The Mold Rush

WE WERE WARNED. The rise of antibiotic resistance is not a surprise. In 1945, when Sir Alexander Fleming accepted the Nobel Prize for his discovery of penicillin, he suggested that society should be careful with antibiotics and that misuse would lead to resistant organisms. He was right, and throughout the last 70 years, he was not the only one to sound an alarm.

Society is now on the brink of losing the ability to fight dangerous infections. Bacteria have gained resistance to our arsenal of antibiotics and have shared their strategies with each other. The once-revered miracle drug penicillin that revolutionized medicine—a compound made by blue-green mold with the express purpose of fighting off its enemy bacteria—is now rarely used, and most of its derivatives are dropping out of commission one by one.

However, while negligent use may fuel resistance, the problem is intensified by the fact that very few new antibiotics have been approved in the last few decades. The high cost of research and clinical trials, coupled with the fact that resistance is a natural phenomenon that will inevitably render new drugs useless after some period of time, makes their development less profitable. In fact, many pharmaceutical companies have not researched new antibiotics since the 1990s.

While many warn of an antibiotic apocalypse, Los Alamos structural biologist Alex Koglin remains hopeful. His passion for understanding the structures of proteins and enzymes has led him to a novel approach for identifying new therapeutics. But that's not all. Using this technique, he and his team mined the genomes of thousands of organisms, and earlier this year they discovered two *completely new* antibiotics.

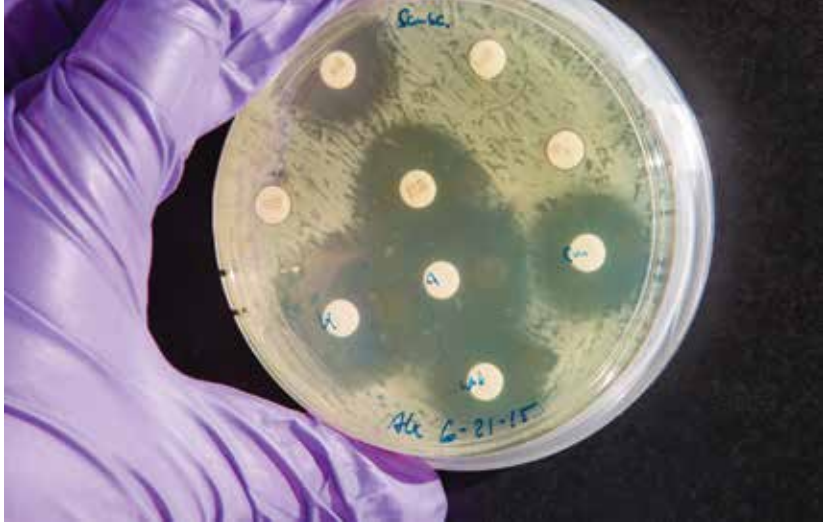
Miracle mold

As the story goes, the discovery of penicillin in 1928 was somewhat accidental when Fleming, an English bacteriologist, returned from vacation to find that a mold called *Penicillium notatum* had contaminated his petri dishes. Upon further study of the situation, he was surprised to find that the mold prevented the normal growth of his *Staphylococcus* bacterial culture.

Microorganisms (microbes), like all living things, must compete for food and space in their ecological niche. Fleming had stumbled upon one of their natural defenses: the mold was secreting a small molecule that could kill its enemy, the staph bacteria. Later work by Howard Florey and Ernst Chain (who shared the Nobel Prize with Fleming, but not the fame) further examined the small molecule they called penicillin and showed it could be isolated, produced, and used as a treatment against infections in humans. The resulting miracle drug was widely used during World War II for stopping infections from wounds and amputations and is credited with having saved thousands of soldiers' lives. Subsequently, penicillin was quickly marketed to the general public as a cure-all for everything from ear infections to gonorrhea.

After the discovery of penicillin, many other antibiotics were isolated from microbes for use in medicine, while others were developed synthetically by slightly altering the structure of existing antibiotics. For decades, new therapeutics were approved at a steady pace, but in the late 1980s and 90s, the numbers began to level off and then dropped dramatically over the last 15 years.

