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Chemical Tools for Dissecting Bacterial Physiology and Virulence[†]

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ABSTRACT: Increasingly, chemical biology is being used in the context of bacterial virulence and the host−pathogen interaction, as small molecule inhibitors provide a number of unique advantages for the study of bacterial pathogens that complement powerful, existing classical genetic approaches. Small molecules have the potential to inhibit targets rapidly and reversibly, with a high degree of specificity. They are therefore well suited for studying the role of essential genes in bacterial physiology and virulence in both genetically tractable and intractable organisms, with the capacity to reveal novel phenotypes and insights into the function of essential factors during infection. The use of small molecule inhibitors during infection is also deepening our understanding of the role that host factors play in bacterial pathogenesis. In the future, the utility of chemical biology will grow as technologies for rapid identification of targets of interesting bioactive small molecules are developed. In this review, we highlight recent work in which small molecule inhibitors are used to study essential genes and genetically intractable organisms, to reveal novel phenotypes related to bacterial physiology, and to probe the role of bacterial and host factors during infection. In addition, we review recent advances in biochemical, genetic, and genomic techniques for target identification.

The discovery of antibiotics in the 20th Century marked a turning point in the struggle against infectious disease, providing the ability to effectively combat infection with bacterial pathogens. At the dawn of the 21st Century, however, we are in danger of losing many of the most important weapons in our arsenal against infectious disease because of bacterial evolution of antibiotic resistance. Most of the antibiotics in use today were discovered in the 1940s and 1950s. Since the 1960s, linezolid and daptomycin represent the only new classes of clinically useful antibiotics that have been discovered and approved for use. As a result, bacterial infections that are difficult to treat using current antibiotics are becoming more widespread, constituting a significant threat to public health.

Methicillin-resistant *Staphylococcus aureus* (MRSA)[1] and *Mycobacterium tuberculosis* are two different pathogens that illustrate the breadth and depth of the problem of emerging antibiotic resistance. MRSA infection is considered to be an emerging infectious disease, and the number of reported MRSA infections has increased significantly in the past decade, more than doubling between 2000 and 2005 (*1*, *2*). It has been estimated that MRSA infection now results in approximately 18650 deaths per year in the United States, killing more people annually in the United States than AIDS (*2*). *M. tuberculosis* is a bacterium that has been a pathogen of humans for millennia and continues to infect an estimated 2 billion individuals, resulting in ∼2 million deaths annually. In the past few years, strains of *M. tuberculosis* that are resistant to many of the first- and second-line TB drugs have emerged. In 2007, an estimated half a million cases of multi-drug-resistant tuberculosis infection were reported, accounting for up to 13% of newly diagnosed cases (*3*). The emergence of drug-resistant *M. tuberculosis* significantly enhances the danger posed by this devastating human pathogen.

The world needs new antibiotics for combating the emerging problem of antibiotic resistance. The ability to find antibiotics using traditional screening approaches has greatly diminished over time (*4*), necessitating the development for more rational approaches to antibiotic discovery. Central to developing these new approaches is a better understanding of the physiology of pathogenic bacteria, which would guide strategies for mitigating the morbidity and mortality caused by infectious disease. To do so will require the creative application of existing and emerging tools and technologies.

GENETIC AND CHEMICAL APPROACHES TO STUDYING BACTERIAL BIOLOGY

Classical and chemical genetics are powerful approaches for understanding and dissecting biological mechanisms. These approaches have different, complementary strengths and limitations and are thus each positioned to reveal novel aspects of biology. Since the advent of tools that allow the manipulation of bacterial genomes, bacterial biology has predominantly been studied using classical genetic approaches. The exception has

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[1]Abbreviations: MRSA, methicillin-resistant *S. aureus*; MDRSA, multi-drug-resistant *S. aureus*; FASII, bacterial type II fatty acid synthesis; OM, outer membrane; CBPV, chlorobiphenyl vancomycin; T3SS, type III secretion systems; QS, quorum sensing; CT, cholera toxin; AHL, acyl homoserine lactone; HTS, high-throughput screening; Stx, Shiga toxin; CT, cholera toxin; PKA, protein kinase A; PKB, protein kinase B; SILAC, stable isotope labeling with amino acids in cell culture.

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been the use of antibiotics to kill bacteria, which is essentially an experiment in chemical genetics in which small molecules are used to perturb bacterial physiology. Recently, chemical biology is beginning to be widely used for studying many aspects of bacterial physiology and virulence. In the following section, we will compare and contrast chemical and classical genetic approaches, highlighting the strengths of chemical biology that can be advantageous when classical genetic approaches are limited.

Classical Genetic Approaches. Classical genetic approaches have been enormously useful and continue to provide much insight into bacterial physiology and virulence. In classical genetic approaches, the standard tool for examining gene function is the manipulation of DNA. A gene of interest is mutated, deleted, or overexpressed, and the phenotype of the resulting mutant is analyzed. Loss of function mutants, including gene deletion mutants (knockouts), are the most commonly used mutants in genetic analysis. It is difficult to use classical genetic approaches to study genes that are essential for growth, however, including most genes targeted by antibiotics, because they inherently cannot be deleted. Point mutations of single nucleotides, including conditional mutations such as temperature sensitive mutations, can help to fill this gap by providing one approach for studying the function of essential genes, with the additional advantage of adding the dimension of time to genetic analysis. Unfortunately, these approaches often meet with limited success. One reason is that it is not possible to isolate a useful temperature sensitive mutation for every gene. Also, some bacterial species grow in a very narrow temperature range, precluding this strategy. Alternatively, the use of promoters that can be regulated and controlled using exogenous inducers allow the study of gene function in a time-dependent, titratable manner, which has some utility for studying essential gene function. Although conditional promoters can be useful for studying essential gene function, they are often not truly conditional, as it can be difficult to turn on and off gene expression on a short time scale in a tunable, reversible manner, particularly in slow-growing pathogens such as *M. tuberculosis* where protein and RNA turnover is slow. The difficulty of applying classical genetic strategies to understanding essential gene function has limited our ability to study the role of essential genes in fundamental processes.

Certain phenotypes regulated by even nonessential genes can be elusive targets for classical genetic strategies. Functions governed by genes for which a number of redundant orthologs exist in a single genome are especially challenging. An additional factor that can complicate genetic analysis is the fact that mutation of genes that are not strictly essential but are required for optimal growth often results in the acquisition of compensatory mutations that can be difficult to identify. Finally, the numerous organisms that are intractable to genetic manipulation, including *Chlamydia*, *Rickettsia*, and *Coxiella* species, are inaccessible by traditional genetic techniques. We have a very limited understanding of bacterial pathogens that have not proved to be amenable to classical genetic strategies.

