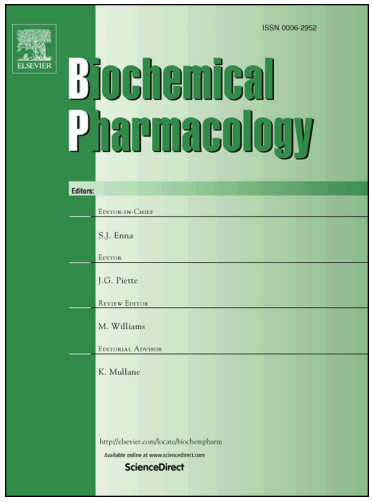
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Review

New Approaches to Antimicrobial Discovery

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New Approaches to Antimicrobial Discovery

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Abstract
The spread of resistant organisms is producing a human health crisis, as we are witnessing the emergence of pathogens resistant to all available antibiotics. An increase in chronic infections presents an additional challenge - these diseases are difficult to treat due to antibiotic-tolerant persister cells. Overmining of soil Actinomycetes ended the golden era of antibiotic discovery in the 60s, and efforts to replace this source by screening synthetic compound libraries was not successful. Bacteria have an efficient permeability barrier, preventing penetration of most synthetic compounds. Empirically establishing rules of penetration for antimicrobials will form the knowledge base to produce libraries tailored to antibiotic discovery, and will revive rational drug design. Two untapped sources of natural products hold the promise of reviving natural product discovery. Most bacterial species, over 99%, are uncultured, and methods to grow these organisms have been developed, and the first promising compounds are in development. Genome sequencing shows that known producers harbor many more operons coding for secondary metabolites than we can account for, providing an additional rich source of antibiotics. Revival of natural product discovery will require high-throughput identification of novel compounds within a large background of known substances. This could be achieved by rapid acquisition of transcription profiles from active extracts that will point to potentially novel compounds.

1. The dual problem: resistance and tolerance.

The rise and spread of antibiotic resistance presents a unique challenge to both science and medicine. Infection is the only disease that threatens not only individuals but societies. Resistance to chemotherapy evolves in only two types of diseases – cancer and infection, and for this reason, both are very difficult to treat. However, cancer has no memory, resistance evolves in somatic cells and is not passed through the germline. By contrast, our pathogens not only develop their own resistance mechanisms, but constantly borrow those that had evolved over billions of years in environmental bacteria. We lack an adequate knowledge base to support rational development of novel antimicrobials to keep up with the threat of resistant bacteria. Today, the result of the standoff between us and our pathogens is epitomized by the spread of multidrug resistant ESKAPE organisms (*Enterococcus*, *Staphylococcus aureus*, *Klebsiella species*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter*) [1]. In the case of some Gram-negative bacteria such as *A. baumannii*, there are strains resistant to all currently available antibiotics [2].

Antibiotics shut down or subvert essential cellular functions, and resistance mechanisms appear to exploit every possible way of preventing a drug from hitting its target. The major types of clinically-relevant resistance mechanisms are destruction of the antibiotic; target modification; and restricted penetration/efflux of the drug [3, 4]. Resistance has been studied for a long time and is fairly well understood. The same cannot be said about tolerance.

It is a given that new antibiotics are needed to combat drug-resistant pathogens [5]. However, we also need antibiotics capable of effectively eradicating an infection. Currently used antibiotics have been developed against rapidly-growing bacteria, and most of them have no activity against stationary state organisms, and even the best have limited activity against dormant persister cells [6]. The relative effectiveness of antibiotics in treating disease is largely a result of cooperation with the immune system, which eliminates non-growing cells and persisters after antibiotics block growth or kill the bulk of a susceptible population. The deficiency of existing antibiotics against ostensibly drug-susceptible pathogens is becoming increasingly apparent with the rise of immunocompromised patients (HIV infected, undergoing chemotherapy, diabetics) and the wide use of indwelling devices (catheters, prostheses, heart valves), where the pathogen forms biofilms protecting cells from the immune system.

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The ineffectiveness of the immune system leads to chronic diseases, which make up approximately half of all infectious disease cases in the developed world.

The main culprit responsible for tolerance of pathogens to antibiotics is a specialized survivor, a persister cell [6, 7]. What we learned about persisters so far can be summarized as follows: persisters are not mutants but phenotypic variants of regular cells produced stochastically in the population, and their relative abundance rises at late-exponential state, reaching 1%[8]; all pathogens form persisters[6]; toxin-antitoxin modules (TA) are the principal mechanism of persister formation in the model organism *E. coli* [9-12]; and a drop in ATP drives persister formation in *S. aureus* [13]; pathways of persister formation are highly redundant [14]; persisters are non-growing [15], dormant [11, 16] cells, which explains their tolerance to bactericidal antibiotics that depend on the presence of active targets for killing the cell [9, 17].

We find that mutants producing elevated levels of persisters are selected for in the course of antimicrobial therapy in infections caused by all pathogens tested - *C. albicans* [18], *P. aeruginosa* [19], *E. coli* [20] and *M. tuberculosis* [21]. In the case of *E. coli* from patients with urinary tract infections, we determined the mechanism by which high persister (hip) mutants form. Mutations in the *hipA* toxin loosen the interaction of the two subunits in the inactive dimer [20], activating this kinase which causes dormancy by phosphorylating glu-tRNA [22]. This heritable drug tolerance is a parallel to the more familiar heritable drug resistance.

Finding of hip mutants underscores the significance of persisters and drug tolerance in the clinical manifestation of disease. An important aspect of tolerance is its link to resistance. Persisters are killed only slowly, if at all, and resume growth when the antibiotic concentration falls. The result is a relapsing infection with a large effective population size which favors development of resistance [23]. The importance of persisters in recalcitrance of infectious diseases raises the bar for drug discovery, we need to develop therapies that effectively kill both regular and dormant cells.

2. Antibiotic discovery - a brief history of a challenging problem.

The genesis of the antibiotic crisis is evident, though not widely appreciated – it is the breakdown of the once successful discovery platform introduced by Selman Waksman in the 40s. The platform was simple, screening soil streptomycetes for antimicrobial activity against a susceptible test organism by detecting zones of growth inhibition on an overlay plate [24]. The method is similar to what Fleming discovered by accident when he saw a *Penicillium notatum* colony clearing a plate seeded with *Staphylococcus aureus* [25]. Streptomycetes are the best producers of antimicrobials, and application of a systematic screen is what made the difference between luck and a discovery platform. Ironically, the *Penicillium* fungus apparently acquired the penicillin biosynthetic operon from streptomycetes by horizontal transmission [26]. The screening of streptomycetes led to the discovery of streptomycin, the first effective compound to act against tuberculosis. This “Waksman platform” was widely adopted by the Pharma industry and produced the major classes of antibiotics over the next 20 years (Fig. 1). The fortunes of the companies, Merck, and Pfizer, and Eli Lilly, among others, rose with the new cures for infectious diseases they were developing. But after 20 years of success, overmining of the soil streptomycetes (and other actinomycetes) resulted in diminishing returns - rediscovery of known compounds, and collapse of the platform.

Resistance to existing compounds was emerging, but modifying antibiotics produced active analogs. This approach was fairly successful for a while, resulting in effective cures [27]. An excellent class of synthetics, the fluoroquinolones, was also developed by the Pharma industry in the 60s, and the tempo of drug discovery seemed to be outpacing the spread of resistance. National resources were shifted indeed, and both the NIH and the Pharma refocused their attention on other problems. However, by the 1990s, it became clear that our victory over the pathogens was an illusion, with resistance spreading faster than discovery of new antibiotics, and we find ourselves in an alarming position of being on the losing side of the war. The story of the most successful class of antibiotics – the β-lactams – is particularly illuminating. Resistance to penicillin was recorded shortly after its introduction in 1947, and traced to hydrolysis of the antibiotic by β-lactamase [28]. Naturally-produced β-lactamase

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inhibitors were discovered, and combined with β-lactams. One of the most successful antibiotics currently on the market is augmentin, amoxicillin + clavulanic acid. Pathogens however continuously develop resistance by modifying/replacing the target, penicillin-binding proteins, and acquiring new β-lactamases, probably from soil microorganisms. As a result, we are witnessing the fourth generation of semi-synthetic β-lactams. Novexell together with AstraZeneca developed a new β-lactamase inhibitor, avibactam, which is active against a majority of β-lactamases [29]. The latest response from Nature is metallo-β-lactamases for which we do not have an inhibitor, and the emergence of the NMD-1 "New Dehli" plasmid coding for a β-lactamase that provides resistance to all β-lactams. The timeframe for introducing a new drug is about 12 years. Pathogens obey no rules when acquiring resistance.

