. 2010; Wei et al. 2011). Similar observations were made with *Mtb*, in which partial genetic inactivation of the biotin synthesis enzyme BioA or DHFR increased its susceptibility toward the novel inhibitors of these enzymes (Shi et al. 2011; Kumar et al. 2012). For many of these mutants, it was also shown that they were most strongly sensitized to inhibitors of the partially depleted protein. These and other data (Glaever et al. 1999; DeVito et al. 2002; Lum et al. 2004; Yin et al. 2004; Donald et al. 2009; Abrahams et al. 2012a; Ollinger et al. 2012) confirmed that partial depletion of an in vitro essential protein often causes compound-specific MIC shifts and can thus provide information on the MOA of a bioactive small molecule. However, as is the case for overexpression, underexpression studies by themselves are not sufficient to claim the underproduced protein as the MIC determining target of an inhibitor, as underexpression could also decrease the MIC by sensitizing other enzymes in the same pathway to chemical inhibition.

That underexpression nevertheless provides a powerful tool for identifying candidate targets is apparent from work with *S. cerevisiae*. Construction of underexpressors is relatively straightforward in *S. cerevisiae* and is often achieved with deletion of one gene copy. Such heterozygous deletion strains are available for almost all *S. cerevisiae* genes including those that are essential for normal growth. This mutant collection has been used extensively to study the MOA of bioactive small molecules by drug-induced haploinsufficiency profiling (HIP). HIP measures the effect of sublethal drug concentrations on the relative growth rates of individual mutants (Fig. 2C). Primarily, these studies confirmed that the mutants of known targets of antimicrobial agents are among those most susceptible to growth inhibition by the respective drug (Giaever et al. 1999, 2004; Lum et al. 2004) and were later implemented in identification of novel candidate targets for established drugs. This included the rRNA processing exosome, which was predicted by HIP (Giaever et al. 2004; Lum et al. 2004) and confirmed by various experiments (Fang et al. 2004; Hoskins and Scott Butler 2007; Hoskins and Butler 2008; Kammler et al. 2008) to be involved in the MOA of the anticancer drug 5-fluorouracil. In the opportunistic pathogen *Candida albicans*, HIP helped to identify the guanine monophosphate synthase (Rodriguez-Suarez et al. 2007), a fatty acid desaturase (Xu et al. 2009), and poly(A) polymerase (Jiang et al. 2008) as targets of novel inhibitors. Experiments conceptually similar to HIP have been performed in bacteria where gene expression can be efficiently reduced by antisense RNAs (Donald et al. 2009; Huber et al. 2009; Xu et al. 2010). Unfortunately, antisense RNAs have only rarely been efficient in *Mtb*. Currently, the generation of a large collection of *Mtb* underexpressors depends on approaches that require homologous recombination during mutant construction, such as transcriptional or proteolytic silencing (Ehrt et al. 2005; Wei et al. 2011).

WCS hits are growth inhibitory by definition and thus should primarily target the essential gene products. Nevertheless, mutations in nonessential genes often modulate a com-

pound's activity. Evidence for this came from several studies including those that used the *E. coli* Keio collection. This library contains almost 4000 mutants each of which has a single gene deleted. Of these 4000 mutants, 283 showed greater sensitivity than wild type to at least one of the 14 different antibiotics tested (Tamae et al. 2008; Liu et al. 2010). Whereas some strains were more susceptible to several classes of antibiotics, others were only sensitized to compounds with the same MOA (Girgis et al. 2009; Liu et al. 2010). The drug sensitivity profiles of the latter mutants can be used to classify a compound's MOA with strategies similar to those implemented in RNA profiling. The discriminative power of these profiles can be enriched with mutations that decrease antibiotic susceptibility, which in *E. coli* are as frequent as those that increase susceptibility (Girgis et al. 2009).