Chemical Genetic Approaches. Chemical genetic approaches, which use small molecules to regulate and inhibit protein function, have risen as a powerful and complementary method to classical genetic techniques. Small molecule probes can inhibit essential gene function in a rapid, reversible, and titratable manner, providing important tools for the study of essential gene function. Small molecules provide the potential for addressing the function of redundant systems, as small molecule inhibitors often inhibit multiple members of a protein family. In addition, chemical genetic approaches can provide an entry into the analysis of the molecular basis of pathogenesis in a large number of previously intractable or genetically challenging bacterial pathogens.

For studying the host side of the host−pathogen interaction, though RNAi can be performed in cultured cells, it is often difficult to achieve complete knockdown of a gene product using RNAi. On a higher level, although it is possible to genetically manipulate a mouse, generating a knockout is laborious and time-consuming, and it can be difficult to study genes that play a role in embryonic development. For this reason, small molecules are useful probes for studying the effect of inhibiting host pathways in both tissue culture and animal models of infection with bacterial pathogens. Small molecule inhibitors of bacterial virulence factors are also extremely useful as in vivo probes and can provide the ability to inhibit virulence factors at specific time points after infection.

Finally, inhibition of a gene product by a small molecule inhibitor can reveal novel phenotypes different from those found through genetic manipulation. Unlike the binary perturbation of a genetic knockout, small molecules have the ability to modulate protein function in a graded manner. Because small molecules are capable of inhibiting single proteins without disrupting larger complexes and networks, they can be finer tools for dissecting protein function. Indeed, small molecules can selectively inhibit a single function of a protein with multiple functions.

For these reasons, chemical biology is proving to be extremely valuable in the study of bacterial physiology and pathogenesis, particularly in combination with classical genetic approaches. The use of small molecules as inhibitors of bacterial virulence factors has been extensively reviewed elsewhere (*5*−*7*). In this review, we describe how chemical approaches best fit into the study of bacterial pathogenesis, focusing on the utility of small molecules for studying essential genes, revealing novel phenotypes, probing genetically intractable organisms, and exploring in vivo infection biology (Figure 1). Below, we discuss a few pertinent illustrations in which chemical biology has been uniquely situated to shed light on fundamental problems in bacterial physiology and pathogenesis.

TARGETING ESSENTIAL GENES

All currently used antibiotics target processes that are required for bacterial growth and/or survival, such as DNA replication, RNA and protein synthesis, and cell wall biosynthesis. Thus, the development of new antibiotics with novel mechanisms of action that can be used to combat resistant infections will likely require the identification and characterization of the essential genes that encode such vital processes. To date, the study of essential genes has been hampered by their intrinsic inaccessibility by traditional genetic techniques. In fact, in recent years, technologies have emerged for genome-wide identification of essential genes using a subtractive strategy that (*8*−*10*) exploits the inability to create null mutants of essential genes. Once identified, however, significant barriers to studying essential gene function using traditional genetic techniques remain. In contrast, small molecule inhibitors have historically been extremely useful for studying fundamental aspects of bacterial physiology. The first examples of chemical biology involved the use of antibiotics as chemical probes for studying essential gene function. These studies include the use of aminoglycoside antibiotics to study ribosome function,

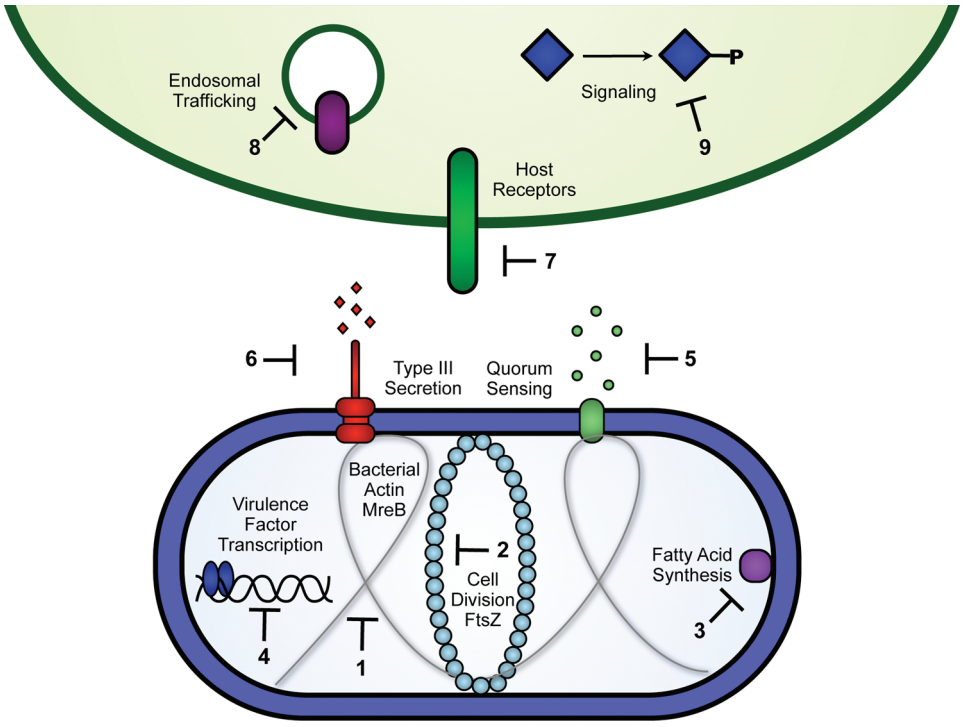

FIGURE 1: Bacterial and host factors targeted by small molecules for inhibition of bacterial pathogenicity. Small molecule inhibitors of bacterial growth and virulence can target bacterial factors and processes such as cytoskeletal elements [MrB, **1** (*17*), and FtsZ, **2** (*23*, *25*)], fatty acid biosynthesis [**3** (*28*, *29*)], virulence factor transcription [**4** (*48*)], quorum sensing [**5** (*59*, *61*)], and type III secretion systems [**6** (*45*, *67*)]. In addition, small molecule inhibitors of bacterial virulence can also target host processes, including blocking host receptors required for pathogen entry [**7** (*76*)], toxin trafficking through the endosomal network [**8** (*82*)], and signaling pathways [**9** (*84*)].

fluoroquinolones to study DNA gyrase and DNA strand breakage, and rifampicin for identification of a subunit of RNA polymerase (*11*, *12*).

