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REVIEW

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Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration?

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Abstract There are few new antibiotics in the pipeline today. The reasons may include starvation at the front of the pipeline due to inadequate sources of suitable compounds to screen coupled with poorly validated discovery methodologies. A successful antibiotic discovery approach in the past, based upon whole cell antibiotic screening of natural products from actinomycetes and fungi, eventually suffered from constipation in the middle of the pipeline due to rediscovery of known compounds, even though low throughput methodology was employed at the front end. The current lack of productivity may be attributed to the poor choice of strategies to address the discovery of new antibiotics. Recent applications of high throughput in vitro screening of individual antibacterial targets to identify lead compounds from combinatorial chemical libraries, traditional chemical libraries, and partially purified natural product extracts has not produced any significant clinical candidates. The solution to the current dilemma may be to return to natural product whole cell screening. For this approach to work in the current millennium, the process needs to be miniaturized to increase the throughput by orders of magnitude over traditional screening, and the rediscovery of known antibiotics needs to be minimized by methods that can be readily monitored and improved over time.

Keywords Actinomycetes · Antibiotics · Natural products · *Streptomyces*

Introduction

The Marcel Faber Roundtable held at the Annual Meeting of the Society for Industrial Microbiology in 2005 in Chicago addressed the question: “Is our pharma pipeline unproductive because of starvation, constipation or lack of inspiration?” I addressed the antibiotic pipeline specifically. Starvation implies problems at the front end of the pipeline (i.e., quality and quantity of compounds to be tested and/or screening methodologies). Constipation implies problems in getting active compounds isolated and characterized (for natural products), further evaluated and confirmed in a timely manner. Lack of inspiration implies problems with the discovery strategy or tactics. I address the question of why the pipeline for new antibiotics is unproductive in this review, and outline some approaches that might help reverse this trend.

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Starvation or problems at the front end of the pipeline

The success of pharmaceutical discovery programs is dependent on two things: (1) having appropriate high quality screens that are predictive of successful clinical outcomes to identify lead compounds to further embellish through medicinal chemistry and other approaches; and (2) having adequate numbers of high quality compounds to screen. The overall quality of the discovery program cannot be good if the quality and quantity of compounds is mediocre, or if the screens are not predictive of successful clinical outcomes. In the past, many potent antibiotics were discovered by screening extracts of fermentation broths from actinomycetes, and to a lesser extent, fungi [2, 3, 5]. This yielded the important classes of antibiotics including macrolides (erythromycin and its derivatives, including ketolides), β-lactams (including penicillins and cephalosporins), aminoglycosides (gentamicin, tobramycin and others), glycopeptides (vancomycin, teichoplanin and related molecules), lipopeptides (daptomycin), ansamycins (rifamycin), tetracyclines and many others. This success indicated that the source of compounds was good and the screening modality was predictive of successful clinical outcomes.

In other words, the overall approach was validated by the successful launch of many antibiotics.

In the 1980s, the pharmaceutical industry encountered diminishing returns from screening fermentation broths for antibiotics [29]. Although a number of recent publications indicate that new antibiotics continue to be discovered [5, 12, 29], the rate of discovery has declined. From 1960 to 1980, the rate of discovery of new antibiotics from *Streptomyces* alone was about 75 per year. The rate declined to about 20 per year from 1980 to 2000, and is projected to level off at ˜15 per year through 2060, provided screening continues at the same pace as in earlier times [29]. In the same timeframe, some companies began focusing more attention on screening natural products for activities in other therapeutic areas, further diminishing the chances for success in antiinfectives.

In the 1990s, high throughput screening against individual targets became the norm in many therapeutic areas, as did the use of genomics to identify new targets for therapeutic intervention. This format was particularly well suited for combinatorial libraries of chemicals, but not for natural products. To accommodate the new format, some companies prepared partially purified natural product libraries for screening. Natural products have had mixed successes in therapeutic areas other than antibacterials [3, 17], and undoubtedly have suffered in recent times from the overall mediocre productivity observed in the new screening paradigm. A number of large pharmaceutical companies have abandoned natural products in their discovery programs, including Eli Lilly and Company, which had many successes in natural products antibiotic discovery, development and marketing [2].