The Industry responded to the rise of resistance by focusing on synthetics, and a number of high-tech approaches were brought together to form the new genomics/combichem/HTS/rational design platform. Essential, conserved proteins identified through genomics were supposed to serve as targets for HTS and rational design, producing novel antibiotics. The end result of the new platform however was the closing of anti-infective divisions at Big Pharma companies. Indeed, not a single drug emerged from this platform [30, 31]. Inhibitors of targets were readily identified, but were restricted by the penetration barrier, which is especially effective in gram-negative species. The outer membrane is a barrier for amphipathic compounds, which essentially all drugs are, since they need to be soluble *and* able to cross the cytoplasmic membrane. The multidrug pumps (MDRs) extrude any compounds that leak in through the outer membrane [32, 33], and recognize chemically unrelated molecules based mainly on polarity, preferring amphipathics. The inner membrane restricts penetration of hydrophilic substances, and the result is then a perfect barrier. Porins in the outer membrane and transporters in the inner membrane allow for uptake of specific nutrients. In the bacterial arms race, some classes of antibiotics evolved to breach this barrier. These are aminoglycosides, β-lactams, tetracyclines, and to a lesser extent chloramphenicols. In the world of synthetics, only one large class able to penetrate well into gram-negative pathogens was developed during 60 years of the global effort, the fluoroquinolones. Metronidazole, effective under anaerobic conditions, and trimethoprim, are broad-spectrum as well. These compounds were developed in small focused projects. The only other new class of synthetics developed in the last 60 years, oxazolidinones, are narrow-spectrum, bacteriostatic compounds, and also resulted from a small focused synthetic program unrelated to HTS. The last useful antibiotic to be discovered, a lipopeptide daptomycin (1986), is also an anomaly – it acts against the membrane with associated toxicity. The last class of antibiotics acting against a specific target to be discovered were streptogramins (1964), narrow-spectrum compounds introduced some 30-odd years later.

Antimicrobial drug discovery presents an unusually difficult intellectual challenge, simply doing more high-throughput screening or adding yet another target to the long list of potential ones will not do.

3. The discovery pipeline and its bottleneck. The vast negative experience of the past half-century with antimicrobial discovery points clearly to the bottleneck: the paucity of compounds that can penetrate into bacterial cells. The HTS approach has been very successful in identifying hits against targets in (and outside of) human cells. Aided by Lipinski rules (≤5 hydrogen bond donors; ≤10 hydrogen bond acceptors; molecular weight ≤500da; logP ≤5) [34], the compound libraries are focused on molecules that will be absorbed after oral administration. These rules however are not useful for identifying good antimicrobial leads which need to satisfy a different requirement – penetration into prokaryotes. But this does not mean that a random unfiltered library has great starting compounds – Lipinski rules simply exacerbate the problem. Earlier libraries did not have that filter. According to the ZINC database, upwards of 13 million compounds are commercially available, and around 10 million have been screened by the Industry in search of antimicrobials. The problem with this global screening effort is that it is very small. The total chemical space – all possible molecules made of 30 atoms of C,H,O,N and S - is 10^{60} [35].

The above analysis paints an extremely bleak picture; if we cannot even discover compounds acting against rapidly growing gram-positive bacteria, what are the prospects of finding broad-spectrum antimicrobials acting against non-growing stationary cells and persisters?

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4. Other problems worth mentioning. Apart from difficulties with penetration and tolerance, antimicrobials face another unique challenge: development of resistance during the course of treatment. In an infected patient, the pathogen produces a population of ≈10^9 cells, which means that the probability of resistance development should be <10^9, otherwise the treatment will fail. Most of the antibiotic classes currently in use hit more than one target, avoiding the instant resistance problem. Fluoroquinolones attack DNA gyrase and topoisomerase, β-lactams inhibit several penicillin-binding proteins, and ribosomal inhibitors such as streptomycin or linezolid bind to rRNA which is coded by multiple genes [36]. The requirement for low resistance frequency has severely limited introduction of compounds acting against single targets. For example, advanced leads with good efficacy in animal models have been developed against a unique bacterial enzyme deformylase, only to fail frequency of resistance testing [37].

Penetration, tolerance and resistance are the main problems, but there are additional barriers as well. Antibiotics are effective at micromolar concentrations, 2 to 3 orders of magnitude higher than a typical drug acting against a eukaryotic target. The main reason for this disparity derives from poor penetration, binding constants of antibiotics to purified targets are comparable to what one finds with other drugs. The need to deliver high amounts of a compound substantially increases toxicity, diminishing the probability of developing good leads.

Apart from these obstacles that science may ultimately overcome, there are also challenges related to market realities specific to antibiotics. Antibiotic therapy is typically short, usually for a duration of several days. Resistance to any antibiotic will eventually develop, limiting its useful lifetime. By contrast, treatment of a typical chronic disease requires taking the drug every day for the rest of one's life. Lipitor, for example, is taken daily and has an annual market of $12 billion, while the best-selling antibiotic, levofloxacin, is taken for several days and had a market of $2.5 billion. However, a $2.5 billion market is attractive enough, if there were a realistic chance of producing a drug to be sold in that market.

5. Approaches and Opportunities.

5.1. Rules of penetration and resuscitation of the high-tech platforms.

It appears that libraries made of random compounds are unlikely to contain well-penetrating molecules with a reasonable spectrum. The penetration problem is also the main obstacle for smaller focused medicinal chemistry projects and for rational drug design based on ligand/target binding. One approach is to take out the major component of the penetration barrier, MDR pumps of gram-negative bacteria. Microcide discovered an effective inhibitor of RND MDRs that are primarily responsible for the high "intrinsic resistance" of gram-negative species to amphipathic compounds [38, 39]. The inhibitor, MC-207,110, discovered in an HTS of commercial compounds, is highly active against different RND pumps of a variety of bacteria [40]. Clinical isolates of *P. aeruginosa* overexpressing the MexAB-OprM pump are resistant to fluoroquinolones and other antibiotics, and MC-207,110 was able to restore susceptibility both *in vitro* and in an animal model of infection. However, polycationic MC-207,110 proved to be nephrotoxic, a common liability of cationic compounds that accumulate in the lysosomes. The positive charges appeared to be essential for function.

While having an effective and safe MDR inhibitor would be great, there no doubt remains a need for novel well-penetrating compounds. The crystal structures of RND pumps were obtained [41-44] but did not prove particularly useful for designing better inhibitors or self-penetrating molecules. The large, poorly structured binding sites of the *E. coli* AcrAB-TolC pump can accommodate a vast variety of chemically unrelated compounds. It seems then that a meaningful path forward is to produce focused libraries based on rules of penetration. These could be obtained empirically [45], similarly to the Lipinski rules that have been highly valuable in selecting orally bioavailable compounds that hit mammalian targets.

The Lipinski rules were formulated by culling the useful properties from a large set of known drugs. Rules of penetration for antibiotics could be similarly established, based on a large number of

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compounds that penetrate into cells of gram-negative bacteria. The missing piece is the availability of such a set of compounds as the existing panel is very small. Obtaining such information is entirely feasible, as penetration of compounds from a random library can be measured using mass spectroscopy and other methods. Some insights into penetration rules can be gleaned even from the small set of good permeators we currently have. One simple consideration is that MDRs have their preferences. We found that hydrophobic cations are preferred substrates [46-48]; highly hydrophobic compounds in general are also well recognized, while relatively hydrophilic compounds of <450da are favored for penetration [49, 50]; anions are not good substrates; and it seems that inclusion of atoms that do not occur frequently in natural compounds (F, B) are good for penetration. Another consideration is target – the broad-spectrum β-lactams are fairly hydrophilic and enter through channels of outer membrane porins and do not need to be amphipathic, since their target is in the periplasm. Fluoroquinolones, the only truly successful class of synthetics, are small, hydrophilic, and carry both an anionic group and a fluorine atom. The recently developed B-containing compounds from Anacor penetrate well into Gram-negative bacteria. The lead compound is a very small molecule, an oxaboral ring containing B and F, which inhibits *leu*-tRNA synthase of gram-negative species [51]. That these useful insights into permeability are based on a very small set of available compounds bodes well for formulating proper rules of penetration based on evaluation of a large sample of molecules.