In the future, small molecules will be instrumental for the identification and characterization of essential genes in the postgenomic era. Further, these same small molecules that are being identified as biological probes for understanding bacterial physiology and essential gene function may represent starting points for the development of new antibiotics. Indeed, these goals are linked and potentially synergistic. We highlight here three recent examples in which small molecules were effectively utilized as research tools for studying the function of essential genes. These studies shed light on novel functions of the bacterial cytoskeleton and on genes essential for bacterial cell wall synthesis.

Bacterial Cytoskeleton. The role of the bacterial cytoskeleton in fundamental processes has recently become an area of intense investigation, as cytoskeletal components were only recently discovered in bacteria. The identification of bacterial homologues of eukaryotic actin, including MreB in various bacterial species, has powered a tremendous leap forward in our understanding of fundamental processes, including chromosome segregation, a process that is poorly understood in bacteria (*13*, *14*). MreB is a recently identified actin homologue that localizes to a spiral that traverses the length of the cell (*15*, *16*). In an elegant example of the power of chemical biology for elucidating specific functions of essential genes, Gitai and Shapiro used A22, a small molecule inhibitor of MreB, to probe the role of bacterial actin in chromosome segregation (*17*).

Because of its essentiality, MreB has been difficult to study using classical genetic techniques. Genetic depletion of MreB is slow and results in pleiotropic effects on cell shape determination, polar protein localization, and cell division (*15*, *16*, *18*). Thus, it has been difficult to determine whether MreB plays a specific role in chromosome segregation. Treatment of cells with the MreB inhibitor A22 (*19*) results in dose-dependent growth arrest, disruption of MreB localization, and prevention of chromosome segregation but has no effect on DNA replication. By adding A22 to synchronized cells at specific time points, the authors were able to achieve temporal resolution of the role of MreB inhibition in segregation of specific regions of the chromosome. Addition of A22 after duplication and segregation of the origin demonstrates that MreB completely blocks the segregation of newly replicated loci near the origin but has no role in segregation of other loci. This degree of temporal resolution of the role of MreB in chromosome segregation could be achieved only with the rapid and specific inhibition achievable using a small molecule inhibitor (Figure 2).

In addition to MreB, many components of the bacterial skeleton have been targeted by small molecule inhibitors. FtsZ is a component of the bacterial cytoskeleton that is structurally homologous to eukaryotic tubulin and undergoes GTP-dependent polymerization and depolymerization to form microtubule-like structures. FtsZ is essential for bacterial cell division, forming a ringlike contractile structure known as the Z-ring at the site of cell constriction (*20*). We have as yet an incomplete understanding of FtsZ polymerization dynamics. Understanding of tubulin function and dynamics in eukaryotic cells was greatly facilitated by the use of the small molecule inhibitors Taxol and nocadazole. Thus, identification of small molecule inhibitors of FtsZ will likely facilitate understanding of this bacterial tubulin-like protein. Also, given the essential role of FtsZ in bacterial cell division, its widespread conservation across bacterial species, and its evolutionary distance from tubulin, FtsZ is considered an attractive target for the development of new antibiotics. This characteristic has provided significant incentive for the identification of small molecule inhibitors of FtsZ.

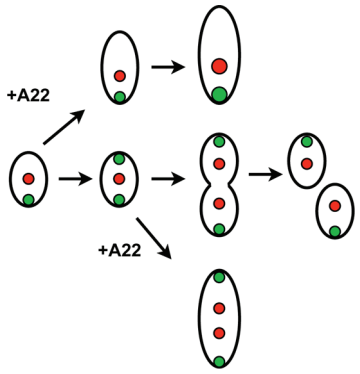

FIGURE 2: Temporal resolution of the role of MreB in chromosome segregation achieved using the MreB inhibitor A22. Green dots represent the origin, and red dots represent a chromosomal region distal to the origin. Addition of A22 to bacteria results in a prevention of chromosome segregation but does not affect DNA replication. If A22 is added after duplication and segregation of the origin, distal regions of the chromosome segregate normally. Thus, MreB is required for segregation of the origin but not other regions of the chromosome. Figure adapted from ref *13*.

Several groups have identified such small molecule inhibitors of FtsZ function (*21*, *22*). In a screen for small molecule inhibitors of the polymerization−GTP hydrolysis−depolymerization cycle of FtsZ, Margalit et al. (*23*) identified five small molecules that fell into two classes: destabilizers of FtsZ polymers and stabilizers of FtsZ protofilaments. Both classes of inhibitors perturb Z-ring assembly in *Escherichia coli* and will be useful probes for studying FtsZ polymerization dynamics, thus contributing to our understanding of the mechanism of cell division in bacteria. Interestingly, both classes of inhibitors also killed a variety of both Gram-positive and Gram-negative bacterial pathogens in broth culture, indicating the potential utility of these inhibitors as chemical scaffolds for developing new therapeutic agents.

In a study that significantly advanced the concept of FtsZ inhibitors as potential antibiotics, Haydon et al. (*24*, *25*) employed fragment-based screening to improve the potency of a previously identified small molecule inhibitor of FtsZ, 3-MBA. The resulting inhibitor, PC190723, was found to have potent antibacterial activity against *S. aureus*, including methicillin-resistant (MRSA) and multi-drug-resistant (MDRSA) isolates. Importantly, PC190723 was also demonstrated to be effective in a murine septicemia model of staphylococcal infection, providing complete protection from a lethal dose of *S. aureus*. PC190723 thus represents an excellent candidate for antibiotic development for combating the growing problem of MRSA and MDRSA infections.

Type II Fatty Acid Biosynthesis. Understanding the function of an essential gene product can allow the evaluation of that gene product as a candidate therapeutic target for the development of antibiotics. For example, FabF is a bacterial enzyme involved in the elongation phase of bacterial type II fatty acid biosynthesis (FASII). Fatty acid biosynthesis is required for cell wall biosynthesis and is considered an attractive target for antibacterial drug discovery (*26*, *27*), in part because of a high degree of conservation of FabF across many significant pathogens. In addition, FASII has been extensively characterized and is known to be essential in vitro, and crystal structures of important enzymes involved in fatty acid biosynthesis have been determined. Currently, no antibiotics in clinical use target FabF.

