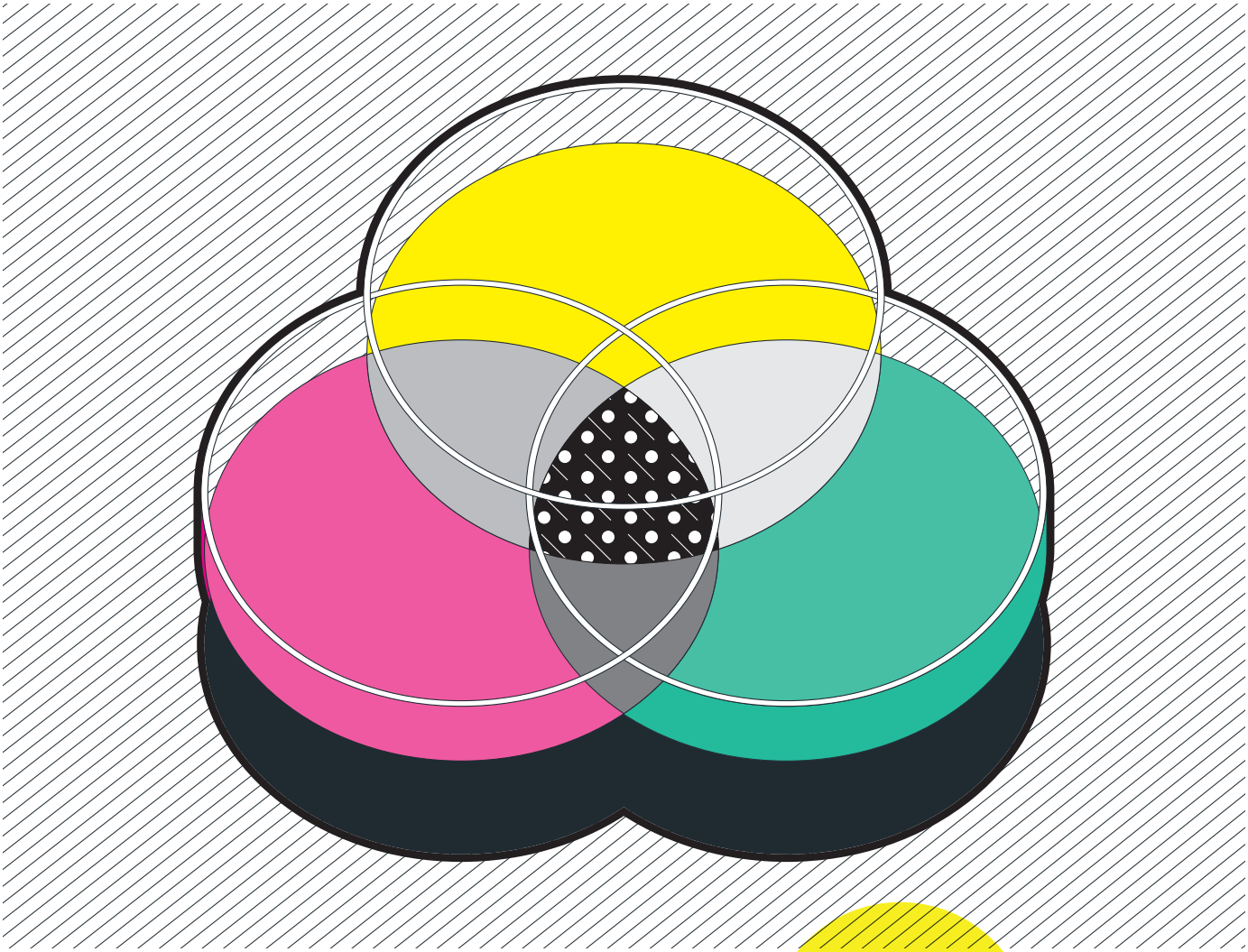


ILLUSTRATIONS BY THOMAS POROSTOCKY

MINING THE MICROBIAL DARK MATTER

Microbiologists are finding new ways to explore the vast universe of unknown microbes in the hunt for antibiotics.

BY CORIE LOK

The first time Robert Heinzen tried to get *Coxiella burnetii* to grow by itself, he failed miserably. The bacterium, which causes an influenza-like illness called Q fever, normally divides only inside the cells it infects — forcing researchers to grow it in mammalian tissue and hampering their efforts to investigate the microbe. When Heinzen tried to find a different way to culture it during his time as a postdoc in the early 1990s, he emerged with only half a book of scribbled notes.

