Antimicrobials from Actinomycetes: Back to the Future

Actinomycetes are the source of most clinically relevant antibiotics in use today and may continue to be so

Richard H. Baltz

The two-faced Roman god Janus could see both forward and backward simultaneously. In describing the "Janus Effect" as it relates to the effectiveness of corporate chief executive officers (CEOs), California business gurus James Kouzes and Barry Posner suggest that CEOs who reflect furthest back are those who also plan ahead most effectively.

In terms of antibiotic discovery, we would do well to reflect on the past as a way of enhancing our forward thinking. Lately the prospects for progress are poor, particularly after several large pharmaceutical companies recently abandoned their natural products and infectious disease discovery programs. Moreover, we are failing to identify new antibiotics despite having powerful experimental tools such as genomics, combinatorial chemistry, and high-throughput in vitro screening at our disposal.

Summary

- Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry.
- Additional actinomycete-produced antibiotics likely could be discovered by subjecting soils and marine sediments to innovative enrichments and whole-cell screening methods.
- Combinatorial biosynthesis methods can generate derivatives of complex antibiotics and other secondary metabolites that would be difficult to generate using medicinal chemistry.
- Sequencing actinomycete genomes may provide insights useful in devising novel antimicrobial agents.

Reflecting Upon the Past

Most antibiotics in clinical use are direct natural products or semisynthetic derivatives from actinomycetes or fungi. Many of those products, including erythromycin and derivatives, vancomycin and teicoplanin, cephalosporins, rifamicin, tetracyclines, and daptomycin, were discovered through whole-cell antibacterial screening procedures. Meanwhile, the more recently established approach of target-based discovery using bacterial genomics, combinatorial chemistry, and high-throughput screening has not yet yielded any antibiotics approved for clinical use, and the prospects for its success are not encouraging.

The traditional method of identifying antibiotics by screening extracts from actinomycetes and fungi against pathogens is no longer considered glitzy science. However, whole-cell screening of pathogens can be impressive.

By contrast, high-throughput in vitro screens depend on finding activity against a particular, sometimes hypothetical target. Typically, it starts with a search for chemicals in libraries that can inhibit a single enzyme from a pathogen. If a chemical shows promising activity, then medicinal chemists modify it to ensure appropriate levels of penetration, stability, toxicity, tissue distribution, and elimination from the body. Such efforts often fail.

Whole-cell screening of broths from actinomycetes or fungi, on the other hand,

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quickly leads to biologically active compounds because the broths are screened against only real, potentially "druggable" targets simultaneously, including many that are not merely single enzymes. Whole-cell screening also tests for whether candidate molecules penetrate the outer barriers of cells and are active against pathogens from the outset.

When we take these key differences from in vitro screening into consideration, this "low-tech" approach sounds surprisingly logical. However, many pharmaceutical companies stopped relying on this logic and, instead, began to apply genomics, combinatorial chemistry, and high-throughput, target-based screening to all therapeutic areas, including the search for novel antimicrobial products. They did so without first validating this approach.

Actinomycetes Antibiotic Metabolic Pathways Vary in Abundance

The erythromycin and streptomycin biosynthetic pathways are at least 500 million years old, while the vancomycin biosynthetic pathway, including the VanA mechanism for vancomycin resistance, is at least 200 million years old. Other antibiotic biosynthetic pathways appear not to be so old, and are distributed less abundantly in soil samples.

In general, newer antibiotic pathways are likely to be poorly dispersed globally and could prove difficult to find. Nonetheless, the actinomycetes that produce antibiotics are abundant in soils. According to some estimates, the top 10 cm of global soil contains 10^{25}-10^{26} actinomycetes, but only about 10^{7} have been screened for antibiotic production in the past 50 years, leaving plenty of room for further screening.

Consider the distribution of antibiotic biosynthetic pathways in soil actinomycetes. Several decades ago, researchers at Rutgers University in New Brunswick, N.J., and Boyd Woodruff and his collaborators at Merck in Rahway, N.J., found streptothricin in about 10% of randomly collected soil actinomycetes, mainly *Streptomyces* species. Streptomycin is found in about 1% of random soil actinomycetes, whereas tetracycline and actinomycin are present at about 0.1%. It is no wonder that these were among the first antibiotics discovered.

The erythromycin and vancomycin biosynthetic pathways are much less abundant—present at frequencies of about 5×10^{-6} and 1.5×10^{-5}, respectively. Even so, they are sufficiently abundant that they could be isolated by low-throughput methods in the 1950s. Many other antibiotics, perhaps as many as 2,000, are isolated at frequencies of $1–2 \times 10^{-7}$, including daptomycin, which was recently approved for clinical use.