To identify inhibitors of FabF, Wang et al. (*28*) engineered a strain of *S. aureus* that expresses antisense RNA against FabF. This strain is thus rendered sensitive to inhibition of FabF and enables the identification of specific FabF inhibitors with whole cell activity in a single assay. Screening of a library of 250000 natural product extracts against the FabF sensitive *S. aureus* strain led to the identification of platensimycin, a small molecule isolated from *Streptomyces platensis*. Platensimycin has broad-spectrum activity against Gram-positive bacterial species in vitro and exhibits no cross resistance to any other classes of antibiotics. Significantly, platensimycin was highly efficacious in a mouse model of disseminated *S. aureus* infection.

More recently, continued screening efforts from the same group resulted in the identification of platencin, a novel natural product related to platensimycin (*29*). Platencin also exhibits broad-spectrum activity against Gram-positive organisms by inhibiting fatty acid biosynthesis. Platencin, however, inhibits two essential proteins involved in fatty acid biosynthesis, FabF and FabH. This enhances the promise of platencin, as it is likely to increase the size of the spectrum of organisms sensitive to platencin and weaken the ability for the evolution of resistance. Platensin and platensimycin are examples of first-in-class antibiotics that resulted from rational target-based screening made possible by knowledge of the function of an essential molecular pathway. These examples demonstrate the importance of studying essential gene function in bacteria for the rational design of new classes of antibiotics.

Recent work, however, raises the question of whether FASII is a suitable target for antibiotic development (*30*). Some bacteria are capable of scavenging and incorporating exogenous fatty acids, which could complement a deficiency in fatty acid biosynthesis during in vivo infection when host lipids might be readily available. Strains of *Streptococcus agalactiae* that lack the FASII enzymes FabF and FabM are capable of growth in vitro in media supplemented with exogenous lipids, including addition of human serum to the growth media, and are not attenuated for growth in several rodent models of infection. These results suggest that FASII enzymes are dispensable for bacterial growth in vivo, likely because of the availability of host lipids. More work is required to determine whether platensimycin and platencin remain viable antibiotic candidates that are efficacious against specific organisms and infection sites.

NOVEL MECHANISMS AND PHENOTYPES

Classical genetic strategies, particularly forward genetic screens that interrogate the genome in an unbiased fashion, have enabled great strides forward in understanding fundamental aspects of bacterial cell physiology, specific mechanisms of virulence, and microbial interactions with host cells during infection. Chemical genetics and classical genetics perturb biological systems in different ways and therefore elicit different phenotypes. Small molecule inhibitors often reveal novel phenotypes in systems that have been extensively studied using classical genetics. The most powerful approach to answering important questions in bacterial physiology and virulence will come from integrating classical genetic techniques with chemical biology.

Regulators of Mitosis in Eukaryotes. The best examples demonstrating how inhibition of a protein target using a small molecule can reveal a different phenotype than the one generated from knocking out that protein using genetic techniques are drawn from the eukaryotic biology literature. For example, small molecule inhibition of the Aurora B kinases, which function as mitotic regulators in eukaryotes, reveals a more subtle phenotype

than RNAi-mediated inhibition (*31*). The discrepancy in phenotypes obtained using the two techniques is explained by the fact that Aurora B is a component of a multiprotein complex that is destabilized upon RNAi depletion of Aurora B, resulting in a pleiotropic phenotype. Chemical inhibition of Aurora B more specifically inhibits Aurora B function without disrupting the entire protein complex of which it is a component. Thus, small molecules can function as finer tools for dissecting specific biological questions.

Bacterial Outer Membrane Biology. In the bacterial world, the power of chemical biology to reveal novel phenotypes was elegantly demonstrated in a series of papers from the Kahne and Silhavey laboratories in which small molecules were used as specific biological probes to help identify novel protein machinery required for outer membrane (OM) biosynthesis in *E. coli* (*32*−*34*). In an attempt to identify the targets of chlorobiphenyl vancomycin (CBPV), a synthetic structural analogue of the clinically relevant antibiotic vancomycin, the authors isolated mutants that were resistant to this compound using a strain of *E. coli* with an OM permeability defect. The only mutations retrieved in this screen all occurred in *yfgL*, a gene encoding an OM protein of unknown function. Interestingly, the lack of YfgL also confers resistance to bile salts and moenomycin, but not to vancomycin or other antibiotics, suggesting that YfgL is not the target of CBPV. The authors then carried out the same screen with vancomycin and other drug controls and found that *yfgL* lies on a continuum of mutations that confer selective permeability and can suppress the effects of various antibiotics. The authors proceeded to characterize YfgL as a component of a novel protein complex of the OM that is essential for OM biogenesis.

Using small molecule tools, the authors of this study identified a completely novel molecular machine of fundamental importance to the biology of *E. coli* that had not been identified in any classical genetic screen. OM biology has been a difficult target for classical genetic analysis. OM defective mutants have not been particularly informative, as they all exhibit general permeability defects. The authors circumvented these obstacles effectively by exploiting the power of small molecules to elicit very specific phenotypes. Although the structural differences are relatively subtle (Figure 3), the small molecules used as probes in these studies have a high degree of specificity for various OM permeability changes. Indeed, despite their overall chemical similarity, CBPV and vancomycin are not sensitive to the same permeability changes (Figure 3). The power of the entire set of analogues, combined with various *E. coli* genetic mutations, allowed for the identification of a novel protein machine involved in OM biosynthesis. This study suggests the exciting possibility that combining the power of classical and chemical genetics will accelerate the identification of novel phenotypes related to virulence in bacterial pathogenesis.

GENETICALLY INTRACTABLE SYSTEMS

Classical genetic techniques have advanced our understanding of many medically relevant pathogens, including species of *Salmonella*, *Vibrio*, *Listeria*, *Legionella*, *Pseudomonas*, and *Mycobacteria*. These bacterial species are readily cultured in vitro in the absence of host cells. In contrast, obligate intracellular pathogens, including species of *Chlamydia*, *Coxiella*, and *Rickettsia*, have thus far remained intractable to traditional genetic techniques, limiting our understanding of the pathogenesis of these important infections.