But the problem kept nagging at him until 2003, when the *C. burnetii* genome was sequenced[1] and he was starting a lab at the US National Institutes of Health's Rocky Mountain Laboratories in Hamilton, Montana. Heinzen thought that the genome could offer important clues to the bacterium's metabolism and growth. Even so, it took his postdoc Anders Omsland almost four years of systematically testing hundreds of combinations of culture conditions to come up with the perfect recipe for cultivating the microbe outside cells[2]. "When he showed me the cultures, I thought, it's got to be a contaminant," Heinzen recalls. But several more months of work confirmed their success.

Coxiella burnetii is still in the minority. An estimated 85–99% of bacteria and archaea

cannot yet be grown in the lab, drastically limiting scientists' knowledge of microbial life and holding back the search for new antibiotics, which tend to be derived from bacteria. That search is becoming more urgent as resistance to existing drugs surges: last month, the World Health Organization approved a global plan to combat antibiotic resistance, and a review panel appointed by the UK government called for a £1.3-billion (US$2-billion) investment from the global drug industry to revitalize antibiotic research. To find new drugs, researchers say that they need alternative ways to investigate the array of uncultured organisms — the mysterious dark matter of the microbial world.

Scientists are already taking steps towards this goal. Advances in cultivation methods and other technologies have helped them to grow previously unculturable microbes, and improved DNA sequencing and bioinformatics are allowing them to examine some microbes without needing to grow them at all. The work has uncovered a breathtaking amount of microbial diversity in samples ranging from soil to permafrost, marine sponges, hydrothermal vents and the crevices of the human body. Some of the discoveries are already pointing to possible antibiotics — and scientists say that they are only just scratching the surface.

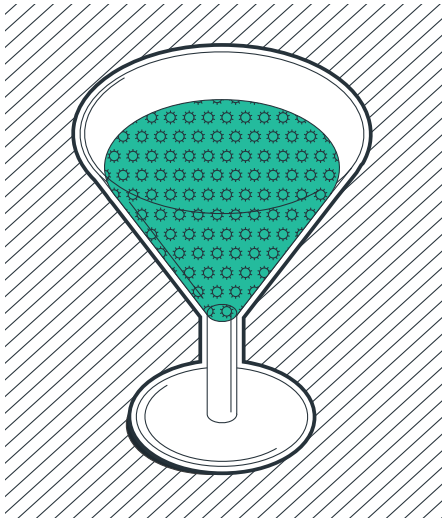
"There is, for sure, high potential for more biodiversity to be discovered," says Ute Hentschel, a marine microbiologist at the University of Würzburg in Germany. "If you look for more, you'll find more."

CULTURE COCKTAIL

Conventionally, biologists have studied microbes by growing pure cultures of a species in fairly standard sets of nutrients. The trouble is that bacteria do not live like that in nature: they inhabit a huge range of environments, usually alongside other organisms, and scientists have struggled to recreate those conditions. But as Heinzen and Omsland showed with their studies on *C. burnetii*, genetic sequences can throw open a door.

Omsland used sequencing to compare the genes expressed when the bacteria were growing successfully inside host cells with those expressed when they were struggling to grow alone. He found a suite of genes involved in protein synthesis that were less active in the struggling microbes, a hint that adding amino acids and peptides to the growth medium might help the bacterium to thrive. But even when Omsland managed to increase the bacterium's protein synthesis 13-fold, it still would not divide[2].

The final clue came from genes suggesting that *C. burnetii* could survive in low-oxygen environments. When the team placed the microbe in 5% oxygen or less, they finally saw it grow. "That was the critical finding," says Heinzen. "It wasn't a nutrient, it was an environmental factor."



"THAT WAS THE CRITICAL FINDING. IT WASN'T A NUTRIENT, IT WAS AN ENVIRONMENTAL FACTOR."

