EXTREME CULTURE

From acid mine drainage to the bowels of the Earth, **Josie Glausiusz** reports how researchers are taking great pains to grow recalcitrant bacteria.

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To reach one experimental site — inside Lechuguilla Cave, New Mexico — Diana Northup and her team must venture 350 metres down into the Earth, hiking along several kilometres of obstacle-strewn passages. For days they rappel down pits, traverse narrow ledges bordering steep drops and pristine lakes, and clamber over boulders — all the while carrying heavy packs of equipment. When they finally arrive at their sampling areas, they must change into clean suits, including bonnets and sterile gloves. "It's hard to convey to you how filthy you can become in four to five days," Northup says. "You're just constantly drenched in sweat. These crusts we study get all over you, gypsum gets all over you, and the sweat helps plaster it all onto you. It's just lovely."

Northup, a microbiologist at the University of New Mexico in Albuquerque, and the inter-university SLIME team (Subsurface Life in Mineral Environments) are looking to find how cave bacteria deposit oxidized iron and manganese crusts on the walls of underground caverns. Her first attempts to grow these microbes in the lab were thwarted. All that appeared on her agar plates were 'weeds', fungi that hikers had tramped into the caves on their boots. Hence the clean suits, to prevent contamination. To get the right incubating conditions, Northup came up with a simple solution: cultivate the bacteria in glass tubes inside the caves themselves, providing the precise environment they prefer: total darkness, low temperatures and high humidity.

Northup's efforts may seem extreme, but they illustrate the lengths to which some microbiologists will go to culture the seemingly unculturable. According to a common estimate, some 99.9% of microbes will not reproduce in Petri dishes lined wth nutrient-rich agar — a culturing technique virtually unchanged since its invention in the early 1880s. Most have more stringent criteria, including rare minerals, specific biochemical signals or the organisms with which they usually cooperate. In other words, they need the comforts of home.

Difficulties in culturing bacteria have helped spur metagenomics, in which researchers descend on a sample — a bucket of sea water, a patch of human skin or a handful of soil — and sequence all the microbial DNA within. Such techniques can start to reveal how many bacterial species can be found in any spot, or how many gene variants appear in the collective population. For example, the J. Craig Venter Institute announced in March 2007 that its *Sorcerer II* Global Ocean Sampling Expedition had discovered 6 million new genes and thousands of new protein families. Mitchell Sogin's group at the Josephine Bay Paul Center of the Marine Biological Laboratory in Woods Hole, Massachusetts, has been sequencing 16S ribosomal RNA from seawater samples to measure species numbers and found microbial diversity much higher than had been reported before.

Exciting as these results are, they also highlight the culture gap. Some biologists remain dedicated to taming recalcitrant bacteria in the lab, challenged and sometimes aided by metagenomic findings. Using specially designed diffusion chambers, mud-filled vessels, microarrays and bioreactors, they mimic the conditions in which the bacteria live naturally. They are growing what no one has grown before, which should reveal more about the organisms than a mere gene census, says evolutionary biologist Lynn Rothschild of NASA's Ames Research Center in Moffett Field, California. "Just because they have a gene, they may not use it. Or we may not learn when they use it," she says. "Unculturability is not a biological characteristic, it's a human failing. They're not unculturable; they're just not cultured yet."

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Some researchers have what Rothschild calls "a green thumb for microbes". Belinda Ferrari of Macquarie University in Sydney, Australia, has developed a novel microcultivation method, using a mud slurry as a medium for growing soil bacteria. Using this system, she has cultivated a wide diversity of bacteria: they include those called TM7, found in soil, bioreactor sludge and the human mouth. Nutrients come only from the mud, a source ideally suited for these microbes, which may suffer in the high sugar levels that suit lab 'weeds'. "If you're in a pot full of jam and not much else, you'll just overdose and get sick from too much sugar," Ferrari explains.