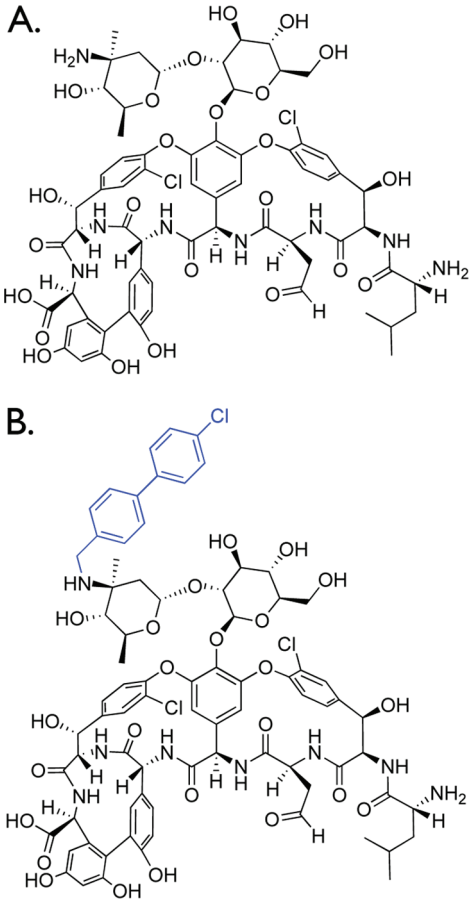

FIGURE 3: Vancomycin and chlorobiphenyl vancomycin. (A) Vancomycin and (B) chlorobiphenyl vancomycin are sensitive to different permeability changes in the *E. coli* outer membrane.

Chlamydia trachomatis and *Chlamydia pneumoniae* are important human pathogens with a significant medical and economic impact (*35*, *36*). *C. trachomatis* is the most common bacterial sexually transmitted disease and is the leading cause of preventable blindness in developing countries where it is endemic. *C. pneumoniae* causes pneumonia, bronchitis, and sinusitis, and infection with *C. pneumoniae* has been associated with chronic diseases such as atherosclerosis (*36*). *Chlamydia* is an intracellular pathogen that replicates in host cells to form large inclusion bodies with many bacteria contained within a host vacuolar membrane. In addition to the obvious difficulty presented by the obligate intracellular lifestyle of *Chlamydia*, other obstacles to genetic manipulation include barriers to DNA transformation at the level of DNA uptake, DNA integration, and clonal selection and propagation (*37*). Thus, *Chlamydia* presents a particularly interesting and important target for chemical genetic strategies.

Many bacterial pathogens have evolved alternative secretion systems for secretion of virulence factors during infection. The type III secretion systems (T3SS) of Gram-negative organisms inject virulence factors directly into eukaryotic cells for manipulation of host cell function. Although T3SS are known to be critical virulence determinants in many pathogenic bacteria, the role of the *Chlamydia* T3SS in pathogenesis has been impossible to test using standard genetic techniques. In a significant example of the utility of chemical biology for studying genetically intractable organisms, "N"-(3,5-dibromo-2-hydroxybenzylidene)-4-nitrobenzohydrazide (C1), a compound that was identified as an inhibitor of the *Yersinia* T3SS (*38*), was recently used to

demonstrate the requirement for T3SS in the developmental cycle of *C. trachomatis* (*39*). Treatment of *C. trachomatis*-infected cells with C1 provided the first evidence of the requirement for T3SS in the pathogenesis of *Chlamydia*.

The CopN protein is predicted to be a substrate of the type III secretion system (T3SS) of *Chlamydia* and has been localized to the inclusion body membrane during infection (*40*). Using a strategy that combined classical genetics in a surrogate organism with chemical screening, Lory and Huang first identified a potential role for CopN by overexpression in yeast (*41*). Overexpression of CopN in yeast results in G2/M cell cycle arrest due to disruption of the mitotic spindle, a finding that was extended to overexpression in mammalian cells. The authors then performed a chemical screen to identify small molecules that relieved CopN-induced cell cycle arrest in yeast and found two inhibitors, 0433YC1 and 0433YC2. To investigate the role of CopN during *C. pneumoniae* infection of mammalian cells, the authors used 0433YC1 and 0433YC2 to create functional knockouts of CopN during infection of mammalian BGMK and Hep-2 cells. Both inhibitors interfere with *C. pneumoniae* replication and inclusion body formation in a dose-dependent manner, demonstrating that CopN is required to support intracellular replication and virulence of *C. pneumoniae*. Thus, the use of small molecule inhibitors allowed the authors to identify the first chlamydial protein required for virulence in this previously intractable obligate intracellular pathogen.

Other Medically Relevant Intractable Bacterial Pathogens. In addition to *Chlamydia*, many other medically relevant bacterial pathogens are intractable to traditional genetic techniques. Thus, our understanding of the pathogenic mechanisms of obligate intracellular bacteria such as *Coxiella burnetii*, the causative agent of Q-fever, and the *Rickettsia* genus, which cause a variety of diseases in humans, including Rocky Mountain Spotted Fever (*Rickettsia rickettsii*), is very limited. The study of these organisms could greatly benefit from the application of chemical biological techniques.

BACTERIAL VIRULENCE FACTORS

In the past five years, there has been a dramatic increase in the number of screens performed with the goal of identifying small molecule inhibitors of bacterial virulence factors. Virtually every common major virulence mechanism of pathogenic bacteria has been targeted, including virulence factor transcription and signaling pathways, T3SS, and quorum sensing (QS). These screens have led to the identification of numerous small molecule inhibitors of virulence factors that are useful probes for testing the role of these factors in animal models of infection.

Toxin Gene Expression. *Vibrio cholerae*, the causative agent of cholera, is responsible for significant worldwide morbidity and mortality. *V. cholerae* elaborates two major virulence factors, cholera toxin (CT), and the toxin coregulated pilus. CT is an adenosine-diphosphate ribosylating toxin that induces elevated cyclic AMP levels in intestinal epithelial cells, thus causing the fluid and electrolyte efflux that characterizes this diarrheal disease (*42*). In a screen for small molecule inhibitors of CT transcription in *V. cholerae* (*44*), researchers identified virstatin, a novel compound that inhibits CT production through disruption of the dimerization of ToxT, a key transcription factor (*43*). Virstatin has no effect on *V. cholerae* growth in vitro but prevents colonization by *V. cholerae* in the infant mouse model of cholera when inoculated with bacteria at the time of initial infection. Even when administered 12 h after inoculation with *V. cholerae*, virstatin dramatically reduces the bacterial burden. Thus, while genetic mutants of ToxT (*45*) can be used to demonstrate its requirement at some point in colonization, virstatin can be used to demonstrate that in fact, there is an ongoing, temporal requirement for ToxT-regulated virulence gene expression throughout infection. This study also demonstrates the potential for using small molecule inhibitors to disrupt virulence in vivo, suggesting the feasibility of this approach for developing this type of drug for use as an antibiotic.