The high throughput formats facilitated screening of single enzyme targets identified by genomics. This was not a particularly good strategy for antibiotic discovery for a number of reasons: (1) Many new targets from genomics were not validated as "druggable", with some exceptions [25]. (2) Certain well validated old targets were not single enzymes amenable to high throughput screening (e.g., the ribosome and the nascent peptidylglycan chain). (3) Combinatorial chemistry is incapable of generating the molecular complexity found in useful natural product antibiotics (e.g., vancomycin, daptomycin, cephalosporin C, erythromycin, rifampin) [5, 12]. (4) Fermentation and partial purification of secondary metabolites is costly, time consuming and incapable of generating enough diversity to compete with traditional approaches. (5) Screening one target at a time in vitro is much less efficient and more prone to false leads than screening all targets simultaneously in a format that demands penetration of the bacterial cell wall and cell membrane and inhibition of the growth of bacteria (whole cell screening). In short, the pharmaceutical industry abandoned a successful (validated) whole cell screening approach from the 1950s through 1980s for an unvalidated new strategy that failed to deliver any new antibiotic in the last decade.

Constipation or problems getting compounds processed

With the recent paradigm of high throughput screening of single anti-infective targets, using primarily libraries of chemicals from combinatorial synthesis, constipation should not have been a problem. There have not been enough quality hits to justify slowing the process down. However, constipation may have been a problem in some instances where low quality hits were advanced to medicinal chemistry programs, generating much work without substantive results.

Processing compounds was clearly a problem with the old paradigm of screening natural product extracts because of the numerous antibiotics already identified in the first 50 years of screening [1, 3, 5], and the time required to isolate and characterize new compounds. I address this issue in more detail below.

Lack of inspiration or problems with the discovery strategy

Successful companies align vision and mission with effective strategy and tactics to develop an appropriate plan, and then execute the plan [7, 14]. For research-oriented companies, this can be extrapolated to state that successful discovery research efforts align vision and mission with effective strategy, tactics, planning and execution. This is easier said than done, and failure can occur at any level: the vision may be unachievable; the mission naive; the strategy inappropriate; the tactics unrealistic; the plan poorly designed; the execution flawed, to name a few. If we look at the current status of antibiotic discovery, the vision seems appropriate: there are new antibiotics to be discovered. The mission seems to be sound: discover new antibiotics to treat unmet medical needs. The tactics of sequencing microbial genomes and identifying genes required for viability are realistic. The plan and execution of high throughput screening has proven to be technically achievable. The problem lies in the strategy of high throughput screening of combinatorial libraries of chemicals against genomics-based individual targets in vitro to discover new antibiotics. This approach was flawed from the onset because the strategy was not fully validated before the plan was developed and put into action. The exploratory research stage that usually precedes the expenditure of major resources was bypassed, based upon overoptimism associated with the new technologies of combinatorial chemistry, genomics and high throughput screening. The strategy was also flawed because it essentially precluded the most successful source of antibiotics, the fermentation broths of actinomycetes. Inspiration without proper validation is risky, and the

pharmaceutical industry learned this the hard way. This dilemma points out a fundamental issue in pharmaceutical discovery: there is no roadmap for operational excellence in discovery research. This is particularly apparent in antibiotic discovery today.

How do we address the current lack of productivity in antibiotic discovery?

Avoid starvation and constipation

The most successful programs to develop new antibiotics in the past employed whole cell screening of fermentation broths from actinomycetes and fungi coupled with chemical modification of natural product lead compounds. A good example of a highly successful program was that of Eli Lilly and Company, which developed and launched more than 30 antibiotics for human medicine and animal health starting with Penicillin G in 1945 [2]. The new technologies of combinatorial biosynthesis [4, 8] and chemoenzymatic modification of natural products [9, 10, 32] add new dimensions to lead optimization not achievable by traditional chemical modification. However, without new natural product leads, lead optimization by any of the available approaches will soon dry up.

There are two main problems with screening actinomycetes or fungi for novel antibiotics: (1) ≥10 million microbes have already been screened and >2,000 antibiotics have already been identified [1, 3, 5, 29]; and (2) the antibiotics yet to be discovered will be produced by microbes less abundant than those that have already been discovered [3]. It has been estimated that new antibiotic producing actinomycetes will be picked at frequencies $\leq 10^{-7}$ per random isolate [3]. To find these strains, we need to be prepared to screen tens of millions of actinomycetes, while excluding or rapidly dereplicating the known antibiotics. This is a tall order! I will discuss some approaches to address these issues below.