The homozygous deletion mutants in *S. cerevisiae* are equivalent to the *E. coli* Keio mutants. Chemical profiling of such mutants (homozygous deletion profiling, HOP) has been particularly useful for compounds whose primary target is not a protein. For example, HOP of ~4700 *S. cerevisiae* mutants revealed that fitness defects caused by deletion of genes involved in DNA repair or in survival during DNA damage can be diagnostic for DNA-damaging agents. In fact, the profiles of these mutants were distinct for chemicals that damage DNA by different mechanisms as they could distinguish compounds that generate inter-strand cross-links from those that do not (Lee et al. 2005). The interpretation of HOP experiments can be facilitated by data from genetic interaction studies. Two genes are defined as interacting if the phenotype of their double mutant deviates from the expected phenotypes of the two single gene mutants (Dixon et al. 2009). In *S. cerevisiae*, many such digenic interactions have been defined. These interactions are valuable for analyses of bioactive small molecules as the genetic interaction profile of a gene can be similar to the HOP profile of a small molecule and thus link the small molecule to the respective gene (Parsons et al. 2004, 2006; Costanzo et al. 2010).

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FACILITATING TARGET-BASED DRUG DISCOVERY

Target-based approaches typically begin with a screen for inhibitors of a purified enzyme. Unfortunately, most inhibitors identified in several high-throughput screenings (HTSs) against bacterial enzymes had no activity against live bacteria (Payne et al. 2007). Nevertheless, target-based approaches have their own advantages. Primarily, MOA studies are more straightforward for a target-based approach than for WCS hits. For example, a bisubstrate inhibitor (BioAMS) designed to inactivate the protein biotin ligase (BirA) was recently found to inhibit the growth of *Mtb*. The study conducted by Duckworth et al. (2011) examined the impact of BioAMS on growth and biotinylation of proteins in *M. smegmatis* and *Mtb*. As expected, the activity of BioAMS (but not of INH or ethambutol) against *Mtb* and *M. smegmatis* directly correlated with expression of BirA, and growth inhibition coincided with the depletion of biotinylated proteins. Together, these straightforward observations confirmed BirA as the primary target of BioAMS (Duckworth et al. 2011). The second main advantage of target-based approaches is that they can survey a much larger chemical space than what can be covered in phenotypic screens, especially if fragment-based approaches are included (Scott et al. 2012). This is significant because the physicochemical properties of most molecules in a typical WCS library are different from those of known antibacterials (Brotz-Oesterhelt and Sass 2010).

The Power and Limits of Genetic Target Validation

Choosing a poor target will doom a target-based drug discovery program from the start, yet this might become obvious only after years of work and millions of dollars have been invested. Identifying appropriate targets is thus crucial. Ideally, the selected protein should be (1) conserved among all isolates of the targeted pathogen(s), (2) susceptible to inhibition by a small molecule, (3) different enough from any human homolog to allow development of low

toxicity inhibitors, (4) amenable to biochemical HTS, and (5) required for bacterial growth (or survival) in a manner that its partial inactivation suffices to improve a patient's health. Showing that a target fulfills all these criteria requires a small-molecule inhibitor that is safe for use in humans (i.e., a drug). Trying to develop drugs against new targets, therefore, always bears the additional risk that comes from working against a target that is yet to be fully validated. This additional risk applies to inhibitors identified from either WCS or biochemical screens and is one of the factors causing the high rate of attrition of compounds with entirely novel MOAs (Kola and Landis 2004).

Druggability assessments evaluate potential binding pockets by computational or chemical methods (Perot et al. 2010; Barelier and Krimm 2011; Fauman et al. 2011); sequence conservation—within bacteria and between bacterial and human homologs—can be assessed with genome sequence data. An important contribution of genetics to the evaluation of potential targets is its ability to determine the importance of a potential target for growth and survival. The most complete source for this information stems from transposon mutagenesis. Many of the genes that *Mtb* requires to grow normally in vitro (Sasseti et al. 2001, 2003), in macrophages (Rengarajan et al. 2005), or in mouse spleens (Sasseti and Rubin, 2003) were defined by *transposon site hybridization* (TraSH). TraSH uses DNA microarrays to simultaneously map and analyze the phenotypic consequences of thousands of transposon insertions. Recently, Tn-seq, which uses next-generation DNA sequencing instead of microarrays, has improved these analyses by providing single-nucleotide resolution and a larger dynamic range (Griffin et al. 2012; Zhang et al. 2012, 2013). TraSH and Tn-seq have contributed more to the phenotypic characterization of gene functions in *Mtb* than any other genetic approach. However, they are not ideal to validate gene products as targets for drug development for two reasons. First, TraSH and Tn-seq can identify in vitro essential genes, but neither approach generates mutants for such genes that could be analyzed under a variety of conditions. In the same man-