Quorum Sensing. Bacteria use quorum sensing (QS) to coordinate the expression of virulence genes as a function of cell density to maximize the likelihood of a successful infection (*46*). In the simplest form of quorum sensing, an increase in bacterial cell numbers and population density results in the accumulation of autoinducer signal molecules above a threshold at which a signal transduction cascade is initiated, culminating in population-wide gene expression changes.

The identification of inhibitors of QS has been an area of intense focus, as QS systems are major regulators of virulence in a diverse group of pathogens. Approaches to disruption of QS using small molecules include inhibiting autoinducer QS molecule synthesis, increasing autoinducer QS molecule clearance, or preventing autoinducer QS molecule binding to its cognate receptor (*47*−*54*). These small molecule inhibitors have been used to demonstrate the requirement for QS during infection. For example, halogenated furones, structural analogues of the QS molecule acyl homoserine lactone (AHL), are able to inhibit exotoxin production in *Pseudomonas aeruginosa* and enhance bacterial clearance in the lungs of infected mice (*48*, *50*). Here we highlight two of the more recent examples in which small molecule inhibitors were used to study the role of QS during infection.

P. aeruginosa is a Gram-negative opportunistic pathogen that causes serious and life-threatening infections in individuals with cystic fibrosis, cancer, major burns, and AIDS. Three distinct regulatory pathways control quorum sensing gene expression in *P. aeruginosa*. Two of these pathways utilize LuxR regulatory proteins that are activated by AHL autoinducers. The third utilizes MvfR, a LysR-type transcriptional regulator that is activated by the 4-hydroxy-2-alkylquinolines (HAQs) 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS). Anthranilic acid (AA) is a primary precursor to HAQ biosynthesis. Recently, the halogenated AA analogues 6-FABA, 6-CABA, and 4-CABA have been shown to inhibit HAQ biosynthesis and, therefore, MvfR-dependent virulence gene expression (*55*). These inhibitors have no effect on *P. aeruginosa* growth in vitro but limit *P. aeruginosa* virulence in two models of infection. Pretreatment of mice with these inhibitors in a thermal injury model of *P. aeruginosa* infection results in delayed kinetics of death and decreased overall mortality. In addition, 6-FABA, 6-CABA, and 4-CABA reduce systemic dissemination in a wound model of infection without restricting bacterial proliferation at the site of infection, presumably as a result of inhibition of virulence factor expression.

Targeting the signaling pathway downstream of autoinducer signaling has also proved to be a successful strategy for identifying inhibitors of quorum sensing (*56*). The quorum sensing sensor histidine kinase QseC responds to both host-derived β-adrenergic signals and the bacterial autoinducer-3 (AI-3) to upregulate virulence gene expression. To identify inhibitors of QseC, Rasko and Sperandio performed a screen to identify small molecules

that inhibit QseC-dependent virulence gene activation in enterohemorrhagic *E. coli* (EHEC) (*57*). *N*-Phenyl-4-{[(phenylamino)thioxomethyl]amino}benzenesulfonamide (LED209) was identified in the screen and inhibits epinephrine and AI-3-dependent virulence gene activation in EHEC with minimal toxicity against bacterial or mammalian cells. LED209 also inhibits virulence gene expression in *Salmonella typhimurium* and *Francisella tularensis* and protected mice from lethal challenges with either organism.

Specialized Secretion Systems. Recently, many groups have performed whole cell HTS to find inhibitors of bacterial T3SS and have identified several classes of inhibitors active against T3SS of a wide range of Gram-negative pathogens, including *Yersinia*, *Chlamydia*, and *Salmonella* (*58*−*62*). In recent work, Felise et al. (*63*) identified a 2-imino-5-arylidene thiazolidinone compound in a screen for inhibitors of the *Sa. typhimurium* T3SS. This thiazolidinone inhibits secretion of type III effectors without affecting growth of the bacterium, or general secretion. This compound inhibits T3SS at the level of assembly of the needle complex and is also active against bacterial type 2 secretion systems (T2SS), suggesting that the target is a component conserved across these secretion systems. The thiazolidinone compound inhibits the function of T2SS and T3SS in multiple Gram-negative pathogens, blocking secretion and virulence in both animal and plant pathogens. In addition, this compound inhibits proliferation of *Sa. typhimurium* in macrophages and inhibits virulence of *Pseudomonas syringae* in a plant model of infection. Thus, this thiazolidinone is useful for probing the functions of T3SS and T2SS across a large spectrum of infection models.

HOST−PATHOGEN INTERACTION

The interface of a bacterial pathogen with its host constitutes an ongoing, dynamic, and complex interaction in which bacteria must create a niche for survival and replication while evading host immune responses. To accomplish these goals, bacterial pathogens redirect host signaling pathways to subvert and manipulate host cell function. Pathogens adapted to an extracellular lifestyle often secrete effector molecules that inhibit phagocytosis by immune cells such as macrophages or kill important immune cells. In contrast, intracellular pathogens often direct phagocytosis and subsequent vesicular trafficking events to create an intracellular niche that is permissive for bacterial growth.

The study of host pathways required for bacterial virulence has lagged behind the study of bacterial virulence factors themselves, in part because of the relative ease of genetic manipulation of bacteria compared to eukaryotic host cells. The use of new genetic techniques such as RNA interference (RNAi) in eukaryotic cell culture models has opened the door to examining the role of host pathways in bacterial pathogenesis. Genetic manipulation of mammalian hosts remains challenging, however, and thus, barriers to translating findings from cell culture to in vivo infection models remain. Small molecules are proving to be extremely useful for studying the role of host factors in bacterial pathogenesis during in vivo infection. In addition, targeting host factors required for bacterial virulence and pathogenesis represents a new paradigm for antibiotic discovery and development.

Perturbing the Host Trafficking System. The host endocytic trafficking system is exploited by a number of pathogens, both to create niches for intracellular survival as in the case of *M. tuberculosis* and for activation and transfer of bacterial toxins. Following endocytosis, the bacterial toxins Shiga toxin (Stx) and cholera toxin (CT) travel in a retrograde direction through the early endosomal system to the trans-Golgi network (TGN), the Golgi apparatus, and the endoplasmic reticulum (ER), where they are translocated through the Sec61p channel into the cytosol (*64*−*66*). The sequential retrograde progression that characterizes the trafficking of these toxins provides a unique and powerful system for probing host endocytic mechanisms. Small molecule inhibitors of toxin trafficking will be useful probes for studying mechanisms of trafficking and may also represent novel therapeutic approaches to treating diseases caused by bacterial pathogens that utilize toxins of this class.