Since adopting the new 'axenic' or host-cell-free culture technique, the *C. burnetii* field has expanded. By selectively turning genes on and off, researchers have learned about how the bacterium interacts with host cells to infect them and divide. "The ability to grow *Coxiella* axenically has, without any exaggeration, completely revolutionized this field of study," says Hayley Newton, a microbiologist and *Coxiella* researcher at the University of Melbourne in Australia. The bacterium is highly transmissible through air and is considered a possible biothreat. Heinzen's lab is now working on making strains in which key virulence genes have been inactivated, in the hope that they might be useful in developing vaccines.

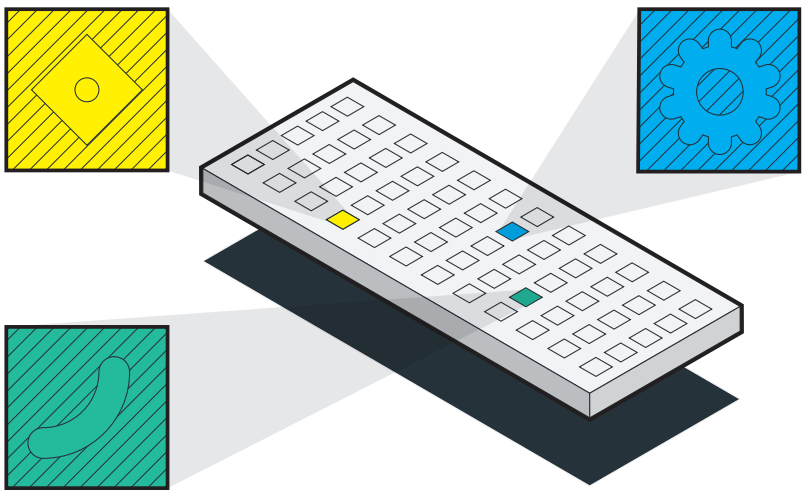
Researchers are now designing culture systems for other microbes that grow only inside cells. Omsland, now at Washington State University in Pullman, has developed a cell-free culture system for *Chlamydia trachomatis*[3], the pathogen behind one of the most common sexually transmitted diseases. He has not yet coaxed *Chlamydia* to divide in his medium, but "I was born optimistic," he says — and his success with *C. burnetii* fuels his hope.

MINIATURIZED CULTURES

One way to speed up the process of finding a culture recipe is to use microfluidic chips — devices with thousands of tiny wells connected by channels that make it possible to run many experiments in parallel. After using this method to cultivate a new microbe[4], Rustem Ismagilov at the California Institute of Technology in Pasadena and his collaborators even named the bacterium isolate microfluidicus 1.

Ismagilov was already working on microfluidics when, in 2012, a group of microbiologists issued a list of 'most wanted' taxa — a call to the research community to grow and sequence microbes that were relatively common in the human body, were distantly related to already-sequenced organisms and had eluded all attempts at cultivation[5].

Ismagilov and his team answered the call with a device that holds 3,200 nanolitre-sized wells and that can fit in the palm of a hand. They scraped samples from the gut lining of a healthy volunteer, and then diluted them so that no more than one cell would end up in each well. By filling so many wells, the researchers increased the chances that their target organism — a human-gut microbe in



the *Oscillibacter* genus — would find its way into at least a few of them. The team used about ten chips to test various conditions, and looked for growth of the microbe by checking its DNA for a key marker gene.

They managed to find their bacterium, and then grow it to larger amounts in Petri dishes. It was one of the first members of the wanted list to be cultivated. Further genetic study revealed that isolate microfluidicus 1 was not actually part of the *Oscillibacter* genus; it had been classified incorrectly and was actually part of a new, related group that the team is now working to characterize.

A key ingredient for growing this bacterium, the team found, was a dash of fluid that had been extracted from the volunteer's intestine. Being able to stretch the use of such a precious sample across thousands of experiments is an important advantage of the microfluidics approach, says Ismagilov. Another is that each starting cell does not have to compete with other species. "Microfluidics allows us to identify culture conditions efficiently and then increase our chances that our target will grow," he says.

Xiaoxia Nina Lin, a chemical engineer at the University of Michigan in Ann Arbor, is using microfluidics to hunt down members of the most-wanted list in human faecal samples. Microbes normally live in complex communities and often rely on other species, so Lin is trying to dissect those relationships by putting two, three or four cells together in myriad combinations on a chip, and working out who is dependent on whom. "It's a good engineering approach," says Vincent Young, an infectious-disease researcher at the University of Michigan who is helping Lin to obtain clinical samples. "You can quickly reduce the complexity."