Revamp the strategy and tactics

To address the current lack of productivity in the discovery of new antibiotics, the industry needs to align the vision, mission, strategy, tactics, plan and execution in a way that can be successful. In other words, the industry needs to use validated strategies and tactics that can be further improved over time, based upon quantifiable inputs and outputs. I will give some examples below. As already mentioned, an approach can be novel, even inspirational, but if it is not validated, it can fail and be quite costly. A potentially viable strategy going forward is to revisit natural products in a way that can deliver candidate molecules, in spite of the issues posed by rediscovery of known antibiotics, and the need to discover antibiotic producing actinomycetes at individual frequencies of $\leq 10^{-7}$.

Back to the future: screening natural products from actinomycetes

Are there more antibiotics to be found?

A number of big pharmaceutical companies were successful in discovering and developing new antibiotics and other secondary metabolites from microorganisms, and the accomplishments of three of these companies (Lilly, Merck and Schering-Plough) have been featured in SIM News [2, 20, 30]. Most of the successes occurred over 20 years ago. In the early years of screening at Eli Lilly and Company (1949–1959), ~200,000 strains were screened, yielding three marketed antibiotics, including vancomycin and erythromycin [2, 13]. It was estimated that vancomycin and erythromycin producers were found within the industry at frequencies of about 1.5×10^{-5} and 5×10^{-6} among random actinomycetes screened, respectively [3]. Many more antibiotics were found later at lower frequencies.

The frequency of discovery of antibiotics from randomly screened actinomycetes has ranged over six orders of magnitude, from 10^{-1} to 10^{-7} [3]. There is only one antibiotic (streptothricin) produced at a frequency of $\sim10^{-1}$, and only a very small number that are produced at frequencies of 10^{-2} to 10^{-3}, including actinomycin D, streptomycin and tetracycline [3, 31]. The number of antibiotics discovered increased exponentially as the frequency of finding them decreased exponentially. For example, about 200 antibiotics were discovered at frequencies of $\sim4\times10^{-7}$, and about 800 (including daptomycin) at frequencies of $\sim2\times10^{-7}$. More than 1,000 antibiotics have been discovered at frequencies of $\sim1\times10^{-7}$ [3]. The exponential expansion of the number of antibiotics with exponentially diminishing frequency suggests that there are indeed many more antibiotics to be discovered at frequencies of $\leq 10^{-7}$ per random actinomycete screened [3]. However, they need to be found among the 2,000 or more [1, 5] antibiotics that have already been discovered, and that are produced by soil actinomycetes at frequencies ranging from 10^{-1} to 10^{-7}. Figure 1a shows a target diagram to represent the discovery frequencies of some well known antibiotics, and others. The concentric circles ranging from red to pink depict frequencies starting at 10^{-1}in the center to 10^{-7} at the periphery. The red to pink zones encompass the 10,000,000 or so actinomycete strains already screened by 2005. The next two (green) layers represent actinomycetes present in soil at lower frequencies which have not yet been screened. It is not known how many concentric zones actually exist, and the current cartoon assumes that ~99% of actinomycetes are yet to be found.

Vancomycin and erythromycin were discovered relatively early using low throughput methodology (~20,000 strains per year) because the producing actinomycetes were present at frequencies substantially higher than 10^{-7}. Waksman [26] won the Nobel Prize for discovering streptomycin, an antibiotic commonly produced by soil