ner as WCS can identify *novip* compounds, genetic screens can identify *novip* targets (i.e., targets whose inactivation kills *Mtb* under a specific in vitro condition but does not prevent persistence or even growth in animals or humans). It is therefore important to evaluate the role of a gene product for survival under a variety of conditions including those that induce nonreplicating persistence. For genes that *Mtb* requires to grow under standard laboratory conditions, which are among the most attractive targets for drug development, these analyses require conditional knockdown mutants.

The construction of conditional knockdown mutants in *Mtb* became possible with the development of chemically controlled genetic switches for mycobacteria (Schnappinger and Ehrt 2014). Many of the switches developed for mycobacteria depend on placing the gene of interest downstream from a modified promoter so that its transcription can be regulated by a tetracycline repressor and a tetracycline derivative (Blokpoel et al. 2005; Carroll et al. 2005; Ehrt et al. 2005; Klotzsche et al. 2009; Boldrin et al. 2010). These regulatory systems facilitated the analyses of several genes in *Mtb* (Schnappinger and Ehrt 2014), but they depend on secondary events, most notably cell division, to deplete the protein of interest. They are therefore less suited to study the importance of gene functions for survival of nonreplicating *Mtb*. Analyses of nonreplicating bacteria can be performed more efficiently with regulatory systems that directly increase protein turnover. In *Mtb*, this can be achieved by modifying a gene's 3' end so that it encodes the sequence of the DAS+4 degradation tag (Kim et al. 2010). Conditionality is achieved by controlling the expression of a protein (SspB) that activates the degradation tag. Recently, transcriptional and proteolytic modes of regulation have been combined in a dual-control (DUC) switch, which can achieve efficient and robust gene inactivation in growing and nonreplicating *Mtb* (Kim et al. 2013). Application of the DUC switch to analyses of the NAD synthetase (NadE) identified this enzyme to be essential for survival of both replicating and nonreplicating bacteria. Its inactivation was further suf-

ficient to rapidly eliminate culturable bacteria from mouse lungs and spleens during acute and chronic infections (Kim et al. 2013). Essentiality of NadE for viability of *Mtb* under all conditions tested so far strongly suggests that this enzyme is also required for growth and survival under most (if not all) conditions that *Mtb* encounters in humans. Whether it allows the development of small-molecule inhibitors that would be safe for use in humans remains to be shown.

The second limitation of TraSH and Tn-seq is that they do not allow measurement of the extent to which a gene product needs to be inactivated to impair growth. Although the impact of a transposon insertion on gene function can vary with its insertion site, the phenotypes reported by TraSH/Tn-seq are often attributable to insertions that create a null allele. Complete inactivation of a protein in live bacteria is rarely, if ever, achieved by small molecules. The phenotypes that are caused by complete deletion are thus less relevant to evaluating a new target for drug development than those that result from its partial depletion. All other factors being equal, a protein that needs to be inactivated by >90% (or less) is a more attractive target than one that needs to be inhibited by 90% before growth is inhibited. That vulnerability of mycobacteria to partial depletion indeed varies among different essential proteins was first shown in *M. smegmatis*. For example, HIV-protease-induced degradation depleted *M. smegmatis* of >97% of DHFR and Alr, but this depletion only slowed growth down. In contrast, even modest depletion of RpoB was sufficient to stop growth entirely (Wei et al. 2011). Unfortunately, the extent of functional inactivation that is necessary to impair the growth of *Mtb* is known only for a few proteins, including BioA and PptT, which are required for the synthesis of biotin and polypeptides, respectively. *Mtb* is not particularly vulnerable to partial inactivation of either enzyme, as both require to be depleted by >90% to affect growth (Woong Park et al. 2011; Leblanc et al. 2012). Applying conditional gene inactivation systems to the characterization of more genes will hopefully identify proteins that need to be depleted >90% to stop *Mtb* from growing.