Why? Some argue that pharmaceutical companies have abandoned the search for antibiotics to focus efforts on more profitable therapies for chronic diseases such as cancer, heart disease, or diabetes—for which patients purchase drugs regularly, year after year. However, others argue that all the easy-to-find antibiotics have already been found, and that developing new antibiotics that are effective against drug-resistant bacteria is a difficult task. Adjusting the chemical structure of an antibiotic to make a new one requires careful attention

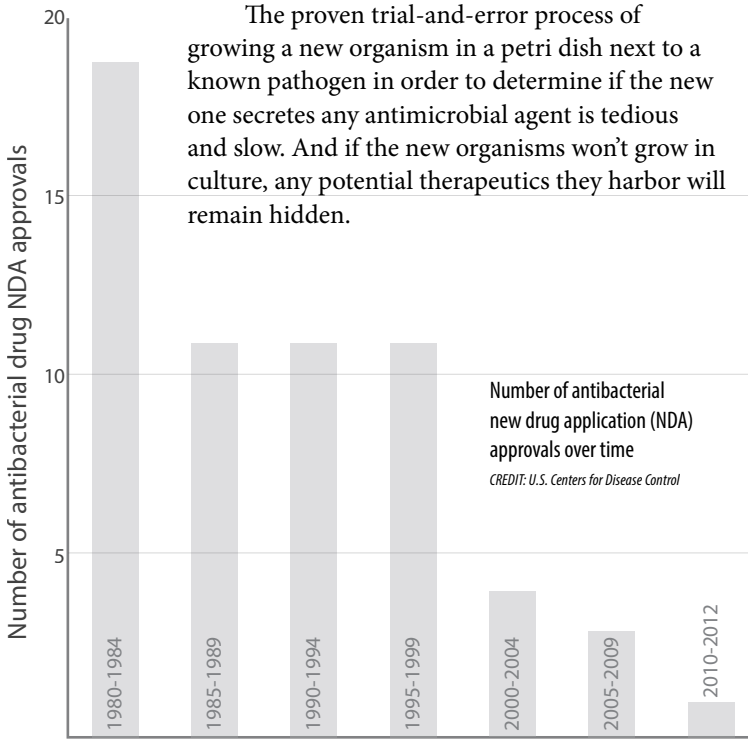


Antibiotics are small compounds made naturally by microorganisms for survival; some are made to kill other organisms that threaten their food supply or living space. (Left) The space between two different molds indicates that one is secreting a compound that inhibits the growth of the other. (Right) How this concept is tested in the laboratory: Small paper disks treated with antibiotics isolated from microorganisms are placed on a plate of bacteria. The area around the paper where no bacteria are growing shows that the antibiotics are working to kill the bacteria, as expected. However, a few antibiotics do not have clearance zones around them, indicating that the microbes are resistant to those particular drugs.

to ensure the new compound is not toxic and still works as desired. But since the resulting structure will be similar to existing drugs, it may also be more susceptible to already-developed bacterial resistance.

On the other hand, finding completely new drugs is also a challenge. There are billions of microorganisms out there in the world (in the soil, in the oceans, in our bodies) and likely all of them have developed defense mechanisms to fight each other. In order to take advantage of this plethora of potential drugs—not only antibiotic agents, but, to name a few, anti-inflammatory, anti-cancer, and anti-viral ones as well—these microbes must be studied in a lab. However, due to the fact that most of them require complex environments in which to grow, scientists have only discovered and cultivated a mere 1 percent of the planet's estimated microbial diversity.

The proven trial-and-error process of growing a new organism in a petri dish next to a known pathogen in order to determine if the new one secretes any antimicrobial agent is tedious and slow. And if the new organisms won't grow in culture, any potential therapeutics they harbor will remain hidden.



Number of antibacterial new drug application (NDA) approvals over time

CREDIT: U.S. Centers for Disease Control

What would Darwin do?

"Nature had and still has endless evolutionary time to develop small bioactive molecules to kill bacteria," says Koglin. "We have to accept that a potential solution for our current public health needs with multi-drug resistance is likely out there, but we have to change the way we look for it."

Koglin's strategy begins with understanding how antibiotics are made by microbes. Antibiotics are small molecules often referred to as secondary metabolites because the cell produces them for a valuable purpose that does not include the primary functions of growth, development, or reproduction. Secondary metabolites might instead be molecules that help the organism adapt to its environment (such as binding to certain nutrients) or protect it by killing enemies.

Since defense molecules such as antibiotics are toxic, they could potentially hurt the host cell that makes them, so they are highly regulated. In order to do this, microbes make antibiotics using special enzyme complexes; one class of these enzymes is called nonribosomal peptide synthetases (NRPSs). This is in stark contrast to the way most other products are made by the cell.

Most cellular products are made by protein complexes called ribosomes following directions from the cell's genomic material (DNA): a transcript (made of RNA) of the directions is made and then translated by a ribosome into protein. Antibiotic compounds are different; they are not directly coded for in the genomic material at all. Instead, the genome contains instructions for the specific NRPS complex needed to make each antibiotic or other secondary metabolite. In other words, the genome encodes the tools, and the tools make the antibiotic (in or near the cell, somewhere the process can be regulated effectively to prevent damage to vital cellular components).