Once the rules are established, they are likely to breathe new life into the failed high-tech platforms. Libraries specifically compiled for antimicrobial discovery can be synthesized that would obey the rules of penetration. This would be a major undertaking, but a doable one. The better-penetrating compounds from a screen of a commercial library could also serve as a basis for expanding the chemical space around these scaffolds and build the first library by following the rules. Similarly, the rules would be very helpful in resuscitating rational design based on the knowledge of the crystal structure of targets. The ligand would not only be optimized for good binding, but for penetration as well. Both traditional and fragment-based design of ligands would benefit from knowing the rules.

5.2. Other platforms worth developing.

Why platforms? Exactly 2 classes of synthetics were developed in the last 50 years, fluoroquinolones and oxazolidinones (linezolid). Only fluoroquinolones are broad-spectrum. This tells us that all other similar efforts focused on a given series of compounds failed, suggesting that the probability of discovering a drug in the absence of a platform capable of churning out leads is 2/50 years, not that different statistically-wise from zero. As we already discussed, the situation with natural compounds is very similar. There were 3 novel natural products introduced as systemic drugs in the last 40 years – daptomycin [52], synercid (quinupristin/dalfopristin) [53], and fidaxomicin [54, 55]. All three were considered insufficiently attractive when they were first discovered. Resurrection of these compounds decades after discovery was motivated by the lack of new leads. Daptomycin is a narrow-spectrum injectable membrane-acting compound, and in spite of toxicity issues, is an important therapeutic for combating serious infections caused by drug-resistant pathogens, with annual sales close to $1 billion. The success of daptomycin apparently derives from its highly bactericidal properties. Synercid combines two protein synthesis inhibitors, is an injectable narrow-spectrum therapeutic with considerable toxicity issues and a market of around $300m. Fidaxomicin is an RNA polymerase inhibitor with a narrow spectrum and poor bioavailability, and because of these properties, it was a good fit for developing a therapeutic against the gut pathogen *Clostridium difficile*.

This analysis suggests that if we want to have a realistic chance of getting new antibiotics, we need to develop reliable discovery platforms – the probability of accidentally discovering a useful novel compound is exceedingly low. There is another argument in favor of platforms – combination therapeutics. The first antibiotics to be introduced, penicillin and streptomycin, were single-acting compounds. The pathogens rapidly acquired resistance, necessitating the introduction of drug combinations. An inhibitor, clavulanic acid, was introduced to block hydrolysis of β-lactams by β-lactamases. Streptomycin was a great drug to treat tuberculosis, until the pathogen acquired a mutation in the ribosomal RpsL protein. As new anti-TB drugs were being introduced, target modifying mutations

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were developed for each one of them [56]. It became clear that a standard course of therapy had to include several drugs to have a realistic chance of success (currently isoniazid, pyrazinamide and rifampin). The misuse of antibiotics has led to MDR- and XDR-TB, with pan-resistance to almost all available compounds [57]. In retrospect, combining 3 antibiotics in a single pill as they became available would have probably kept them effective for decades with little or no resistance developing. While we have the benefit of hindsight, there are no proposals to introduce combinations of several new antimicrobials - this is unrealistic given the virtual absence of novel compounds. Platforms have the potential of changing this and largely solving the resistance problem. Once there is a reliable method for discovering new compounds, this will enable development of drugs acting against multiple as well as single targets, currently outside of our reach (due to resistance development), and produce combination therapies with lasting efficacy. Focusing on compounds with very low probability or resistance development, as we will discuss below, is another attractive option.

As we argued above, lack of good starting compounds is the main problem, thus our suggestion to identify rules of penetration to revive the high-tech platforms. But is it possible that existing libraries harbor useful compounds that have been missed? Are there other approaches to rational discovery apart from designing ligands that bind to, and block targets? Is it possible to resuscitate Waksman's platform? These are the questions we shall examine in the sections below.

5.3. Back to Domagk. Is it not peculiar that the first useful antibiotic, the sulfa drug prontosil [58], was discovered in a screen of available dyes (no more than several hundred), while a screen of the current global library of ~10^7 compounds produced nothing at all?

As the libraries grew, a number of innovations were introduced aimed at improving the screening outcome; thus we have *in vitro* screening, targeted screens, Lipinski rules, and specificity test. It is possible that each time we tried to improve things, the result was to discard valuable compounds. The first screen was also perfect: Domagk tested compounds against mice infected with *Streptococcus*. An *in vitro* screen would have missed prontosil - the compound is cleaved in the intestine by gut bacteria, releasing the active sulfonamide moiety, which inhibits dehydropteroate synthase in the folate pathway. There are obvious advantages to testing compounds *in situ* - this automatically eliminates the significant burden of toxic molecules and demonstrates efficacy, again automatically eliminating substances with problems of action in an animal, such as serum binding, instability, or poor tissue distribution. In addition, different types of compounds may be uniquely uncovered, such as those requiring activation in situ and those hitting targets that are only important in an infection but not *in vitro*. While this would theoretically be the perfect way to go, testing in 10^7 mice is not an option for a variety of reasons, including ethical considerations and large amounts of required test compound. We therefore considered an intermediate between *in vitro* and a mammal—an animal which, unlike mice, can be dispersed in microtiter wells. *Caenorhabditis elegans* can be infected with human pathogens by simply ingesting them, and we found that the worm can be cured by common antibiotics such as tetracycline and vancomycin, and at concentrations typically achieved in human plasma [59]. Dead worms infected with a pathogen such as *Enterococcus faecalis* can be detected by typical eukaryotic vital dyes. An automated approach based on vital staining was developed, and a pilot screen produced hits, some of which had no activity *in vitro* [59, 60]. *C. elegans* lacks serum and other important properties, but is probably a useful intermediate between mice and humans, pointing us in the right direction—back to Domagk.

5.4. Species-selective compounds. The history of treating tuberculosis provides a compelling case for species-selective antibiotics. The disease is caused by a single pathogen, the diagnosis is unambiguous, and testing random compounds against *M. tuberculosis* produced antimicrobials which act primarily against mycobacteria. The first anti-TB compound to be discovered was streptomycin, a broad-spectrum natural antibiotic. Subsequent testing of synthetic compounds resulted in the discovery of isoniazid, pyrazinamide, ethionamide and ethambutol [61], and all turned out to be selective against mycobacteria, and especially active against *M. tuberculosis*. Increase in resistance of the pathogen due

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to target modification mutations necessitated continuous additions of new antibiotics, and these were then borrowed from the growing arsenal of compounds with a broader spectrum: other aminoglycosides (apart from streptomycin), rifampin and moxifloxacin. Direct screening against *M. tuberculosis* was largely abandoned. However, a screen against a rapidly growing surrogate, *M. smegmatis*, was performed relatively recently by Janssen Pharmaceuticals and again resulted in a compound that happens to be selective against mycobacteria. The compound, R207910 (also known as TMC207), turned out to be an inhibitor of the well-conserved F1Fo ATPsynthase [62]. Several important lessons can be derived from this empirical quest for TB therapies. It appears that a screen against a pathogen produces primarily compounds acting selectively against this pathogen (or against closely related members of a group). One type of these compounds hit group-specific targets. For example, ethambutol is an inhibitor of arabinosyl transferase [63], part of a biosynthetic pathway for mycolic acids which are specific to mycobacteria. The other type of compounds affects conserved enzymes, but is only effective against mycobacteria. A minor difference in the sequence of the C subunit of mycobacterial ATPsynthase affords binding of R207910 which has no activity against other bacteria. Similarly, isoniazid, a prodrug, is activated by a well-conserved catalase, and the active substance then forms an adduct with NADH. The catalase is essential only in mycobacteria, and the adduct is an effective inhibitor of inhA, a mycolic acid biosynthetic enzyme [64]. It does makes sense that identifying a compound that inhibits a single protein is easier as compared to finding a compound that inhibits many members of an orthologous group. We noted above, citing Payne *et al*, that not a single antimicrobial with a reasonable spectrum came out of HTS campaigns [30]. R207910 was obtained through an HTS of a commercially available library and has been introduced into the clinic. This is the first antimicrobial that came out of an HTS and perhaps it is not surprising that the compound is narrowly selective.