NATURE'S INCUBATOR

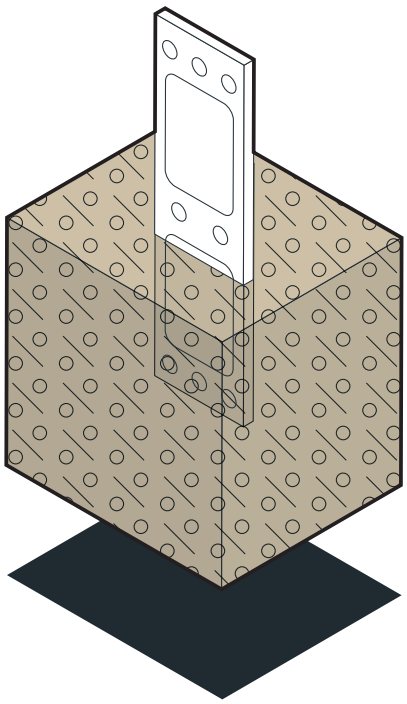
When Slava Epstein and Kim Lewis started to collaborate 15 years ago, they realized that they might not need to coax recalcitrant microbes into growing in the lab. If a bacterium already grows happily in its natural environment, they reasoned, then why not just cultivate it there? So the two microbiologists, from Northeastern University in Boston, Massachusetts, started working on a simple device that they could stick in the ground. They called it the iChip.

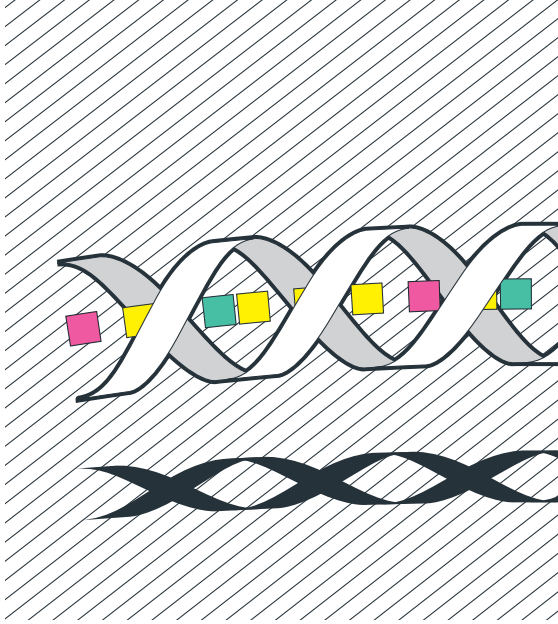
The approach paid off earlier this year, when Lewis, Epstein and scientists from their start-up company NovoBiotic Pharmaceuticals in Cambridge, Massachusetts, reported that they had used the iChip to isolate a new bacterial species from soil[6]. The thumb-sized device is less sophisticated than a microfluidics chip: it consists of 384 tiny wells that were filled with samples of soil that have been mixed with agar and diluted to ensure that only one cell ends up in each chamber. The chip is sealed with a membrane that traps the bacteria but allows molecules to diffuse back and forth, and was planted in a grassy field in Maine — the same soil from which the sample had been taken.

After a month, the researchers transferred colonies from the chip to Petri dishes in the lab, took extracts from them and screened those for antibiotic activity. They had grown 10,000 types of bacterium — many more than if they had just put the soil sample on an agar plate. They homed in on a new species that they called *Eleftheria terrae*[6] and found that the bacterium produces an antibiotic, called teixobactin, that kills various human pathogens in the lab, including drug-resistant strains of *Staphylococcus aureus*. "To me that's a phenomenal result, that they can find really neat new molecules from groups of organisms that pharma largely hasn't focused on," says Sean Brady, a chemical biologist at Rockefeller University in New York.

But what generated headlines was the discovery that other bacteria did not develop resistance to teixobactin[6], as they do to most other antibiotics. That is because teixobactin binds to molecules that have important roles in cell-wall synthesis; bacteria are not known to modify these molecules to evade the effects of antibiotics. The clincher, Lewis says, is that although *E. terrae* is inherently resistant to the teixobactin, it does not seem to have resistance genes that could be transferred easily to other bacteria. This does not mean that resistance will never emerge, but that it could take 20 or 30 years.