Threats such as food competitors, predatory organisms, or environmental changes (pH, temperature, food availability) trigger the production of NRPS complexes through the normal genome-transcription-translation process. Then NRPS

enzymatic components assemble in a highly specific order determined by the genetic sequence. Once assembled, the complexes begin producing metabolite compounds—in assembly-line fashion—to respond to the threat. Each enzyme has a specific operation. Step by step, some enzymes build the backbone of the molecule, while another enzyme might add a hydroxyl (OH) group, or a methyl (CH_3) group, or, as in the case of a recent discovery by Koglin's team, an aldehyde (CHO). The chemical structure of the metabolite compound produced at the end is based entirely on the order of the enzyme complex: each NRPS cluster has a specific order and therefore a specific product.

As a result, an organism keeps instructions for an entire toolbox of NRPS complexes within its genome. This requires tremendous effort by the microbe, and an individual organism might not always have the correct NRPS tools to respond to the present threat. However, as Koglin explains, bacteria function

In the 1940s and 50s, penicillin was marketed to the general public as a cure-all for everything from ear infections to gonorrhea—and it worked. Unfortunately, decades later, gonorrhea is making a comeback as some strains have gained resistance to multiple antibiotics. These resistant strains are already becoming a major problem in Australia, France, Japan, Norway, Sweden, and the United Kingdom.

CREDIT: NIH

Resistance Matters: Too Much of a Good Thing

Just as some microbes evolve the ability to produce antibiotics for self-defense, those microbes and others can also evolve resistance to the antibiotic. The more antibiotics are used in humans and animals, the more bacteria become resistant. In the United States today, antibiotic-resistant bacteria cause about two million serious infections and 23,000 deaths per year.

In Industrial Agriculture

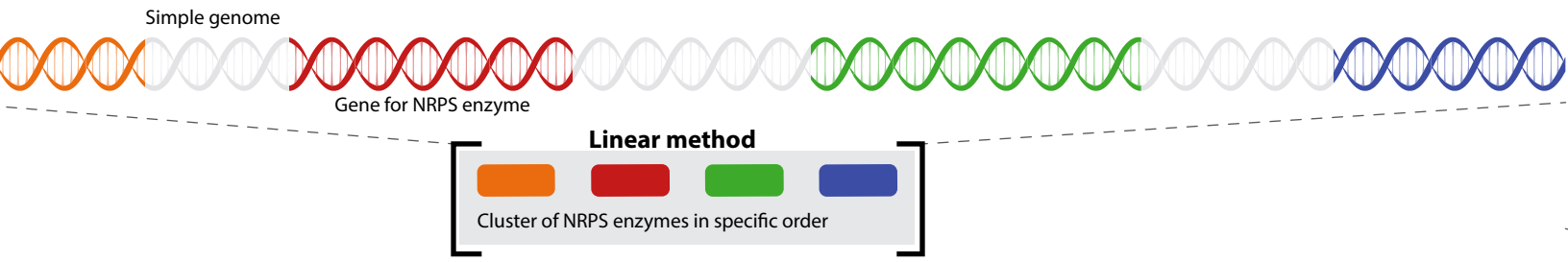
In the 1940s, as industrial agriculture began to expand, requiring animals to live in close quarters in large numbers, farmers began using antibiotics to keep them healthy. They quickly discovered that the drugs actually increased growth in the livestock and poultry, allowing the production of larger animals using less food, thus pushing down food costs. In 1977, the Food and Drug Administration (FDA) wrote a proposal to withdrawal approval for administering penicillin in animal feed, but it did not pass. Instead, meat production increased over the last few decades and a large portion of it has included the use of antibiotics such as tetracyclines, penicillin, streptomycin, and bacitracin, to name a few. In fact, the majority of the antibiotics sold in the United States (70 percent, according to the Pew Charitable Trust and PBS Frontline) are given to livestock.

This widespread use of antibiotics—especially for animals that are not sick—is creating large populations of resistant bacteria that can cause disease in humans. Furthermore, resistant bacteria from animals that have been fed antibiotics can spread if the animal manure is used to fertilize fields or is washed into waterways by rain. Although some agricultural representatives argue that the direct correlation of use in animals and disease in humans is difficult to prove, many studies have shown there is an impact. Likely with this in mind, the FDA issued a call in 2013 for the voluntary reduction of the use of antimicrobials for growth enhancement in livestock—followed by Tyson foods and McDonald's announcing earlier this year that they will begin to limit the use of antibiotics in their chickens.