To identify small molecule inhibitors of Stx retrograde trafficking in host cells, Saenz and Haslam performed a luciferase-based high-throughput screen to identify inhibitors of Stx-mediated inhibition of protein translation (*67*). The hits from the screen were subjected to a number of secondary assays that allowed the authors to identify compounds that specifically inhibited trafficking mechanisms upstream of protein translation itself. Two compounds were identified that provide strong protection against the effects of Stx. 4-Bromo-2-[6-(2,6-dimethylphenylamino)-3*H*-imidazo[1,2-*b*][1,2,4]triazol-5-yl]phenol (compound **75**) was found to inhibit Stx trafficking at an early stage, preventing transport of Stx to recycling endosomes. (*E*)-2,2-Dimethyl-4-styryl-2,3-dihydro-1*H*-benzo[*b*][1,4]diazepine (compound **134**) inhibited toxin trafficking at a later stage, preventing Stx from transiting from recycling endosomes to the TGN. These compounds specifically inhibit retrograde transport, as normal anteretrograde transport was demonstrated to be unaffected by compound treatment. Thus, these compounds are potentially useful as tools for elucidating the differences between anteretrograde and retrograde trafficking.

Interfering with Host Signaling. Protein kinases are important signaling proteins that control many complex processes in mammalian cells and thus may play a significant role in bacterial pathogenesis. Kinases are exploited by many viral and bacterial species for the promotion of pathogenesis. For example, c-Src protein kinase inhibitors have been shown to block assembly and maturation of dengue virus (*68*). To identify host protein kinases required for *Sa. typhimurium* virulence, Kuijl and colleagues (*69*) screened a library of kinase inhibitors to identify small molecules that restrict *Sa. typhimurium* replication in human breast cancer cell line MCF7. H-89, a known inhibitor of protein kinase A (PKA), was found to inhibit growth of *Sa. typhimurium* in MCF7 cells without affecting growth of MCF7 cells or *Sa. typhimurium* in broth culture. Interestingly, H-89 limits proliferation of multiple intracellular bacteria, including *M. tuberculosis*.

By testing H-89 for inhibitory activity against a panel of kinases, they found that H-89 inhibits both protein kinase A (PKA) and protein kinase B (PKB) family members in vitro. The PKB family members AKT1, AKT2, and AKT3 play important roles in mammalian cell biology and are well-known regulators of many processes, including cell survival and growth regulation (*70*). To define the specific kinases involved in controlling intracellular replication of *Sa. typhimurium*, a siRNA library containing siRNA pools targeting the complete human kinome was tested in a HTS for inhibition of *Sa. typhimurium* growth. The kinases identified clustered in one network around AKT1, confirming that the effect of H-89 likely results from inhibition of AKT1. To identify a more specific inhibitor of AKT1, a series of

H-89 analogues was synthesized, resulting in the identification of ETB067. ETB067 selectively inhibits AKT1 and not PKA and limits proliferation of intracellular *Sa. typhimurium* growth at levels comparable to that of H-89.

Although *akt1*−/− mice have been generated (*71*), these mice display impairments in both fetal and postnatal growth that limit the utility of these mice in examining the role of AKT1 in infection with *Sa. typhimurium*. To test the role of AKT1 in *Sa. typhimurium* infection, the compound ETB067 was administered to *Sa. typhimurium*-infected mice upon the first signs of illness. Administration of ETB067 prolonged survival of infected mice, confirming the importance of AKT1 for *Sa. typhimurium* virulence during infection and illustrating the feasibility of using AKT inhibitors as antibiotics.

TARGET IDENTIFICATION

The manipulation of biological pathways using chemical probes has enormous potential to provide fundamental insights into bacterial physiology and mechanisms of virulence in bacterial pathogens. To fully realize the power of chemical biology, however, we need rapid and reliable strategies for identifying the targets of small molecules with interesting biological activities identified from whole cell phenotypic screens. Historically, the difficulty of target identification has been the limiting factor in the utility of chemical biology. In the past few years, many novel technologies have emerged that greatly facilitate small molecule target identification and hold promise for a new era in which target identification becomes straightforward and routine.

Biochemical Approaches to Target Identification. Traditionally, protein targets of bioactive small molecules have been isolated on the basis of their affinity for small molecule ligands. In a biochemistry-based approach, a small molecule is conjugated to a solid matrix to form an affinity column used to capture protein targets from cell extracts. This approach can be extremely effective and has facilitated many significant discoveries in chemical biology, including the identification of the cellular targets of the calcineurin inhibitors CsA and FK506 (*72*). Another important example is the identification of histone deacetylase as a target of the small molecule trapoxin, which significantly advanced our understanding of the role of histone deacetylases in the repression of gene transcription (*73*). Classic affinity-based approaches to target identification are often unsuccessful, due to a couple of significant pitfalls. First, the availability of reactive groups suitable for cross-linking a small molecule of interest to an affinity matrix can be limiting. Second, a high background of nonspecific protein binders to the small molecule of interest can lead to a large number of false positives, particularly when the affinity between the small molecule and protein target is low.

Recently, techniques for distinguishing nonspecific binders from true targets have been developed that compare proteins retained by the bioactive small molecule with a structurally related but inactive molecule. One such approach utilizes SILAC (stable isotope labeling with amino acids in cell culture), a technique developed for quantitative proteomics (*74*, *75*) (Figure 4) to minimize numbers of false positives and reduce the time required to identify the true target of a bioactive small molecule.