The NovoBiotic team went on to grow larger quantities of the bacterium. It is now generating grams of the drug using a fermenter, doing extensive preclinical testing of this and other drug candidates, and seeking more leads from uncultivated microbes in soil and marine samples. Epstein is using the iChip to culture new microbes from soil and water in Greenland,





and says that he has received more than 200 requests this year for the device and advice on how to use it.

DON'T CULTURE, SEQUENCE

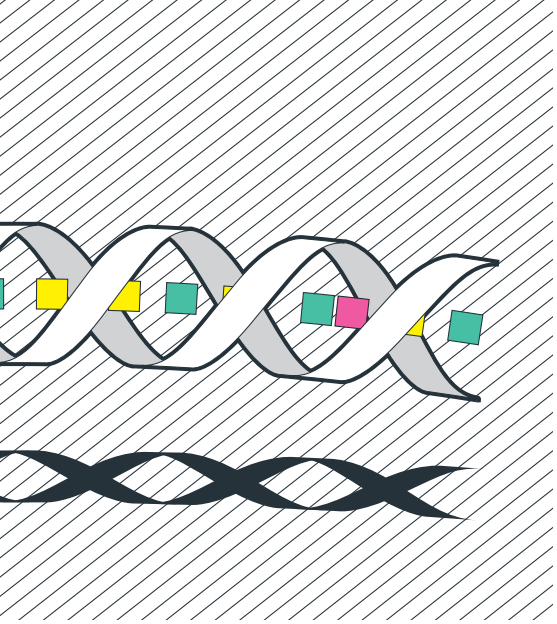
Despite these successes, culturing microbes is still a complicated, hit-and-miss affair, so many researchers are bypassing it altogether and instead learning what they can from DNA. Advances in sequencing methods mean that scientists can now analyse genomes from individual uncultured microbial cells — rather than, as before, typically sequencing a community of many different types of microbe en masse and then trying to piece the sequences back together.

Tanja Woyke at the US Department of Energy's Joint Genome Institute in Walnut Creek, California, first got interested in single-cell sequencing not long after the key discovery ten years ago that an enzyme from a bacteria-infecting virus could be used to make many copies of a bacterial cell's genome[7]. Woyke wanted to use the tools to fill out the microbial tree of life.

She and her group collected samples from nine different habitats, including sediment from a Nevada hot spring and water near a Pacific hydrothermal vent. They isolated some 200 cells, sequenced the genome of each one and classified the cells into more than 20 new lineages that do not have any cultivated representatives[8]. "They were the first to really take single-cell genomics to the next level, in terms of the number of sequences and single cells analysed," says Hentschel.

Last year, Jörn Piel of the Swiss Federal Institute of Technology in Zurich and his colleagues reported that they had used single-cell sequencing and other techniques to identify uncultured bacteria in marine sponges[9]. These filter-feeding creatures have long been of interest to scientists because they produce a rich set of chemicals with anticancer, antibiotic and other medicinal properties. They also harbour dense microbial communities that contribute up to 40% of the sponge's mass and were suspected to be the source of these chemicals. But the members of those communities had not been cultured.

Piel and his group focused on the sponge



"THEY WERE THE FIRST TO REALLY TAKE SINGLE-CELL GENOMICS TO THE NEXT LEVEL."

Theonella swinhoei, which harbours about 1,000 types of bacterium and generates dozens of known bioactive compounds. In 2011, they started to sequence DNA from individual bacterial cells isolated from sponge samples and looked for two gene clusters known to be involved in the production of biologically active molecules. They found these genes in a bacterium called *Entotheonella*[9].

What was most surprising to Piel, however, was that this one organism was responsible for nearly all of the bioactive compounds linked to the sponge — something that became clear when sequence data showed that the bacterium harboured all the necessary genes. When Piel received the key data from his collaborators, "I almost fell out of my chair," he says; it was the first evidence that an uncultivated microbe can be such a 'talented' producer of bioactive chemicals. "The ability to create many distinct compounds in a single strain, this is not that common," he says.

Piel's lab is now trying to engineer gene clusters from *Entotheonella* into a culturable organism such as *Escherichia coli* so that the host can churn out the compounds, something that is not likely to be easy given that biosynthetic genes can be large. He is also mining the genomes of microbes in sponges from Japan, Papua New Guinea and Israel in search of other bacterial super-producers.