In Medicine

According to the U.S. Centers for Disease Control, over half of the antibiotics prescribed for patients are inappropriate. This means that in many cases, a patient suffering from a viral infection will be given an antibiotic, which will not do anything to fight the infection. Instead, it will give any bacteria in the patient's body a week's worth of exposure to the drug, which can fuel resistance. In one study, only 10 percent of adults who had a sore throat actually had strep (a streptococcal bacterial infection that requires antibiotics); however, 60 percent of patients with sore throats were given antibiotics. Conversely, if a patient is correctly given antibiotics for a bacterial infection but does not finish the full prescribed course, there is an increased possibility that some bacteria will survive and may become resistant through the limited exposure.

Changing this situation requires educating both the public and the medical community. However, another improvement could come with advanced diagnostic tools. Doctors can test a patient with a sore throat to find out if it is indeed caused by streptococcal bacteria, but such tests cost time and money, and there are not reliable, affordable tests for all possible infections. Research and development of new, less-expensive diagnostic tools that can rapidly screen for multiple diseases at a time could give doctors the information they need to treat patients more effectively and responsibly.



together as a species, and the survival of the individual is not the priority. Through a process called horizontal gene transfer, bacteria can exchange genes for their NPRS complexes to other bacteria to help their colony survive. And the genes are reorganized in the recipient organism's genome to create novel clusters that will produce new compounds. Indeed, this serves as a good example of survival of the fittest because if the compounds created don't work to eliminate the threat, the bacteria are more likely to die. However, if at least one bacterium in the colony creates a compound that allows it to survive, it may spawn a new colony equipped with its successful NRPS tools.

Because the antibiotic produced can be highly toxic to the organism that made it, that organism must sometimes evolve resistance to its own defense mechanisms. This resistance is also transferred to others in the colony, which is helpful so long as the resistance doesn't get transferred to the organism the antibiotic is supposed to kill, in which case it is no longer useful. So alternatively, a microorganism might defend itself against its own dangerous metabolites in other ways. Koglin and his recent postdoc Matthais Strieker (who now holds a faculty position at the University of Braunschweig in Germany) actually discovered examples of secondary metabolites that are so toxic they cannot be entirely produced inside the host cell. Rather, only precursor molecules are made in the cell and then immediately exported so their biosynthesis can be completed outside of the host.

Targeting the toolbox

Not surprisingly, many researchers in academia and industry have focused on examining the NRPS toolbox to find new antibiotics. Fascinated by the structures of NRPS clusters and their ability to create different compounds based only on the order of their components, Koglin and Strieker began to seek an understanding of the driving forces for NRPS enzyme cluster assembly. Their hope was that if they could understand the clusters better, they might discover how to find novel ones that make novel antibiotic compounds.

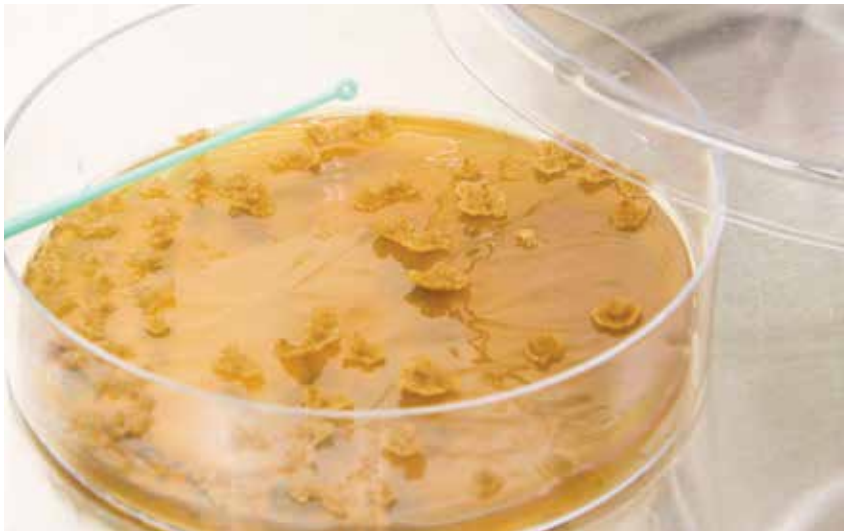
To do this they needed to determine which NRPS assembly lines exist in which organisms, so Koglin and Strieker turned to their Los Alamos colleagues' expertise in genomics, bioinformatics, database development, and microbiology to screen the microbial, fungal, and plant kingdoms. Those colleagues included bioinformatics scientists Jean Challacombe and Scott Hennelly, the Bioscience Division genome team, and microbiologist Chris Yeager.

Nature had and still has endless evolutionary time to develop small bioactive molecules to kill bacteria.