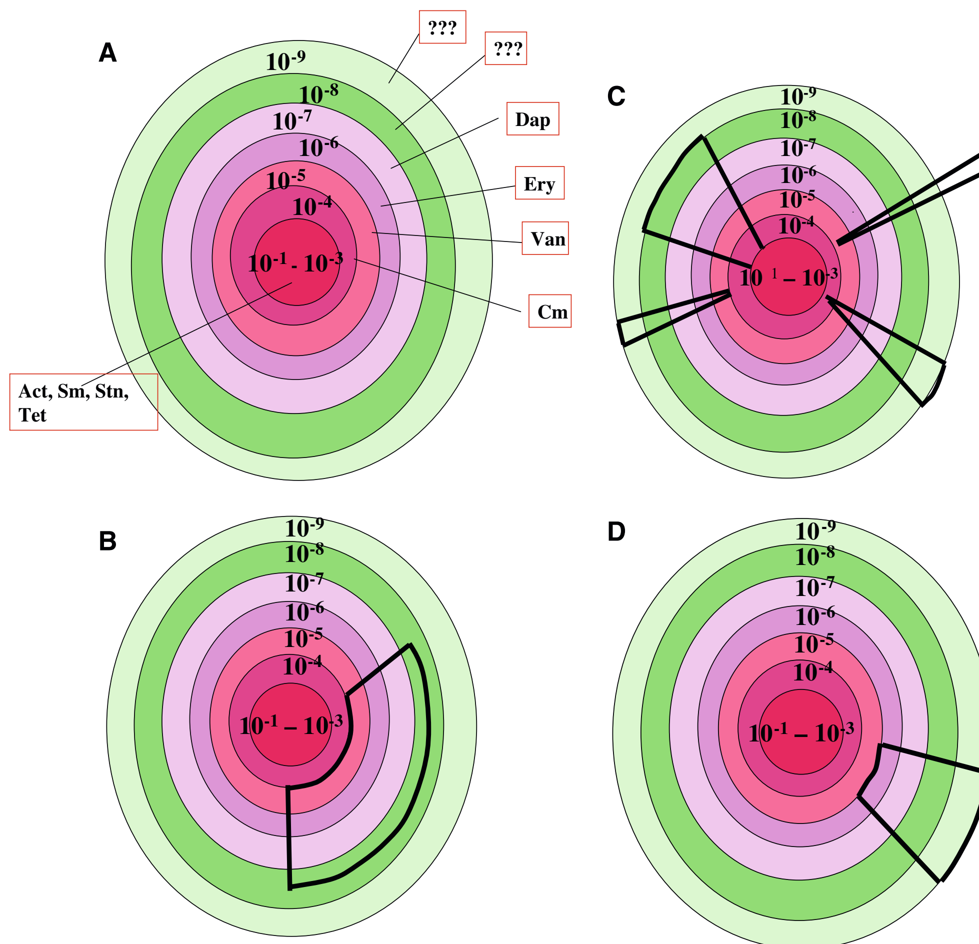

Fig. 1 Frequencies of actinomycetes that produce antibiotics. The *inner red circle* of the target diagram represents actinomycetes that are randomly present in soil at frequencies of 10^{-1} to 10^{-3} among total actinomycetes. The outward expanding concentric *circles* represent actinomycetes that are present in successive tenfold lower abundances. The *red to pink* zones encompass those strains that are present at frequencies spanning 10^{-1} to 10^{-7}, including ˜10,000,000 or so (the reciprocal of 10^{-7}) that were screened for antibiotic production over the past 50 years [3]. **a** The frequencies of discovery of some antibiotics. *Act* actinomycin D; *Cm* chloramphenicol; *Dap* daptomycin; *Ery* erythromycin; *Sm* streptomycin; *Str* streptothricin; *Tet* tetracycline; *Van* vancomycin. **b**. The hypothetical actinomycete screening space using an *E. coli* strain that excludes common broad spectrum antibiotics produced by actinomycetes present at frequencies of 10^{-1} to 10^{-4}. c The hypothetical actinomycete screening space using "deep selections" with antibiotics or other agents. **d**. The hypothetical actinomycete screening space using an engineered *E. coli* screening strain coupled with shallow or moderate antibiotic selection

actinomycetes. Streptomycin producers can be found by screening ˜100 random soil actinomycetes. In contrast, the pharmaceutical industry screened ˜5,000,000 actinomycetes before Lilly discovered daptomycin [3]. It is likely to take the actual or virtual screening of ≥10,000,000 actinomycetes to find the next new clinically useful antibiotic (the green zone in Fig. 1a). For an individual company to be successful, these are the numbers that need to be addressed.

More on the numbers game

Only a miniscule fraction of soil has been sampled for actinomycetes, and only a fraction of actinomycete genera appear to have been isolated and characterized [3]. It has been estimated that only 1–3% of the antibiotics produced by *Streptomyces* species have been discovered [29]. There is also good historical evidence that rare actinomycetes can produce novel compounds not produced by the *Streptomyces* majority. Examples include erythromycin and spinosyns (*Saccharopolyspora* species), vancomycin and rifamycin (*Amycolatopsis* species), teicoplanin (*Actinoplanes* species), and tobramycin and apramycin (*Streptoalloteichus* species). It is also apparent that the use of specific enrichments and selections can enhance the discovery of new species of rare to very rare actinomycete genera [15, 18, 21–24], and previously unknown new genera [16, 28].

How to address the numbers

The projection of a discovery rate of 15 new antibiotics per year [29], using the low throughput screening

methodology of the past, is not sufficient to justify screening for novel antibiotics. It may take the discovery of about 50 new antibiotics to identify a structural class that ultimately is developed into a marketed antibiotic [2, 13]. There are three potential ways to address the issue of low productivity with low throughput screening: (1) increase the throughput dramatically while excluding many of the most common antibiotics; (2) screen only those actinomycetes that have not already been exhaustively screened (i.e., exclude the most common actinomycetes species); and (3) couple high throughput screening with exclusion of the most common streptomycetes.