GENE PROSPECTING

Michael Fischbach, a biochemist at the University of California, San Francisco, has developed a different way to analyse microbial sequences: rather than isolating single cells, he sifts through the growing banks of bacterial genomic data.

Fischbach and his group developed a machine-learning algorithm that is trained to recognize key patterns associated with bacterial genes that synthesize interesting molecules such as antibiotics. Then they let it loose on a large set of bacterial genomes to look for new gene clusters with similar features.

Their targets included bacteria from soils and oceans, which are known to harbour an incredible diversity of microbes. But the algorithm generated a surprising number of hits from microbes that live on or in the human body, known collectively as the human microbiota. Fischbach was both excited and intimidated when he got the first results. Excited, because the bioactive compounds made by the microbiota were largely uncharted territory, and could have important roles in human health and disease. Intimidated because Fischbach mostly worked on microorganisms in soil.

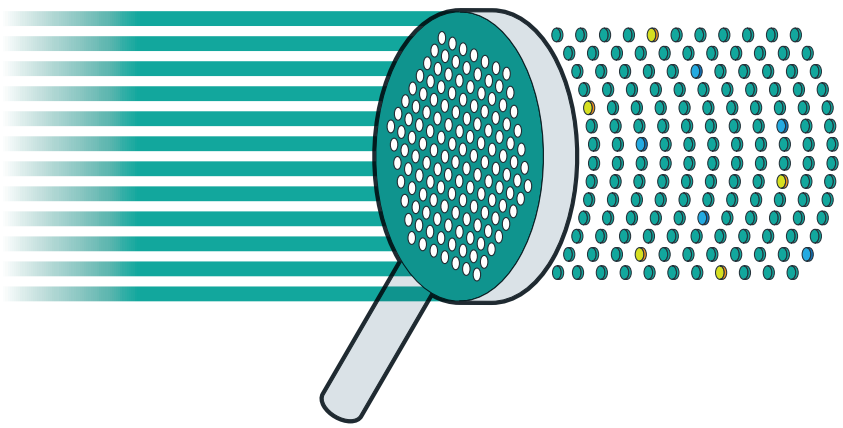
Still, Fischbach decided to take the plunge, and has since switched his entire lab's focus to the human microbiome. Using an improved version of the algorithm, his team went prospecting in the genomes of almost 2,500 organisms in the human body[10], and found more than 14,000 biosynthetic gene clusters. "It has been remarkably easy to find interesting molecules from the human microbiome," says Fischbach. "It's so easy that I can tell it has nothing to do with our ability to find them, it's more that there's just a lot to find."

The group narrowed down the list to more than 3,000 common gene clusters, and found that one generated an antibiotic, lactocillin, made by a microbe commonly found in the vagina. It is one of only a handful of bioactive chemicals isolated from the human microbiota. Lactocillin blocks the growth of common vaginal pathogens such as *S. aureus*, but not of other bacteria that normally inhabit the vagina. Fischbach is now generating the molecules from the gene clusters he is finding, solving their structures, and working with collaborators to learn more about their function.

The natural-products field has tended to focus on soil and marine microorganisms rather than on human ones, says Gerry Wright, a chemical biologist at McMaster University in Hamilton, Canada. "I think it's a great idea to look at those genes and clusters," he says. However, turning such compounds into usable drugs will require a lot of preclinical work, says Wright. "Just by looking at a molecule, it's almost impossible to tell whether it's going to be suitable as a drug." And even if it seems promising, the barriers to commercializing a new antibiotic are high (see M. Woolhouse and J. Farrar *Nature* **509,** 555–557; 2014).

But Lewis takes hope from the recent progress. With all the burgeoning efforts to culture and analyse uncultured microbes, he is already imagining what could be discovered if such efforts were scaled up. He hopes to see a level of drug discovery to match that in the Waksman era, the time in the 1940s and 1950s when Nobel-prizewinning microbiologist Selman Waksman discovered more than 20 antibiotics by systematically screening thousands of soil microbes for their ability to block the growth of other bacteria.

"The fact that we're finding compounds with such remarkably interesting modes of action that we haven't seen before, that's the most interesting part," says Lewis. "We've scratched only a tiny part of Mother Earth." ■



Corie Lok *is* Nature's *Research Highlights editor.*

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