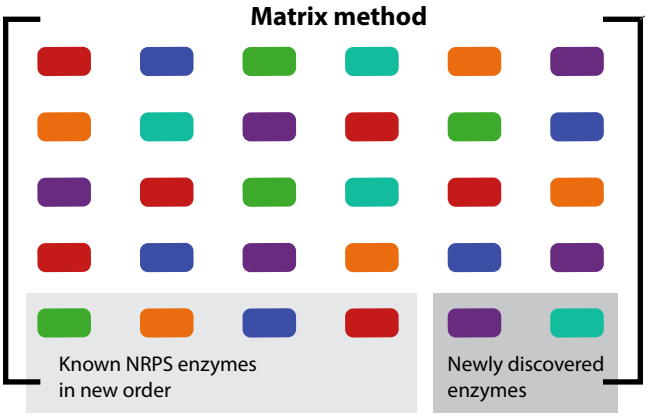
Over the last decade, genome sequencing has made significant advances, in particular with the invention of metagenomics—a method that enables entire communities of microorganisms to be sequenced at once without having to culture them individually. The resulting explosion of widely available genome-sequence data ensures an ample supply of newly discovered organisms that can be mined for novel compounds.

With metagenomic sequence data and expert collaborators at hand, Koglin and Strieker set out to study the clusters. Koglin explains that many people look at the genes for NRPS enzymes linearly, but that he and Strieker wanted to find a cluster that was truly different, with the hope that it might be less susceptible to resistance. So they needed a new approach.

Streptosporangium roseum and *Catenulispora acidiphila:* two organisms the Los Alamos team is investigating for new drug candidates.



Nonribosomal peptide synthetases (NRPSs) are clusters of enzymes that make antibiotic compounds. In an effort to find new antibiotics, scientists search the genomes of new microorgansims for the genes that encode NRPS enzymes. It is relatively easy to find the genes if they always exist in the same linear order in the genome; however, in complex organisms that organize their DNA on multiple chromosomes, the genes might be spread out and more difficult to identify. In an effort to find NRPS clusters that are truly different, with the hope that they might be less susceptible to resistance, Los Alamos scientists developed a matrix method to find the genes for those NRPS enzymes wherever they might be in the genomic data—even if they are not found together. The result: they were able to discover known enzymes in new orders as well as new enzymes that together produce a completely new class of antibiotic compounds.



First, they examined the known functions of each of the enzymes necessary to create an antibiotic-type, or bioactive, molecule. With this information, they developed an algorithm to find the genes for NRPS enzymes wherever they might be in the genomic data—even if they are not found together. For instance, in a complex organism like a plant that has its genes organized on chromosomes, the NRPS genes for one cluster could be found spread out on different chromosomes. And once they found the pieces of a cluster, they began to examine which other genes were nearby that might be new and potentially involved in NRPS synthesis.

It's kind of like looking for all the words in a sentence, but not requiring that they be in the right grammatical order—just whether certain words are there, within certain proximity parameters. This led to a matrix-type approach that allowed Koglin and Strieker to screen vast amounts of data—genomes from thousands of unknown microbes—to determine which organisms have the right tools encoded in their genomes for producing bioactive molecules that have potential as new antibiotics.