I have estimated that many other antibiotics remain to be discovered at lower frequencies of about 10^{-7} per random actinomycete screened. A challenge in finding such antibiotics is that other far more common and already known antibiotics also appear among these searches at high frequencies, and they need to be weeded out. In other words, among all known antibiotics, the frequency distribution of the most common antibiotics far exceeds that of the least common antibiotics (Fig. 1). Besides not rediscovering well-known antibiotics, more than 10^{7} actinomycetes need to be screened to find any one antibiotic whose abundance is below that threshold. These search efforts thus require either high-throughput screening or specific sampling methods and selections that enrich for unexamined subsets of actinomycetes.

Macrodroplets Simplify, Miniaturize Screening for Novel Antimicrobials

At Cubist Pharmaceuticals, we screen more than 10^{7} separate actinomycetes per year, using simplified steps for isolating and growing them. For instance, rather than germinating spores, spores are packaged into Ca^{2+}-alginate macrodroplet beads that are 2 mm in diameter and contain nutrient media plus a cocktail of naladixic acid, trimethoprim, nystatin, and cycloheximide to inhibit any nonactinomycete bacteria and fungi. The actinomycete spores germinate, and the mycelia grow, differentiate, and produce secondary metabolites in the beads. We use this method to screen millions of actinomycete spores per year with minimal automation.

When screening millions of spores per year, it is critical to exclude the most common antibiotics. At Cubist, we screen against *Escherichia coli*, looking for any compounds that are active against this gram-negative pathogen. Because more than 60% of known antibiotics are active only against gram-positive bacteria, our use of *E. coli* simplifies the screening, enabling us to

Baltz: Eschewed Molded Plastics To Seek Antibiotics and other Natural Products

If Richard Baltz had any doubts about finishing college, they vanished the summer he worked in a plastics factory. His job was to remove plastic from hot injection molding machines every 45 seconds, working in a room where the temperature sometimes climbed to 115°F. Working under those conditions confirmed his desire to "use my brain to make a living," he says.

His choice to pursue science was in part a response to advice from several sources, including his father, his high school basketball coach, and a favorite teacher. His father told him, "Do something interesting with your life." His basketball coach told him, "Success in basketball is 10% raw talent and 90% hard work," an ethic he now applies to his research. And his teacher encouraged a science project on Einstein's theory of relativity, showing him that "science is fun."

Today Baltz, 62, is a scientific fellow at Cubist Pharmaceuticals, in Lexington, Mass. "I have been involved in developing new methods to identify actinomycetes that produce new or novel antibiotics and other pharmacologically active metabolites," he says. "These areas represent potential solutions to the current low productivity, and low level of interest of the pharmaceutical industry in natural-product approaches to antibacterial discovery and development."

Baltz finds it challenging to pursue both scientific and commercial success within industry, and has developed three rules to help. "First, work on a combination of short term/low risk and long term/higher risk reward projects," he says. "Second, do experiments that have two or more potential positive outcomes. Third, surround yourself with outstanding scientists—they will make you successful while following rules 1 and 2."

One of eight children, Baltz was born in an Army hospital south of Indianapolis, Ind. At the time, his father was serving with the U.S. Army in France in World War II, and they didn't meet until Baltz was two years old. The family lived in Indianapolis until Baltz was nine, and then moved to Cambridge, Ohio, where his father worked for RCA Victor, a manufacturer of televisions, radios, phonographs, recordings, and Minuteman missile launch control centers.

"Growing up in the small town—15,000—was great for me and my sibs, but more challenging for my parents, who left friends and a bigger city," he says. "I am writing a book about many of my adventures in Cambridge." He won a *Certificate of Achievement and Potential* award from the Engineering Society in Cambridge during high school. "This was the time of Sputnik, and I was encouraged by the engineering society to pursue engineering, which I did for one year at Ohio State University before changing my major to pre-med," he says.

He received his B.S. in microbiology from Ohio State University in 1966. Although he considered medical school, he instead followed his father's advice to apply for a job at Eli Lilly and Company before committing to medical school. "I took his advice, and that was my first step toward a career in science," he says. After two years at Lilly, he began graduate studies in microbiology at the University of Illinois, Urbana-Champaign, completed his Ph.D. in 1971, and remained at the University of Illinois to do postdoctoral research. He rejoined Lilly in 1974 to resume a career in antibiotic research and development.