Genetic Approaches to Target Identification. Genetic techniques for target identification are particularly powerful in genetically tractable systems such as yeast or bacteria. A myriad of genetic techniques have been successfully employed for

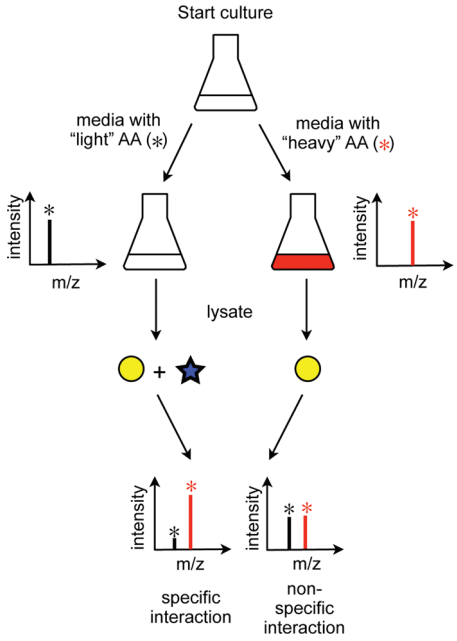

FIGURE 4: Identifying targets of small molecules using quantitative proteomics. SILAC identifies specific interactions with small molecule baits. Cells are labeled with light (black) or heavy (red) amino acids (AAs), and lysates are incubated with bead-conjugated small molecule baits with and without a soluble small molecule competitor (blue star). After being washed, bound proteins are eluted from beads and are digested to yield peptides that are analyzed by mass spectrometry. Proteins that interact specifically with the small molecule will be enriched in the heavy state over the light and will be identified by a high ratio of heavy to light peptide fragments. Nonspecific interactions are characterized by a 1/1 ratio of heavy to light. Figure adapted from refs *83* and *84*.

the identification of the target of small molecules, including identification of the genetic basis for resistance to small molecules, three hybrid systems, and enhancer and suppressor screens (*76*).

Investigating the genetic basis for resistance to a bioactive small molecule has been a particularly fruitful method for target identification. This method is applicable when the effect of the small molecule results in growth inhibition or killing. A classic example is the identification of the target of rapamycin (TOR), which was identified in a genetic screen in budding yeast (*77*).

More recently, the generation of resistant mutants was used to identify the target of a novel drug active against *M. tuberculosis*. Researchers identified a diarylquinoline compound (R207901) that was highly active against *M. tuberculosis* in vitro (*78*). To identify the target of this compound, mutants resistant to the compound were selected and subjected to total genome sequencing. Point mutations in *atpE*, a part of the F0 subunit of ATP synthase, were found to confer resistance to R207901. Subsequent experiments confirmed that the ATP synthase is the target of R207901.

Identification of targets of bioactive small molecules by total genome sequencing of resistant mutants is becoming increasingly feasible as technologies for rapid and inexpensive total genome resequencing have been developed (*79*). These technologies include sequencing by hybridization, nanopore sequencing, and sequencing by synthesis (*80*). In particular, sequencing by synthesis approaches pioneered by companies such as Solexa (Illumina) and 454 are becoming widely available. These technologies are particularly powerful for identifying single-nucleotide polymorphisms (SNPs) but are also capable of identifying

insertions and deletions that may also engender a resistance phenotype. The capacity for rapid and inexpensive total genome sequencing increases constantly with the emergence of new technologies. In addition to being used for target identification, next-generation sequencing technologies will revolutionize classical genetics. The ability to associate phenotypes with point mutations and other subtle genetic changes that are currently difficult to detect will soon become routine through the use of new genome sequencing technologies.

Gene dosage manipulation in the form of suppressor and enhancer screens has also been exploited as a method for identifying drug targets. Overexpression of the target of a small molecule often engenders a shift in the minimum inhibitory concentration of the small molecule, whereas a decreasing level of expression of the target often sensitizes the target to the effect of the small molecule. Recently, both techniques have been successfully used for identifying the targets of small molecule inhibitors of bacterial or fungal growth. In a proof of principle experiment, Li and Brown (*81*) used a multicopy suppression library in *E. coli* to identify suppressors for three well-known antibiotics, trimethoprim, cycloserine, and fosfomycin. In each case, overexpression of the known targets of the antibiotics led to a suppression of bacterial growth inhibition. The authors then used the same library to identify dihydrofolate reductase as the target of two novel small molecule inhibitors.

Drug-induced haplo-insufficiency has been shown to be effective for identifying targets of well-characterized compounds in yeast. This strategy utilizes a competitive growth assay in which pools of bar-coded genome wide heterozygous yeast strains are grown in the presence of a growth inhibitory drug. Strains with decreased dosage of the drug target drop out of the pool (*82*−*84*). In principle, this strategy should be applicable to bacteria using libraries of clones engineered for low-level expression of essential genes (*85*).

Application of techniques using gene dosage manipulation should greatly facilitate the process of target identification. One benefit of these strategies is that they are potentially applicable to a larger number of phenotypes than the generation of resistant mutants, which is limited to small molecules that affect growth and survival.

Genomic Approaches to Target Identification. The ability to collect extremely large data sets and mine them computationally has led to the development of biological pattern analysis as a tool for small molecule target identification. Hughes and Friend demonstrated the use of a large compendium of gene expression data in response to various chemical and genetic perturbations to identify genes and pathways affected by bioactive compounds in yeast (*86*). In this approach, the pattern of gene expression engendered by treatment with a bioactive small molecule was compared to a collection of 300 gene expression profiles. The drug treatment patterns were matched with yeast mutants deficient in target proteins. A similar approach is being developed by for use in mammalian systems. The Connectivity Map project is a large-scale collection of genome-wide transcriptional data from cultured human cells treated with bioactive small molecules. For pathogenesis, this will be useful for identifying the targets of host-directed inhibitors. Currently, the Connectivity Map contains more than 7000 expression profiles representing 1309 compounds. The Connectivity Map has been demonstrated to correctly identify the target pathways of small molecules that do not act immediately proximal to transcription in addition to small molecules with direct effects on transcription (*87*).

The organismal target of a small molecule inhibitor (host or pathogen), the phenotype affected, and the relative potency will determine which of the strategies outlined above is most likely to succeed. In the future, parallel application of multiple strategies for target identification should allow for rapid and reliable identification of pathways and targets affected by interesting bioactive small molecules.

CONCLUSION

Chemical genetics is becoming an increasingly important strategy for studying mechanisms of bacterial physiology and virulence. One of the major advantages of small molecule inhibitors is that they are excellent tools for probing aspects of biology that are not easily accessible to traditional genetic techniques. The study of essential gene function, genetically intractable organisms, and the role of both bacterial and host factors in virulence during animal infection will be greatly enriched by the use of small molecule inhibitors. Because small molecule inhibitors can inhibit a target through a variety of mechanisms, often with exquisite sensitivity and specificity, the use of small molecule inhibitors often reveals novel phenotypes. Thus, the synergy of chemical biology with classical genetics will provide the greatest power for understanding the depth and complexity of bacterial systems and the host−pathogen interaction. Ultimately, the use of small molecule inhibitors as tools for probing biological mechanisms will open up new ideas for taking more directed approaches to the development of new antibiotics.

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