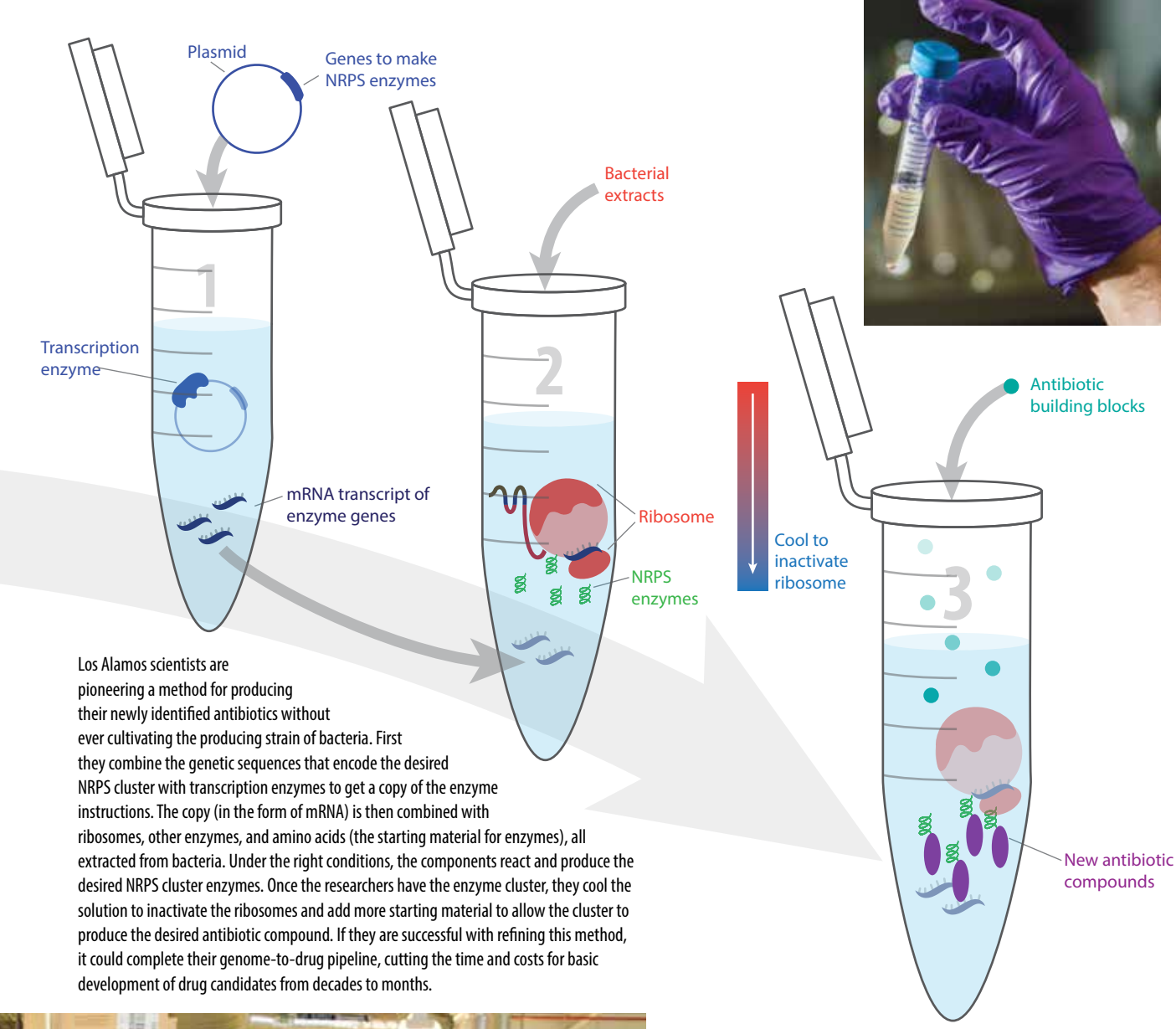
To accelerate the screening process, Challacombe has been developing a relational database to enable parallel searches and alignments. "The goal is to put all the powerful tools in one place to do the analysis," she says. "That way, if you find one NRPS cluster in one organism, you can determine if it looks the same in another."

The team used this matrix approach to screen 30,000 clusters of enzymes in over 4,000 plant, fungal, and bacterial genomes. From this, it was able to identify 16,000 clusters that would make something completely new. The team further narrowed its results by searching for very specific criteria, such as certain amino acids or side chains, the ability to integrate into a membrane, the activation of key enzymes, etc. In the end, the researchers found five new bioactive compounds they predicted could be useful as antibiotics.

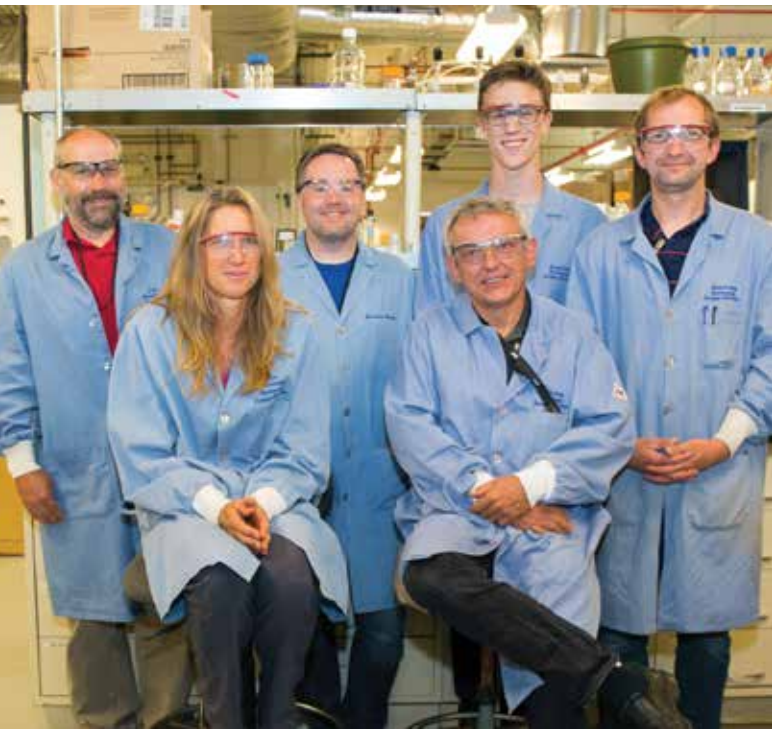
Among the NRPS clusters that produced these five compounds, two clusters caught the team's attention by the fact that they produced practically identical compounds but had evolved independent biosynthetic pathways to do so in completely different organisms: one, an anaerobic hyperthermophile called *Clostridium thermocellum,* and the other, a cold-lake predatory bacterium called *Herpetosiphon aurantiacus*.