The above analysis suggests that we may have a validated discovery platform waiting to be applied to other species. There is a growing consensus that species-selective compounds are the future of antimicrobial discovery [27]. Species-selectivity eliminates an important uncertainty at the beginning of the discovery process – nuisance compounds that present a major obstacle are automatically eliminated. Indeed, if a compound is active only against a particular pathogen, it is unlikely to be a detergent or a DNA intercalator. By the same token, species-selective compounds will have a lower probability of being toxic to humans - a target harbored by a particular species of prokaryotes is unlikely to be present in a mammal.

Another important advantage of species-selective compounds is that they will not harm our gut symbionts. We are becoming increasingly appreciative of the potential role of our microbiota in health, not only in disease. The composition of the microbiome seems to affect everything from gastrointestinal diseases, diabetes and obesity to the general state of the immune system to mental health [65-68]. Taking broad-spectrum antibiotics may result not only in antibiotic-induced diarrhea, which is unpleasant but treatable, but have more problematic, lasting consequences - the microbiome recovers poorly from antibiotic treatment [69]. An additional important advantage of selective compounds relates to resistance. Resistance to a broad-spectrum compound may emerge in any bacterium including commensals (each of us carries 10^{14} cells of those), and then move into a pathogen. This will not happen with a narrow-spectrum compound which only targets a specific pathogen.

Apart from protecting the microbiome by creating specific antibiotics, manipulating the microbiome with selective compounds presents an intriguing opportunity. The rise of the microbiome field liberates microbiology from the confines of infectious diseases. Effects of gut commensals on health or disease have been proposed through correlation studies or by introducing specific microorganisms into sterile animals, usually mice. These studies provide useful starting points for investigation. In order to properly establish causality between a particular species or group and a state of disease or health, we will need compounds to selectively eliminate these organisms from an animal with a normal microbiome. Compounds that provide benefits by eliminating or suppressing unfavorable species could then be developed into drugs.

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5.5 Natural products: reviving the Waksman platform.

The crisis in antibiotic discovery, and especially the disappointing results from the massive effort aimed at developing synthetic compounds, prompted many experts to call for a revival of natural product drug discovery [27, 70, 71]. The enormous background of known and/or nuisance compounds however presents a serious barrier to discovery. Accessing the chemical diversity that has been hidden - prospecting in the bottom of the ocean, growing previously uncultured organisms or turning on silent operons all make sense, but the background of knowns remains an almost unbreachable obstacle.

Advances in analytical methods – mass spectrometry and NMR – allow for "dereplication", determination of an accurate mass and general structure to find an exact match to a known or at least make a reasonable guess as to the potential novelty of a compound. However, this often requires analysis of a pure fraction and is far removed from the initial screen by agar overlay that can result in tens of thousands of producing colonies a week. For now, it seems that a biological approach is our best bet for resolving the enormous bottleneck of knowns.

Transcription profiles of antimicrobials enable a fairly good assignment into known; unknown hitting a known target; and unknown hitting a novel target. This capability existed for some time now, and the dramatic (and continuing) drop in cost makes sequencing of a large number of mRNA (reverse transcribed) samples realistic. We will describe the sources of natural products, but first, will make a necessary detour and consider antibiotic targets.

5.5.1. The good targets. The majority of currently used antibiotics hit only 3 classes of targets: the ribosome; penicillin-binding proteins; and DNA gyrase/topoisomerase. These targets are well-conserved, and many of the antibiotics acting against them are broad-spectrum. But the total number of genes in the core, conserved essential bacterial genome is about 250 [72]. One common feature of the 3 preferred targets is that they are in fact multiple targets [36]. A single β-lactam will typically act against several related penicillin-binding proteins responsible for peptidoglycan synthesis. The ribosome is coded for by multiple copies of rRNA genes, and fluoroquinolones act against related DNA gyrase and topoisomerase. In all cases, resistance development due to multitargeting is $<10^{-9}$. There are other attractive properties these targets have. The ribosome is by far the most prolific attractor for different classes of antibiotics. The ribosome is a hybrid protein/RNA machine, a remnant from the RNA world. The ribosome no doubt originated in a world free of antibiotics and its complex structure has been set; it seems that it cannot easily mutate to avoid binding by inhibitors. For example, 16S RNA is the most conserved component of bacteria, and for this reason, the preferred target for both antibiotics and taxonomists. Many of the ribosomal inhibitors corrupt the process, for example aminoglycosides cause mistranslation, producing toxic peptides that kill the cell [73]. Penicillin-binding proteins are located outside of the cytoplasmic membrane, which obviates the need to penetrate into the cell of gram-positive species. Not surprisingly then, the MIC for β-lactams is comparable to their k_i for the enzymes, in the low nanomolar range, making them some of the most potent antibiotics. β-lactams also induce autolysis of growing cells and effectively kill [74]. In gram-negative species, β-lactams only need to cross one membrane, the outer one, so hydrophilic compounds of this class are broad-spectrum antibiotics. Fluoroquinolones, the only successful synthetic class of broad-spectrum antibiotics, inhibit the ligase step of DNA gyrase/topoisomerase, converting these enzymes into endonucleases [75, 76]. Fluoroquinolones are highly bactericidal, adding to their attractiveness.

Apart from the classes that act against these major targets, there is a variety of compounds that each act against a particular target. The common limitation of compounds from this group is a relatively high probability of resistance development due to single-target action. For example, rifampin, an inhibitor of the RNA polymerase, has excellent bactericidal activity (mechanism of killing unknown), but resistance develops at a rate of 10^{-8}, necessitating its use in combinations.

5.5.2. **Sources of natural products**. The first antibiotic producer was discovered in a laboratory in St. Mary's Hospital in London after a Penicillium spore landed on a partially opened Petri dish [25]. The next producers were obtained in a focused isolation from soil of Rutgers University in New Jersey [24].

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As discovery efforts broadened, so did prospecting, and companies would ask their employees to bring soil samples from their travels. Some of the producers of currently-used antibiotics come from far-flung locations, vancomycin from Southeast Asia, for example. After the golden era of discovery ended, a more concerted effort was made to prospect outside of North America and Europe, but this did not pay off; it does not seem that soils of the Southern Hemisphere or Asia have a diversity of secondary metabolites much different from the better explored locations of the North. Instead of traveling wide, some explorers ventured deep, to the bottom of the oceans. A plethora of secondary metabolites has been discovered, especially from marine invertebrates such as sponges and corrals. Interestingly, it appears that most of these compounds are actually made by bacteria colonizing these animals. Some of the novel compounds are antimicrobials. For example, sporolide A and B are compounds from *Salinispora* Actinomycetes living in the marine sediment [77]. Toxicity has been a problem for secondary metabolites from the sea, precluding (so far) their development as antibiotics.