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Target-Directed WCS

Classical WCS often requires complex studies to define the MOA of hits; biochemical screens rarely identify inhibitors that can penetrate the bacterial cell envelope. Both difficulties can be overcome with target-directed WCS. The principle of this approach was first established in *Staphylococcus aureus*. Overexpression of a *fabF* antisense RNA caused the 5' end of the *fabF* mRNA to be degraded. This decreased the expression of FabF, an enzyme required for fatty acid biosynthesis, and increased the sensitivity of *S. aureus* to FabF inhibitors (Young et al. 2006). Agar diffusion assays were then used to screen small-molecule libraries against wild-type *S. aureus* and the FabF underexpressor. Molecules that were more active against the underexpressor included platencin and platensimycin, two novel inhibitors of fatty acid biosynthesis with broad-spectrum activity against Gram-positive bacteria (Wang et al. 2006, 2007; Jayasuriya et al. 2007; Fischbach and Walsh 2009). In *Mtb*, transcriptional repression instead of antisense RNA has been used to generate mutants expressing lower than wild-type levels of pantothenate synthase (PanC), diaminopimelate decarboxylase (LysA), isocitrate lyase (Icl1), or the type I signal peptidase (LepB). In addition, these recently constructed mutants show target-specific changes in their susceptibility to different small-molecule inhibitors (Abrahams et al. 2012a; Ollinger et al. 2012). WCS with these strains promises to identify new inhibitors that are able to reach the cytoplasm of *Mtb* and inhibit a desired target.

2000 million (Adams and Brantner 2006; Paul et al. 2010). Late-stage attrition can therefore drain the resources of commercially unattractive areas, like antibacterial drug development, and cause them to fail entirely over a long period of time. The two main causes of late-stage attrition are lack of safety and efficacy in people (Kola and Landis 2004; Bennani 2012). Predicting clinical efficacy will remain imprecise. However, if we understand the MOA of the drug candidates that are being tested in the clinic, it should be possible to avoid trials with chemically different compounds that fail for the same mechanistic reasons. The genetic approaches discussed here have helped to characterize the MOA of several antibacterial compounds. However, the MOA of many antibacterials is complex and new insights are still gained into the MOA of drugs that have been in clinical use for decades (Chakraborty et al. 2013). No single approach will therefore be sufficient to define the MOA of most antibacterials. Studies that combine several approaches—for example, underexpression, overexpression, resistance selection, and biochemical studies (Huber et al. 2009)—promise to be the most successful. Drug candidates with novel MOAs are attractive because they could improve TB chemotherapy fundamentally (e.g., by reducing its duration). But they bear a higher risk of late-stage failure if they inactivate targets that have not yet been clinically validated. Measuring the effects that can be achieved by inactivating such novel targets (e.g., by genetic means) in a variety of assays, including animal models, seems prudent.

Second, genetics can also contribute to drug development by providing single-gene underexpressors that help increase the sensitivity of WCS and bias hits toward a desired target. Underexpressors can furthermore be used in a secondary assay to evaluate biochemical HTS hits. This distinguishes compounds that penetrate the bacterial cell envelope poorly (and are not active against wild type but active against an underexpressor) from those that do not penetrate at all (and are active neither against wild type nor the underexpressor). Such compounds could be starting points for medicinal chemistry efforts to improve compound efficacy against wild-type

CONCLUDING REMARKS

None of the approaches discussed in this review existed during the golden age of antibiotic discovery, and antibacterial drug discovery can be pursued without them. However, modern genetics can help improve a process that has delivered few novel molecular entities during the last 50 yr. One important contribution would be to help reduce late-stage attrition (i.e., failure in clinical trials). The cost of introducing a new drug to the market varies between US\$500 and

bacteria, which could expand the chemical space into the area that is effective against bacteria and underrepresented in most screening libraries.

ACKNOWLEDGMENTS

I thank Kathryn O'Brien, Shipra Grover, and Sabine Ehrt for critically reading the manuscript. I am grateful for past and present support from the National Institutes of Health, the Bill & Melinda Gates Foundation, The Wellcome Trust, and the Ellison Medical Foundation.

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Cold Spring Harb Perspect Med 2015; doi: 10.1101/cshperspect.a021139 originally published online February 13, 2015

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