Baltz is married and enjoys family activities. He also believes "in maintaining a balance between the scientific and the esthetic aspects of life." He enjoys art, blues music, and contemporary literature. For exercise he likes to split logs for his wood-burning stove, and "play full-court basketball with players half [his] age."

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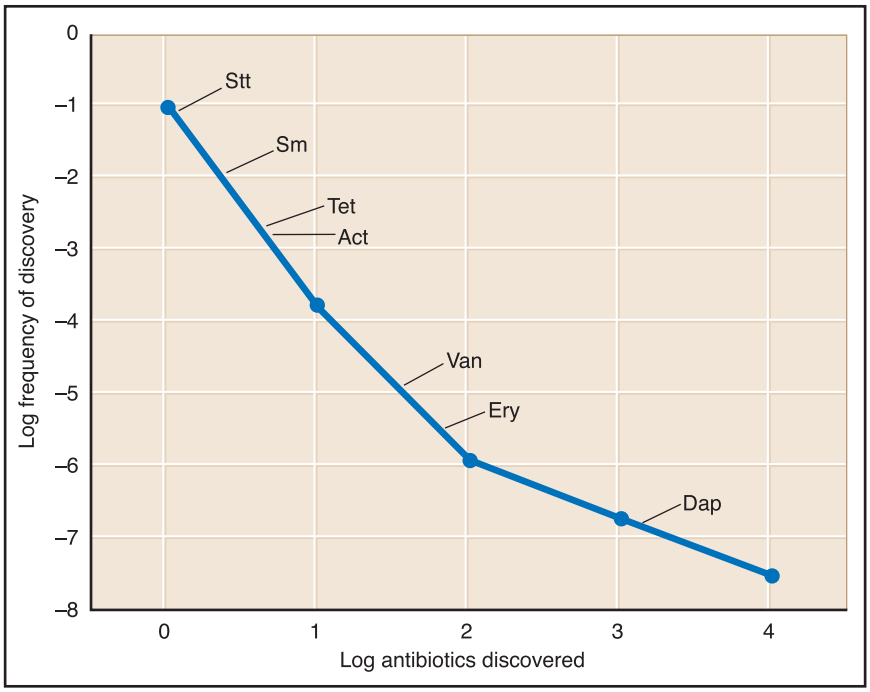
focus on what we consider to be the critical and most clinically important challenge.

E. coli enables us to exclude the most common, broad-spectrum antibiotics by using genetics. For example, well-characterized genes conferring resistance to streptothricin, streptomycin, tetracycline, chloramphenicol, bleomycin, ampicillin, rifampin, and multiple aminoglycosides were inserted into the *E. coli* K-12 chromosome, while a specific deletion conferred resistance to albomycin. Additionally, by inserting resistance to naladixic acid and trimethoprim, we kept our *E. coli* screening strain from becoming inhibited by antibiotics added to

FIGURE 1



Frequency of discovery of new antibiotics as a function of the total antibiotics discovered. Several antibiotics are shown with arrows pointing to their frequencies of discovery among random actinomycetes. Act, actinomycin D; Dap, daptomycin; Ery, erythromycin; Sm, streptomycin; Stn, streptothricin; Tet, tetracycline; and Van, vancomycin.

growth media to inhibit nonactinomycete bacteria.

Inserting multiple resistance genes into the *E. coli* chromosome makes our test strain easier to maintain while reducing our overall hit rate to less than 0.1% because we can exclude so many known antibiotics. In turn, this approach makes subsequent structural analysis of promising antimicrobial candidates more manageable.

Problems Applying Metagenomics when Searching for Novel Antimicrobials

Metagenomic methods are useful for characterizing microbes that cannot be easily cultivated and can also be used to isolate their genes. Although researchers at Aventis, TerraGen, Diversa, and in some academic laboratories use metagenomic methods to seek novel antibiotics, they reported no practical successes.

Why has metagenomics failed so far to uncover new antibiotics? The problem lies largely with technical limits and, secondarily, with the notion that many uncultivable microbes will produce novel antibiotics. Simply surveying uncultivable microbes for genes encoding large type I polyketide synthase (PKS) and nonribosomal peptide synthetases (NRPS) can be helpful for addressing the latter shortcoming. The genes for these enzymes, which are the hallmarks of complex antibiotic biosynthetic gene clusters, are not found at high frequencies in random DNA cloned from soil samples.