Another untapped source of antimicrobials is hidden in the silent operons of producing microorganisms. Sequencing of the first genomes of Actinomycetes showed that their potential for producing secondary metabolites far surpasses their known ability. Enzymes producing antimicrobials – the polyketide synthases and non-ribosomal peptide synthases – are encoded by large operons with homologies to known genes. Sequencing of the model Actinomycete, *Streptomycetes coelicolor*, showed that it codes for 20 secondary metabolites, while it is known to produce only 3 antimicrobials [78]. *S. avermitilis*, the industrial producer of the antihelminthic avermectin, does not make antimicrobials at all (at least not in the lab), but according to its genome sequence, harbors 30 operons for secondary metabolites [79]. This general theme of numerous silent operons holds for the many additional actinomycetes genomes that have since been sequenced. At least some silent operons do code for antimicrobials. One approach is to simply try many different growth media. Ecopia was an early pioneer in genome mining for biosynthetic clusters, and producers selected by bioinformatics were then fermented in 40 different media [80]. This resulted in discovery of several novel secondary metabolites including new enediynes, highly reactive compounds that were advanced as anti-cancer agents but ultimately failed. One obvious reason for failure was the limited ability of eliciting production of the hidden wealth of secondary metabolites by these empirically composed fermentation conditions.

There have been notable advances in our understanding of what makes bacteria turn on production. Antibiotic production usually peaks when the culture approaches stationary state. The first quorum sensing factor, butyrolactone, was actually discovered in a study aimed at identifying a factor activating production of streptomycin by *Actinomyces streptomycini* (also known as *Streptomyces griseus*) [81]. Since that early study, butyrolactones were found to induce production of a number of antibiotics in a variety of actinomycetes, and in *S. griseus*, a regulatory pathway linking butyrolactone to streptomycin synthesis has been identified [82, 83]. However, starvation and quorum sensing factors are not universal elicitors of antibiotic synthesis, it is unfortunately not that simple. What the missing factors are is unknown. One straightforward approach is to ignore the natural design and force production by cloning the silent operons in a suitable host or engineering expression in the original producer by disrupting putative repressors or overexpressing activators [79, 84-86]. Several compounds a year are reported from studies focused on manipulating silent operons [87]. This is interesting and important research, but the current throughput cannot support a drug-discovery pipeline. Large-scale induction of silent operons for antibiotic production remains a significant challenge.

Uncultured bacteria are perhaps the most promising source of secondary metabolites [70, 71], organisms that previous generations of prospectors would not see on their Petri dishes. Based on metagenomic analysis of soil and marine samples, 99% of all microbial species on the planet are “uncultured”, do not grow under laboratory conditions [88, 89]. Several groups in Academia and the Industry, realizing that this new source could revive the Waksman platform, established metagenomic approaches to access the enormous hidden potential of uncultured microorganisms. Biotech companies such as Terragen, Xoma, Diversa, Millenium, and at least one of the Big Pharma, Rhone Rourer Poulenc, went after antimicrobials of uncultured bacteria. There was an appealing elegance in their approach: the metabolites of uncultured organisms would be accessed by cloning the producing

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operons from soil DNA, without the need to know whom those sequences belonged to [90]. Cloned DNA was transformed into *E. coli*, *S. coelicolor*, or another suitable host. Proof-of-principle was established for this approach, and several antimicrobial compounds resulting from soil DNA were described [91]. But as with silent operons, the throughput never surpassed several compounds per year, and the approach was largely abandoned. More recently there has been a revival – Warp Drive (MA) is working on total synthesis of biosynthetic operons with the goal of producing compounds in a suitable host; and Lodo Therapeutics (NY) is working on prospecting by metagenomics.

Growing uncultured bacteria is an obvious alternative, and a general method for this has been developed [92-94]. The gist of the approach is to grow bacteria *in* their natural environment. This is done by introducing a marine sediment or soil sample diluted with agar in between two semi-permeable membranes, and placing this "diffusion chamber" back into the natural environment (Fig. 2). Compounds diffuse freely through the chamber, and bacteria are tricked into perceiving it as their natural environment. This approach dramatically improves growth recovery, up to 40% of cells seeded into the chamber produce colonies.

A common cause of "uncultivability" is a need for growth factors produced by neighboring bacteria. Iron-chelating siderophores have been identified as an important group of such factors in the marine sediment environment [95]. Repeated reinoculation from chamber to chamber results in "domestication" of a considerable proportion of uncultured microorganisms, an ability to grow on a Petri dish or in a fermenter [96]. NovoBiotic, has been using in situ cultivation for antibiotic discovery.

So far, several interesting compounds came from uncultured bacteria (Fig. 3A). Lassomycin was identified in a specific screen of a collection of uncultured bacteria against *M. tuberculosis* and is produced by *Lentsea*, an actinomycete [97]. Extracts were counterscreenced against *S. aureus*, and only specific hits against *M. tuberculosis* were followed. Since virtually no natural products are specific against mycobacteria, this simple screen allows one to determine extracts containing novel compounds before doing any chemistry. This resolves the bottleneck of known and toxic compounds that slows down natural product discovery. Lassomycin is a lasso-shaped peptide which inhibits the essential ClpP1P2C1 protease of *M. tuberculosis* while increasing its ATPase activity (Fig. 3A). This dual mode of action kills growing and dormant cells of the pathogen. The compound also serves as proof-of-concept for species-selective screening as a new antimicrobial discovery platform.

Teixobactin, a novel inhibitor of cell wall biosynthesis, was discovered in a screen of uncultured bacteria [98] (Fig. 3B). It is produced by *Eleftheria terrae*, a β-proteobacterium belonging to a new genus. Teixobactin binds the lipid-pyrophosphate-sugar moiety of lipid II, precursor of peptidoglycan, and lipid III, precursor of wall teichoic acid in Gram-positive bacteria. There is no resistance development to teixobactin, since its targets do not mutate. Sophisticated resistance mechanisms such as destruction of the antimicrobial usually originate in the producing organisms, but *E. terrae* is a Gram-negative bacterium which protects itself by exporting teixobactin across the outer membrane, and there is nothing for target Gram- positive organisms to borrow. It appears that teixobactin evolved to be essentially free of resistance. The discovery of teixobactin challenges the dogma of inevitable resistance.

Fungi have been an important source of antimicrobials and secondary metabolites in general. Penicillin and later another class of β-lactams, cephalosporins, have been isolated from fungi. As we have mentioned, fungi apparently borrowed these antibiotic-coding genes from actinomycetes, and perhaps not surprisingly, discovery of useful antibacterial antibiotics from fungi has been limited to these two classes. Fungi are very good at making generally toxic antimicrobial compounds (Lynn Silver, personal communication), and secondary metabolites that attack eukaryotes, including other fungi. Caspofungin, an inhibitor of β-glucan synthase that inhibits fungal wall synthesis, has been discovered fairly recently in an extract of *Aspergillus* species [99]. Caspofungin is an excellent broad-spectrum antifungal which kills growing cells; it is in many respects analogous to antibacterial β-lactams. Humans lack caspofungin target, and the drug is very well tolerated. Caspofungin is also the only natural product antifungal originating from fungi that made it to the clinic. Whether there are additional useful antifungals to be discovered from this source is unclear - fungi unlike bacteria share many conserved

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targets with mammals, and finding a specific antifungal is difficult. The flip side of the homologous targets is the opportunity to use “toxic” antifungals for treating human diseases. Fungi are the source of the highly successful cholesterol lowering compounds, statins [100], and the immunosuppressors cyclosporine [101] and rapamycin [102]. Fungal inhibitors of Hsp90 are currently in development [103]. It seems likely that additional useful drugs will come from exploiting fungi, but whether they are a good source of antimicrobials is unclear.