High throughput screening of actinomycetes

One way to increase the probability of finding new antibiotics is simply to screen at a higher rate. To accomplish this, the fermentation stage of the initial screen needs to be miniaturized to accommodate millions of actinomycetes per year rather than the tens of thousands screened per year as in the past. At Cubist Pharmaceuticals, we have addressed this by developing a fermentation system the uses calcium alginate macrodroplet beads. The beads, which are ˜2 mm in diameter, contain media for growth of actinomycetes, and antibiotics that inhibit the growth of fungi and single cell eubacteria commonly found in soil, but not actinomycetes. Actinomycete spores are extracted from large pools of soil samples from diverse locations to assure the screening of diverse actinomycetes, and individual spores are packaged into the macrodroplets along with media and antibiotics. This technology has been scaled to screen millions of actinomycetes per year.

A daunting issue associated with the screening of millions of actinomycetes is exclusion of or chemical dereplication of known antibiotics. At Cubist Pharmaceuticals, we have begun to address this by constructing a screening strain resistant to many of the most common antibiotics. Of the antibiotics discovered to date, the majority has activity against Gram-positive pathogens and not Gram-negative pathogens [1, 5]. The use of a Gram-negative screening strain would substantially reduce the number of hits by known antibiotics. However, some of the most common antibiotics are broad spectrum, including streptothricin, streptomycin, actinomycin D, and tetracycline. Fortunately, many antibiotic resistance genes have been characterized, including ones for the most common antibiotics. To rapidly engineer a Gram-negative screening strain, we chose *Escherichia coli* K-12 which has the following advantages: (1) it is non-pathogenic; (2) its genome has been sequenced [6], thus facilitating chromosomal insertions of resistance genes and (3) it is readily manipulated genetically by a variety of methods. We have engineered ˜15 antibiotic resistances in this strain by chromosomal insertions to exclude common broad spectrum antibiotics produced by actinomycetes. The antibiotic hit rate with the engineered strain is low, so screening of millions of actinomycetes, fermentation follow-up of hits, and chemical isolation and dereplication are feasible. Figure 1b shows the hypothetical screening space addressed by using the engineered *E. coli* screening strain. Screening >10,000,000 actinomycetes should facilitate the partial sampling of the "green zone", which may include hundreds of previously undiscovered broad spectrum antibiotics, while excluding the Gram-positive only antibiotics. As observed in the recent past, the majority of new antibiotics will be derivatives of known classes, but a fraction will undoubtedly fall into novel structural classes amenable to further modification by medicinal chemistry, combinatorial biosynthesis or chemoenzymatic modification for drug development.

Selection of rare actinomycetes by antibiotic and other selections (virtual screening of millions of actinomycetes)

Another way to enrich for novel antibiotic producers is to select against common antibiotic producers while selecting for a variety of uncommon actinomycetes. This can be done empirically by using antibiotics or other toxic agents, or by enriching particular genera by other techniques [18, 21–24]. For instance, if an antibiotic inhibits colony formation by a random mix of actinomycete spores from soil by four to six orders of magnitude (I refer to this as "deep selection"), then by definition it has selected against many of the most common soil actinomycetes. The survivors of antibiotic selection can then be screened for phylogenetic diversity by sequencing the 16S rDNA genes from a small set of randomly chosen actinomycete colonies. This gives two key pieces of information. First, it establishes if the most common streptomycetes, including those that produce common antibiotics, have been reduced in frequency relative to the untreated control population. For example, a random sampling of actinomycete spores usually yields about 90% *Streptomyces* species, with certain species virtually always present (e.g., *Streptomyces griseus*, the streptomycin producer). If *S. griseus* were present at 1% in the random mix of spores, then any antibiotic that reduced the colony counts by four to six orders of magnitude would by necessity inhibit the growth of *S. griseus* strains. The 16S sequence analysis would confirm this, as well as confirm the reduction of other common streptomycetes. Secondly, the 16S sequence analysis gives a snapshot of what genera and species have been selected, and if that group is interesting, diverse and potentially productive. There may be certain rare or low abundance genera that have a good track record in producing important secondary metabolites not produced by streptomycetes. An example is the genus *Saccharopolyspora*. Erythromycin, a historically important antibiotic which is also