Along with Los Alamos chemist Jurgen Schmidt, the team began to further examine the enzyme clusters in these two organisms and the compounds the clusters produced. Although the scientists still do not fully understand the mechanisms of the clusters or their order, they sought to elucidate the chemical structures of the metabolite products using stable-isotope labeling (adding a heavier carbon-13 atom instead of a normal carbon-12 to allow tracking), mass spectrometry, nuclear magnetic resonance, x-ray crystallography, and neutron scattering. ("The convenience of having this comprehensive suite of capabilities at our fingertips—as well as genomic sequencing and computation—is a unique advantage for scientists at Los Alamos," says Schmidt.) In the end, they isolated and named an antibiotic compound called thermocellomycin from *C. thermocellum* and one called aurantiamycin from *H. aurantiacus*.

Once the new compounds were isolated and characterized, the team could test thermocellomycin and aurantiamycin for antimicrobial activity. The results have been impressive. Both new compounds were shown to inhibit the growth of 13 pathogen species, encompassing over 20 strains. This list included known, highly problematic resistant strains such as MRSA (methicillin-resistant *Staphylococcus aureus*, which causes deadly skin infections) and *Clostridium difficile*



Los Alamos scientists are pioneering a method for producing their newly identified antibiotics without ever cultivating the producing strain of bacteria. First they combine the genetic sequences that encode the desired NRPS cluster with transcription enzymes to get a copy of the enzyme instructions. The copy (in the form of mRNA) is then combined with ribosomes, other enzymes, and amino acids (the starting material for enzymes), all extracted from bacteria. Under the right conditions, the components react and produce the desired NRPS cluster enzymes. Once the researchers have the enzyme cluster, they cool the solution to inactivate the ribosomes and add more starting material to allow the cluster to produce the desired antibiotic compound. If they are successful with refining this method, it could complete their genome-to-drug pipeline, cutting the time and costs for basic development of drug candidates from decades to months.



Left to right: Scott Hennelly, Jean Challacombe (seated), Michael Humbert, Jurgen Schmidt (seated), John M. Gordon, and Alex Koglin

(which causes a life-threatening gastrointestinal disease), as well as *Bacillus anthracis* (which causes anthrax) and *Yersinia pestis* (which causes the plague).

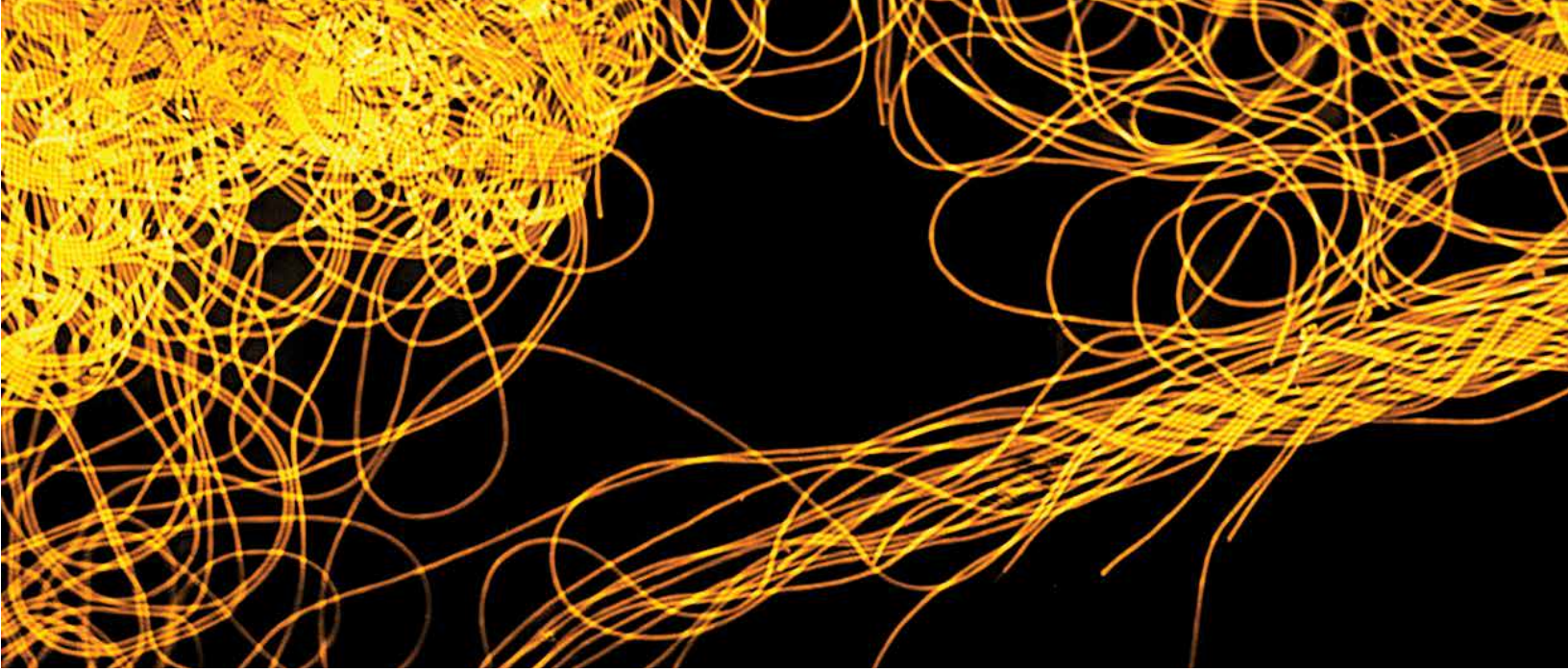
"What we've found here is a completely new class of antibiotics," says Koglin.