The last on our list of potential sources of antimicrobials are plants – the original source for remedies to treat all diseases, including infection. A large number of drugs are of plant origin, derived from toxins evolved to prevent grazing by animals, and useful when taken in moderation. For example, glucoside inhibitors of Na+/K+-ATPase are used to treat cardiac arrhythmias and certain kinds of heart failure [104]. There are also two plant antimicrobial therapeutics, both borrowed from ethnobotany, and both are antimalarial – quinine; and artemisinin. Quinine comes from the bark of the cinchona tree [105] and was used by Amerindians, and artemisinin is produced by *Artemisia annua* [106], and is used in traditional Chinese medicine as an antimalarial. No antibacterial antibiotics came from plants, which is surprising, given the very large effort aimed at mining this source, and the fact that plants have numerous chemical defenses against their microbial pathogens [107]. It appears that plants do not generally make highly potent inhibitors of specific microbial targets. The known exception is coumarins, inhibitors of bacterial DNA gyrase [108]. But even in this case, novobiocin, a systemic therapeutic produced by Streptomyces is a more potent inhibitor of the same class. Microorganisms rapidly acquire resistance, which probably forces plants to rely mainly on non-specific, and therefore toxic compounds [48]. A good example is berberine alkaloids which are membrane-acting hydrophobic cations, and also DNA intercalators. They hit two immutable targets – the membrane and DNA. The only defense against these compounds is efflux by multidrug pumps, and it is probably not an accident that hydrophobic cations are preferred substrates of MDRs [46]. Plants harbor a variety of MDR inhibitors, producing an effective synergistic combination with berberine [47, 48]. The known inhibitors however act only against the MF MDRs of Gram-positive species; efforts to find inhibitors of RND MDRs of Gram-negative species have not been successful (Lewis, unpublished), and small molecule plant antimicrobials with high activity against Gram-negative species are virtually unknown. More widespread and less sophisticated than berberines are generally-toxic plant phenols, catechols and tannins.

6. Other platforms. Several additional approaches to antibiotic discovery have been considered, including virulence as a target and antimicrobial peptides and we will review these briefly.

The rationale behind targeting virulence factors is based on the ostensible lack of resistance development towards a therapeutic that will not inhibit cell growth. As we have mentioned already, the frequency of resistance for single-target compounds is high, and presents a barrier that will eliminate most compounds (and targets) from consideration. If blocking a virulence factor will not readily lead to resistance, this class of targets becomes fairly attractive. There has been a considerable effort aimed at developing compounds against virulence targets in Academia and small Biotech over the last two decades, but was not particularly successful, in part due to poor choice of targets. The first promising compound that comes from this approach, M64, was recently published [109]. M64 is an inhibitor of MvfR, the transcriptional activator of the *P. aeruginosa* quinolone quorum sensing (QS) regulon. M64 is an optimized analog of a compound identified in a high-throughput screen of commercial libraries (the Harvard ICCB collection). QS controls virulence in this pathogen. M64 decreased persister formation in an exponentially growing culture of *P. aeruginosa* and improved survival of mice with a lung and wound infection. The in vivo efficacy of M64 was especially pronounced in combination with ciprofloxacin. The fact that it was discovered in an HTS suggests that commercial libraries, which failed to produce compounds with a broad spectrum, may harbor useful molecules acting against a single target. Discovery of M64 bodes well for two emerging platforms – specie-specific compounds; and antivirulence factors.

Antimicrobial peptides (AMPs) are probably as numerous and varied as small molecule antimicrobials. Animals, plants and bacteria all make AMPs [110, 111]. While these compounds are not

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homologous, there is a general theme – these are primarily small (3-5 kDa), alpha-helical, amphipathic, positively charged membrane-acting molecules. Their mode of action presents the main problem, toxicity. Humans make numerous AMPs, including defensins found in the bloodstream, an important component of non-adaptive immunity. One would imagine that defensins should be potent and non-toxic, given their location. This is not the case, at concentrations needed to cover a broad range of pathogens, defensins show considerable cytotoxicity. A concerted effort over the past decades aimed at developing natural, semi-synthetic or synthetic AMPs has not resulted in a therapeutic. Leads with efficacy in an animal model of systemic infection have been conspicuously lacking as well, that is, until a recent publication of a "star"-shaped fully synthetic AMP [112]. Made of randomly polymerized arms of lysine and valine attached to a core, these ≈40 kDa nanoparticles showed excellent efficacy against a systemic murine infection of *A. baumannii*. The discovery is surprising; it is yet unclear why these AMP nanoparticles have excellent selectivity against bacterial cells that surpasses those of all previously tested natural and synthetic compounds of this type. Further research will hopefully substantiate this finding which may point the way towards developing a new type of antimicrobials.

While star AMPs are only making their entrance, there is a single approved membrane-acting antibiotic, daptomycin, which could be classified as an AMP compound – it is a cyclic amphipathic peptide produced by *Streptomyces roseosporus*. Daptomycin increases K^+ conductance of bacterial membranes and has a reasonably good selectivity, sparing human cells [52], but the compound does have toxicity issues, and is used against serious infections as a second-line therapeutic. The mechanistic basis for daptomycin selectivity is not well understood. The examples of star AMPs and daptomycin suggest that there is promise for this class of compounds.

7. Conclusions. With the collapse of antibiotic discovery from cultivable soil actinomycetes due to overmining, we lost the only platform we had, thus the antimicrobial resistance crisis. A decade ago it was unclear what to do about antibiotic discovery; both the Industry and Academia were validating targets and screening synthetic libraries, which did not produce reasonable leads. The publication by Payne and co-authors in 2007 was a watershed event – they detailed the failure of a massive screening program at GSK that was aimed at both pure targets and whole cells [30]. It became clear that the discovery of antimicrobials is uniquely challenging [113], primarily due to poor penetration into bacterial cells, and the bottleneck is in the lack of good starting compounds. Without good leads, especially against Gram negative pathogens, there was virtually nothing to develop. A consensus is gradually building around the ideas for moving forward by resolving the bottlenecks in antimicrobial drug discovery. For synthetics, this means understanding the rules that guide the penetration of compounds across the envelope of Gram negative bacteria. For natural products, it is improving access to uncultured bacteria, turning on silent operons, and developing rapid dereplication to eliminate the background of known and nuisance compounds [114, 115]. These goals are now entirely achievable; we finally know what to do, and introduction of new and effective platforms for antibiotic discovery is within our reach. It is also encouraging that there is consensus of experts on the nature of the main problem – lack of leads, and the need to focus on discovery (see, for example, the recent Pew Roadmap for Antibiotic Discovery [116]). The acute problem of AMR has led the European Union (the IMI initiative), the US Government (https://www.whitehouse.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf) and a consortium of Wellcome Trust, NIAID (NIH), BARDA, and the UK Government - the CARB-X (http://www.carb-x.org) [117], to dedicate substantial funds to fight the problem. Surprisingly however, these funds are directed at development of non-existing leads. Exacerbating this miscalculation is the plain fact that an attractive lead with efficacy in animal models and good safety will be picked up for development by the Pharma Industry, there is no need to expend public funds for this. Hopefully, reason will prevail and resources will be redirected to address the core problem of AMR by building drug discovery platforms. Once that happens, we will be in good position to tilt the balance in the standoff with pathogens in our favor.

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Figure Legends.

Fig. 1. The timeline of antibiotic discovery and introduction. Top panel, year of introduction into clinical practice; bottom panel, year of discovery of a major antibiotic class. Sulfa-drugs, quinolones and the oxazolidinones are synthetic. The last class to be discovered, lipopeptides (daptomycin) is also an exception, the compound does not hit a specific target, but rather increases membrane permeability.

Fig. 2. Diffusion chamber. Design and application of a diffusion chamber (A) and a microbial trap (B). Upper panel: schematic of the growth chambers. Lower panel: side view of chamber/trap interior. (C) iChip for high throughput microbial cultivation *in situ*. The iChip is a plastic platform with miniature holes which is dipped into a suspension of soil bacteria. Each well captures on average one cell. The iChip is covered with polycarbonate membranes and placed into soil.

Fig. 3. Compounds from uncultured bacteria. (A) Lassomycin, a lasso-fold peptide produced by *Lentsea*, inhibits the ClpC subunit of the mycobacterial ClpP1P2C1 protease and activates its ATPase activity. (B), Teixobactin, a depsipeptide produced by a β-proteobacterium *Eleftheria terrae.* Teixobactin binds lipid II, precursor of peptidoglycan, and lipid III, precursor of wall teichoic acid. PG: peptidoglycan; CM: cell membrane; CW: cell wall; WTA: wall teichoic acid.