the starting material for the semi-synthetic antibiotics azithromycin, clarithromycin, dirithromycin, roxithromycin, and telithromycin, is produced by *Saccharopolyspora erythraea*, but not by *Streptomyces* species. Similarly, spinosyns, important insecticidal macrolides used in agriculture and animal health [2], are produced by *Saccharopolyspora spinosa* [27] and *Saccharopolyspora pogona* [11], but are not known to be produced by *Streptomyces* species or other actinomycete genera. An antibiotic that selects against *Streptomyces* species and for *Saccharopolyspora* species might lead to the discovery of novel *Saccharopolyspora* strains that produce additional important compounds not produced by the abundant streptomycetes. It might also enrich other rare actinomycete genera as a side-benefit. The same argument can be made for the selection of other rare genera of actinomycetes by different antibiotics or other selective agents. A good example of a deep selection was the use of streptomycin to select the very rare *Streptoalloteichus* strain that produces tobramycin and apramycin [2]. Figure 1c shows some hypothetical examples of how deep selection can exclude the common antibiotic producers (the red zone) and enrich the uncommon new antibiotic producers (the green zone).

A good antibiotic selection should not only enrich multiple rare actinomycete genera but also lead to the discovery of new genera and new species of existing genera. This can be readily documented by 16S rDNA sequencing. If this is achieved, then screening thousands of strains from the specific antibiotic selection should yield novel compounds. This approach can be carried out by traditional low throughput fermentation, and is, therefore, particularly well suited to identify novel compounds active against Gram-positive pathogens, since a higher initial hit rate is easily tolerated when screening thousands rather than millions of actinomycetes.

Coupling high throughput screening with antibiotic selections

A potentially powerful way to search for novel antibiotic producers is to couple high throughput miniaturized screening with moderate or "shallow" antibiotic selection (one to two orders of magnitude reduction in actinomycete colony forming units). For example, if the actinomycete colony forming units were reduced by 99%, then screening 1,000,000 actinomycetes would be the equivalent of screening 100,000,000, but discarding the 99,000,000 most abundant. Figure 1d depicts how this might exclude actinomycetes from the "red zone" and enrich those in the "green zone". This approach should facilitate the screening of some actinomycetes that are present in soil at frequencies $< 10^{-7}$. The quality of the antibiotic selection can be evaluated by the same criteria for the "deep selections" described above using 16S rDNA sequencing: (1) reduction of common streptomycetes; (2) enrichment of rare actinomycete genera, including ones of known value; and (3) enrichment of a diverse population that includes new species of known genera, and possibly new genera. Another advantage of coupling high throughput screening with "shallow selection" is that many different antibiotics or combinations of antibiotics can be evaluated before committing major resources to the most promising selections.

Mining the biosynthetic capacity of slow growing actinomycetes

The high throughput macrodroplet screening (with or without shallow selection) and the deep selection methods can be adapted to facilitate the isolation and screening of slow growing, rare actinomycetes. Even though these strains may not be suitable for large-scale fermentation, their genetic capabilities to produce novel compounds might be transplanted into more robust production strains. It is now clear that antibiotic biosynthetic pathway genes are generally clustered, and can be cloned into bacterial artificial chromosome (BAC) vectors. BACs can be transferred from *E. coli* by conjugation into robust, relatively fast growing *Streptomyces* species, and integrated into the chromosome by site-specific integration mediated by bacteriophage ϕC31 integration. A good example of this is the insertion of a BAC vector containing a 128 kbp insert containing the A21978C (daptomycin) gene cluster in *Streptomyces lividans* [19]. After minor medium optimization, the recombinant produced 60 mg/l of the A21978C lipopeptide factors, which is about 60% the amount typically produced by the *Streptomyces roseosporus* wild type strain.

Novel chemistry and novel antibiotic outputs

The three antibiotic screening approaches outlined above should present opportunities to explore actinomycetes that have not already been screened by the pharmaceutical industry during the last 50 years. The 16S rDNA sequence analysis can give a snapshot to determine if you are on the right track before committing major resources to screening, fermentation analysis and chemical dereplication. The right track(s) ultimately should yield novel compounds with antibacterial activities against key pathogens. The ultimate success will be measured by the ratio of new and novel compounds that become leads to the number of known compounds dereplicated. Having the ability to rapidly dereplicate and discard known antibiotics, and to chemically and microbiologically characterize new and novel compounds, is important to avoid the next bout of constipation.

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