The road to the medicine cabinet

In the 1940s, when society began to produce penicillin on a large scale, the challenge was to grow enough *Penicillium notatum* mold in containment so that the drugs could be siphoned off. Today, large-scale production of antibiotics generally still relies on growing microbes.

If the new Los Alamos drug candidates thermocellomycin and aurantiamycin are ever to be approved for widespread use, they will have to be produced *en masse* as well. However, the Los Alamos team has not yet been able to culture these organisms in large quantities, nor has it been successful in producing thermocellomycin and aurantiamycin using alternative host organisms.

To address this aspect of the challenge, the team is developing a method to produce the newly identified antibiotics without cultivating the producing strain of bacteria.



Los Alamos scientists recently isolated the new antibiotic aurantiamycin from this bacterium, *Herpetosiphon aurantiacus* (shown here in a photo taken with a specialized type of optical microscope). Aurantiamycin has already demonstrated effectiveness against 13 pathogen species, including highly problematic resistant strains such as methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile*, and is currently being tested for toxicity in animals before consideration by the Food and Drug Administration.

CREDIT: Marine Biological Laboratory/Irina Arkhipova and Michael Shribak

The method relies on combining all the necessary components (the genetic sequences that encode the NRPS cluster, transcription enzymes, and ribosomes) in a vial at the right temperature and pH so that the components react to produce the desired NRPS cluster. Once the team has the enzyme cluster, it should be able to add amino acid building blocks so that the NRPS cluster can produce the antibiotic compound—all without the need for a live host organism.

"It would be a tremendous shortcut not to have to grow the organism," says Michael Humbert, a guest scientist working with the Los Alamos team. Humbert, who is a specialist when it comes to host-free biosynthesis, explains that the ribosomes have to be especially stable in order to survive the lengthy process of creating large amounts of enzyme clusters. The team had been using *E. coli* enzymes, but they are not holding up to the stress of the production line, so it is now trying ribosomes from different thermophilic (heat-loving) organisms because of their increased stability.

What we've found here is a completely new class of antibiotics.

In the meantime, while the Los Alamos team is working on improving its production method, the United States Army Medical Research Institute of Infectious Diseases is busy testing thermocellomycin and aurantiamycin for toxicity in animals and doing further analysis of the drugs' effectiveness against biothreat strains. If all goes well, the new antibiotics could be headed for Investigative New Drug Approval, a critical requirement preceding clinical trials as part of the process to gain Food and Drug Administration approval.

However, the real advancement here is that if Koglin and his collaborators can successfully produce thermocellomycin and aurantiamycin in the laboratory without the need for growing the organisms, then they will have developed a complete genome-to-drug pipeline, cutting the time for basic development of drug candidates from decades to months.

In contrast to Fleming's serendipitous discovery of penicillin, the Los Alamos scientists demonstrated a new strategy to selectively screen the microbial kingdom for the tools needed to create antibiotics. This strategy enabled their discovery of two new potential drugs, thermocellomycin and aurantiamycin, but it also showed how a targeted analysis of the NRPS toolbox could be used to find therapeutics. This approach could be used again and again, so as bacteria develop resistance to new drugs, scientists will have a reliable way to stay ahead of the game by continually discovering more new compounds. Moreover, the team's efforts to produce new antibiotic compounds without ever cultivating an organism stand to greatly accelerate therapeutics production in general. Together, these achievements have the potential to revolutionize drug development. And there is nothing accidental about that. LDRD

—Rebecca McDonald

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