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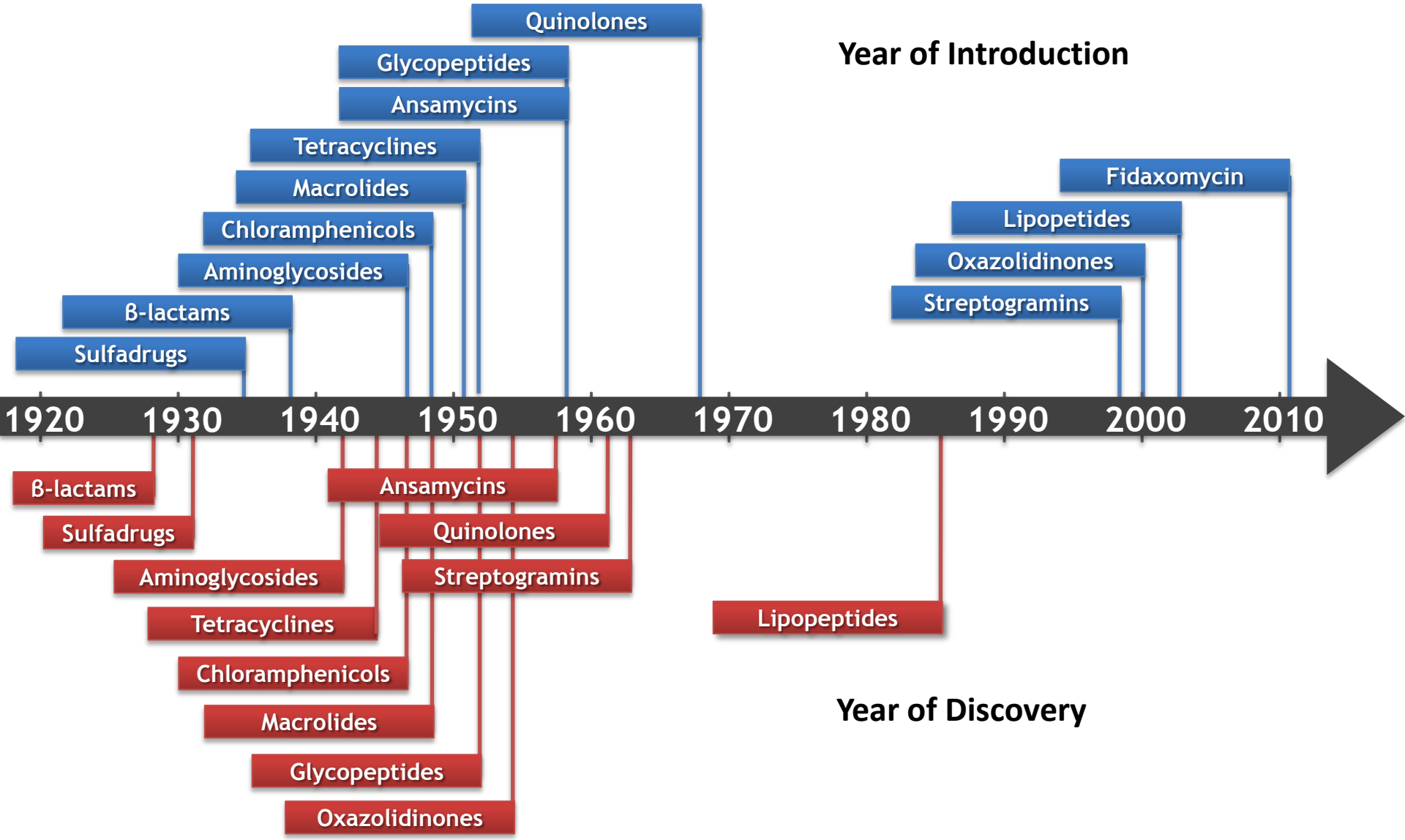
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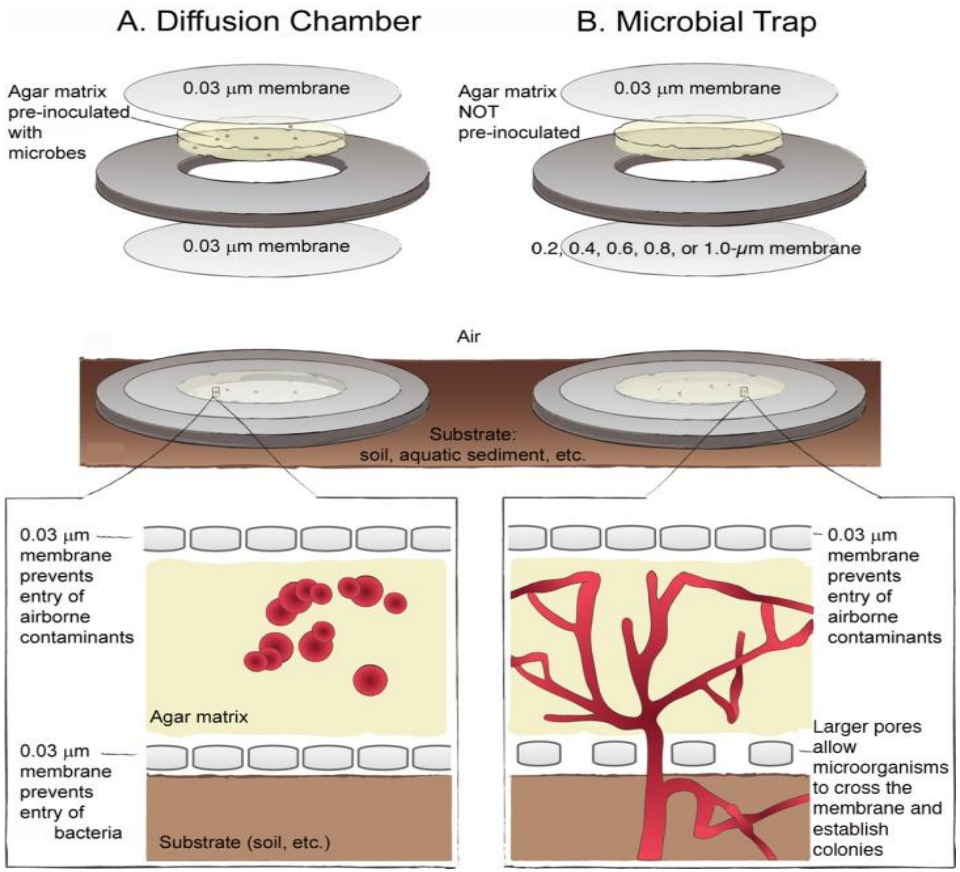
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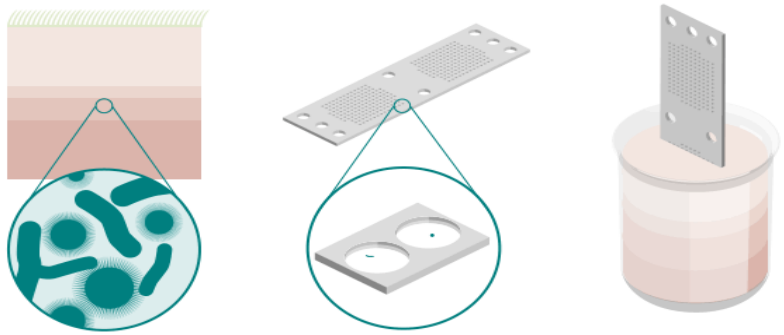
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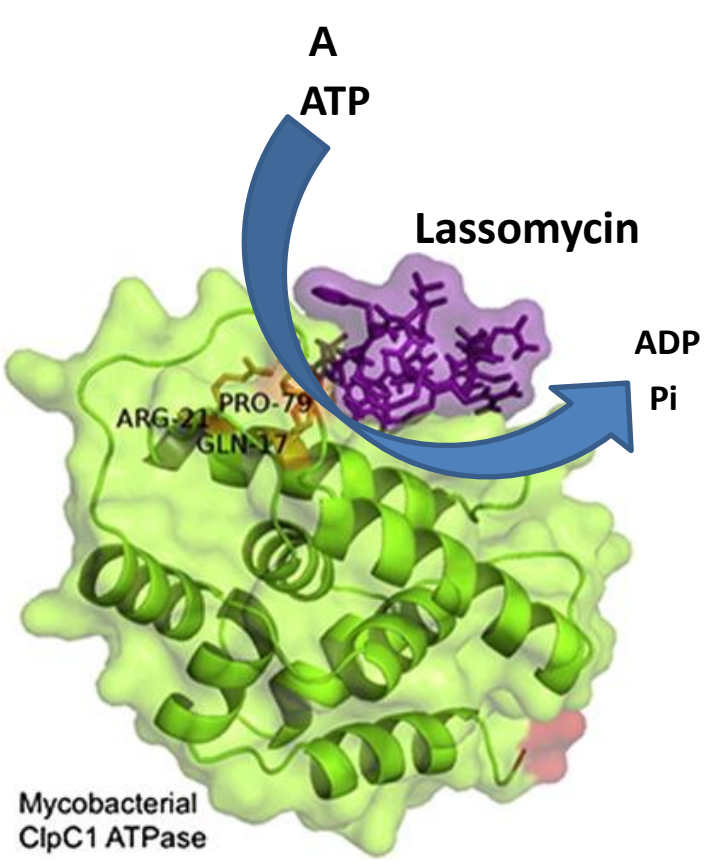
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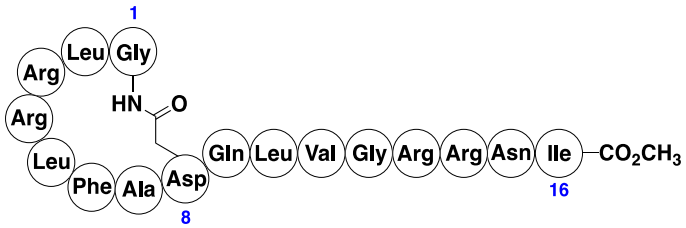