Meanwhile, consider how the metagenomic approach might be used to isolate a novel antibiotic biosynthetic pathway. A gram of fresh soil contains about 10^9 colony-forming units of bacteria, about 10^7 of which are actinomycetes, the most prolific producers of antibiotics and other important secondary metabolites. If 10^9 cultivatable bacteria represents 1% of the microbes in the soil, a ratio that is based upon metagenomic estimates, then 10^{11} uncultivable microbial genomes are present per gram of soil.

Working with soil samples, we can make cosmid and BAC libraries to isolate genes encoding known antibiotic biosynthetic pathways as a proof of principle. For example, streptothricin remains the most commonly isolated antibiotic from soil actinomycetes. Based on Boyd Woodruff's accounts from several decades ago, we estimate that streptothricin-producing actinomycetes are present at about 10^6 CFU per gram of soil.

The streptothricin biosynthetic gene cluster is small enough to clone on a cosmid vector. Since streptomycete genomes are about 8 Mb, a library of about 400 clones should contain about one copy of the isolated cluster. Because there are about 10^{11} uncultivable microbes in our 1-g soil sample, streptothricin producers should represent about 1 in 10^5 of the microbial genomes there. To isolate this streptothricin gene cluster, we need first to prepare a cosmid library of 400×10^5 (or 4×10^7) clones, then transfer the library to a streptomycete host that is capa-

Approaches to accelerate antibiotic discovery from actinomycetes

High-throughput whole cell screening of terrestrial actinomycetes
- Global soil sampling
- Pooling of soils and extraction of spores
- Miniaturized fermentations starting with spores
- Screening organism resistant to common antibiotics
- Improved throughput with automation

Enrichments and selections for uncommon terrestrial and marine actinomycetes
- Antibiotics and taxon selective media
- Untapped random and exotic soils
- Untapped marine sediments

Genome mining
- Sequencing multiple common actinomycetes
- Sequencing rare and slow growing actinomycetes
- Expression of new pathways in robust streptomycete hosts

Combinatorial biosynthesis
- NRPS pathways
- PKS pathways
- Glycosylations and other modifications

ble of expressing all of the promoters and other regulatory elements, and then screen the clones for streptothricin.

The problem with this scheme is evident from looking at the unfavorable numbers. First, it is not technically feasible to make cosmid libraries of 4×10^7 clones from DNA extracted from soil. The current limit is off by about 1,000-fold. Second, cosmids can be transferred from *E. coli* to streptomycetes by conjugation at efficiencies of only about 10^{-4} to 10^{-5}, further reducing the likelihood of success. Thus, it is not surprising that no one has yet cloned the streptothricin biosynthetic gene cluster—meaning this particular metagenomic proof of principle remains beyond reach.

A second metagenomics scenario calls for isolating an antibiotic biosynthetic pathway encoding a novel antibiotic. According to estimates, actinomycetes that produce new antibiotics are likely to be present at quantities of 1 in 10^7 or less. Moreover, any particular cluster of antibiotic-encoding genes is not likely to be captured on a single cosmid, judging from the daptomycin example. Instead, a bacterial artificial chromosome (BAC) vector will be needed to clone the 100 kb of DNA that typically are needed for such complex pathways.

Unlike the streptothricin-producing actinomycete, the genome for any undiscovered antibiotic-producing actinomycete is likely present at about 1 in 10^{11} per gram of soil, and a BAC library of about 2×10^{13} clones would be needed to find that biosynthetic pathway. Although BAC libraries of 2×10^4 clones with insert sizes from about 12 to 85 kb have been prepared from soil DNA, this technology is still at least nine orders of magnitude too inefficient to work for antibiotic biosynthetic gene clusters.

The only currently conceivable way that metagenomics might work in this capacity is if some very common uncultivable microbes—those more abundant than the most common cultivatable microbes—could produce antibiotics when their genes are cloned into *E. coli*. Since a BAC library of 10^4 clones represents about 100 genome equivalents of coding information, however, the probability of this approach being successful is vanishingly small.

Hence, it makes better sense to focus on developing methods for growing supposedly uncultivatable microbes and then screening millions, not hundreds, of them per year. For instance, researchers at NovoBiotic Pharmaceuticals in Cambridge, Mass., are making progress isolating and learning how to grow rare actinomycetes that cannot be cultivated on standard laboratory media.

Lower Genome-Sequencing Costs Make Actinomycete Genome Mining Feasible

The genomic sequences of *Streptomyces avermitilis* and *Streptomyces coelicolor* are providing insights into the abundance of potential secondary metabolic pathways present in individual streptomycetes. Both contain tens of potential PKS and NRPS pathways.