Year of Introduction
Quinolones
Glycopeptides
Ansamycins
Tetracyclines
Macrolides
Chloramphenicols
Aminoglycosides
β-lactams
Sulfadrugs
Fidaxomycin
Lipopetides
Oxazolidinones
Streptogramins
1920
1930
1940
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1990
2000
2010
β-lactams
Sulfadrugs
Aminoglycosides
Tetracyclines
Chloramphenicols
Macrolides
Glycopeptides
Oxazolidinones
Ansamycins
Quinolones
Streptogramins
Lipopeptides
Year of Discovery

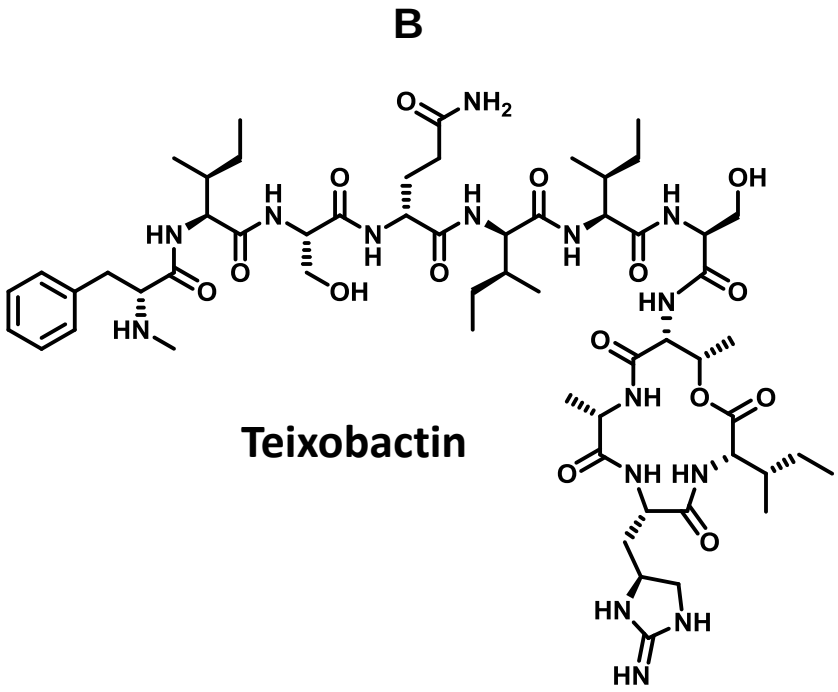

A. Diffusion Chamber
B. Microbial Trap
Agar matrix pre-inoculated with microbes
0.03 μm membrane
0.03 μm membrane
Agar matrix NOT pre-inoculated
0.03 μm membrane
0.2, 0.4, 0.6, 0.8, or 1.0-μm membrane
Air
Substrate: soil, aquatic sediment, etc.
0.03 μm membrane prevents entry of airborne contaminants
Agar matrix
0.03 μm membrane prevents entry of bacteria
Substrate (soil, etc.)
0.03 μm membrane prevents entry of airborne contaminants
Larger pores allow microorganisms to cross the membrane and establish colonies

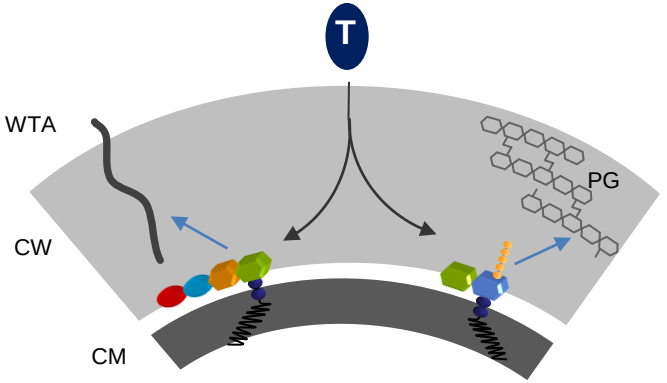
C. iChip.

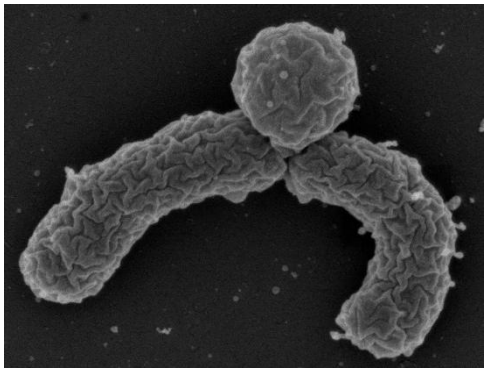



A
ATP
Lassomycin
ADP
Pi
ARG-21
PRO-79
GLN-17
Mycobacterial
ClpC1 ATPase


1
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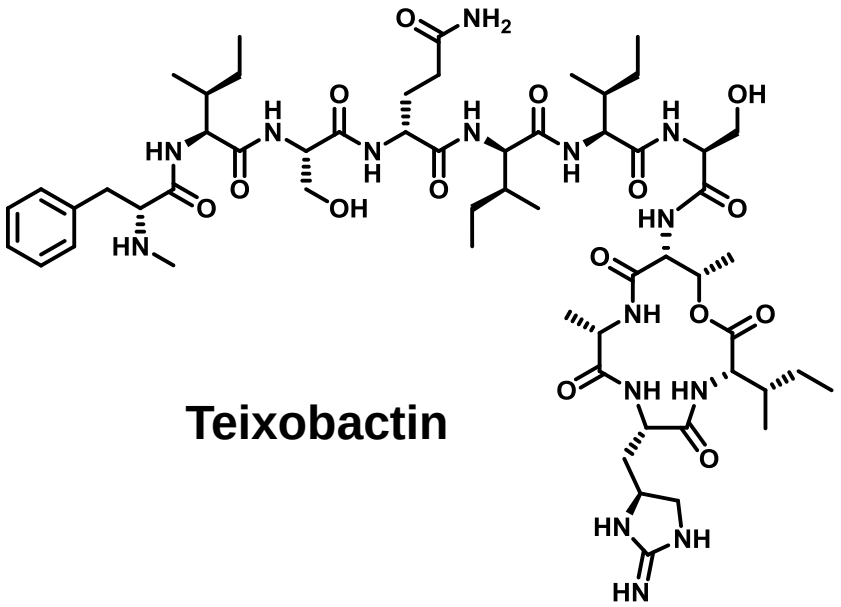

B
Teixobactin


T
WTA
CW
PG
CM



Uncultured *Eleftheria terrae*





Teixobactin