Meanwhile, researchers at Ecopia BioSciences in Saint-Laurent, Quebec, Canada, use microbial genomics to predict structures of previously unidentified secondary metabolites from actinomycetes. In turn, they optimize fermentation conditions for producing those compounds. With genome sequencing methods becoming less expensive, it might be worthwhile to sequence many different actinomycete genomes, including those of rare and new genera from terrestrial and marine sources. The data from such efforts then could lead to new secondary metabolites, including some with antimicrobial activities. The data would also lead investigators to new genes for use in combinatorial biosyntheses.

Marine Actinomycetes Are a Source for Novel Antimicrobials

More than 70% of the surface of the earth is covered by water. Actinomycetes comprise about 10% of bacteria colonizing marine aggregates, and can be isolated from marine sediments, including those obtained at depths of 10,898 m from the deepest part of the Marianas Trench. Many actinomycetes isolates from this deep-ocean source contain NRPS and PKS pathways, the hallmarks of secondary metabolite production. Despite their abundance, however, marine sediments and marine invertebrates are relatively untapped sources for new secondary metabolites.

Interesting examples of recently isolated novel secondary metabolites include abyssomicin C and salinosporamide A. Abyssomicin C is produced by a *Verrucosispora* strain isolated from the Sea of Japan at a depth of 289 M; hence its name, the antibiotic from the "abyss." This polycyclic polyketide antibiotic acts by inhibiting *para*-aminobenzoic acid biosynthesis in the folic acid pathway. It and its analogs are being evaluated as candidates for treating drug-resistant gram-positive pathogens.

Salinosporamide A is a β-lactone-γ-lactam proteosome inhibitor produced by the novel obligate marine actinomycete *Salinispora tropica*. This compound is in a phase-I clinical trial to treat solid tumors and lymphomas that is being sponsored by Nereus Pharmaceuticals of San Diego, Calif.

Combinatorial Biosynthesis Also Can Lead to Novel Antimicrobials

Researchers have been developing methods for genetically engineering biosynthetic pathways over the past 25 years. These efforts parallel fundamental biochemical studies on mechanisms for assembling complex secondary metabolites by giant multisubunit, multienzyme NRPSs and PKSs. Together, the mechanistic knowledge and streamlined engineering approaches make combinatorial biosynthesis workable for generating derivatives of antibiotics and other secondary metabolites that would be difficult or impossible to generate by medicinal chemistry.

Antibiotic biosynthetic pathways evolve over billions of years, whereas project timelines in industry extend from 1 to 10 years. Hence, combinatorial biosynthesis projects are called on to speed the process of novel antibiotic development by 10^7- to 10^9-fold. This approach probably will not work for generating wholly novel antibiotics.

However, combinatorial biosynthesis is now well suited for making improved versions of known antibiotics in ways that are not adequately addressed through traditional medicinal chemistry approaches. For instance, combinatorial biosynthesis was used to generate derivatives of the reduced macrolide erythromycin by modifying the polyketide structure and then adding sugars and methylating them. The macrolide biosynthetic pathways of tylosin and spinosad were also successfully engineered, yielding novel spinosyns with improved insecticidal spectrums.

Meanwhile, several glycopeptide antibiotics that are related to vancomycin and teicoplanin were modified in vitro and in vivo by combining chemoenzymatic, fermentation feeding, and genetic engineering methods. Similarly, daptomycin-like lipopeptides were generated using combined chemoenzymatic and combinatorial biosynthetic approaches. Other examples where such approaches were used successfully include the development of hybrid antitumor antibiotics by combining components of the staurosporin and rebeccamycin biosynthetic pathways, and of hybrid gyrase-topoisomerase IV antibacterials by mixing components of the novobiocin and chlorobiocin pathways.

In general, researchers working in academic

laboratories and biotechnology companies account for these advances in combinatorial biosynthesis. However, this patchwork approach is not ideal, and a more concerted effort could help to realize the full potential of this technology. Indeed, to discover genuinely new antibiotics requires a more visionary, long-term, internationally coordinated approach.

These efforts also should include the sequencing of multiple actinomycete genomes to further our knowledge of the extent and variety of secondary metabolite pathways present in actinomycetes, and to provide additional genes to exploit in combinatorial biosynthesis (see table). Such an endeavor will undoubtedly lead to discoveries and new uses of secondary metabolites in other therapeutic areas such as cancer and immunosuppression, two areas where natural products from actinomycetes already have made substantial contributions.

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