From culture to cash

There are commercial spinoffs for extreme culture. In the Netherlands, microbiologists Mike Jetten and Marc Strous of Radboud University Nijmegen have enriched anaerobic ammonium-oxidizing (anammox) bacteria and nitrate-dependent anaerobic methane-oxidizing bacteria. The latter, collected from a canal contaminated with agricultural runoff, were grown in a bioreactor with a nutrient-limited supply of carbon dioxide, nitrate and methane. The two succeeded in culturing colonies of these microbes after a year of trial and error. Anammox bacteria are now being used to clean waste water on a large scale in the Netherlands and Japan.

Meanwhile, Kim Lewis and Slava Epstein of Northeastern University in Boston, Massachusetts, are trolling the depths of the uncultured for new antibiotics using a diffusion chamber in which microbes are suffused in the conditions of their natural environment — soil, for example, or sea water plus marine sediments. Lewis and Epstein have founded the company NovoBiotic to capitalize on their cultures. "The practical benefits are enormous," Lewis says. "If you want to discover new stuff, you want to go to organisms you haven't seen before. It's reasonable to assume that 99% of the remaining bacteria will have at least some useful antibiotics."

The spelunking Northup has begun to find antibiotics in her samples. Initially, though, she strove to grow crust-forming cave bacteria to understand their basic biology. "One of the reasons we culture rather than do DNA sequences is because we want to catch them in the act of precipitating the minerals, so that we can say definitely, 'These guys can do it'," she says.

While underground she and her team scrape crust off the cave wall and stab it into a glass tube filled with 'sloppy' agar. An iron carpet tack or reduced manganese at the bottom of the tube provides metal, and rock dust from limestone in the caves provides trace minerals that the microbes need. The bacteria form bands in the agar at different oxygen levels and precipitate iron and manganese deposits.

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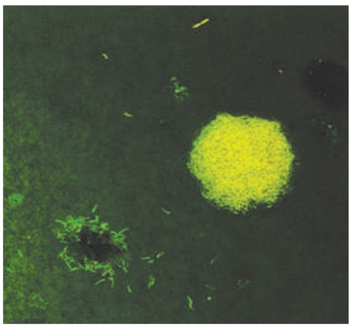

B. FERRARI, N. TUJULA

SLIME's lab in a cave (above); Ferrari's colonies of bacteria around soil particles (left).

Because these microbes grow so slowly, Northup and her colleagues usually leave their cultures percolating for years. She has already cultivated a variety of microbes from New Mexico caves, including iron- and manganese-oxidizing species of *Bacillus*, *Caulobacter* and *Alcaligenes*: odd-looking bacteria whose strange morphology she refers to as "beads on a string" and "hairy sausages". She has also found samples of *Actinomycetes*, bacteria that are known to produce more than 4,000 antibiotics, as well as enzymes that could be of use in biotechnology.

Antibiotics and ecology

Northup now has a student searching her *Actinomycetes* samples for novel antibiotics. "Because this is fairly unexplored habitat, we're hoping to find some rarer ones," she says. "We'd also maybe like to characterize what habitats in caves tend to be richer in organisms that produce antibiotics — to test the hypothesis that in low-nutrient environments they're more likely to produce these secondary metabolites to keep their neighbours at bay."

Such ecological questions could have widespread implications. A group led by Steve Giovannoni, a microbiologist at Oregon State University in Corvallis, has cultivated at least 11 strains of SAR 11, a marine microbe that is not only the smallest independently replicating bacterium known but also has the smallest genome ever seen in a free-living cell. What's more, SAR 11 "is possibly the most abundant organism in the oceans", says Giovannoni. It converts organic matter into carbon dioxide, and therefore plays a huge role in global biogeochemical cycles. All the more reason, therefore, to understand its basic biology — something Giovannoni thinks is best done through pure culture. "Metagenomics is fantastic," he says, but "with an organism like this, it's vastly superior to have a culture. If hypotheses emerge from a genome sequence, you can then test them. Also, at least 30% of the genes in every microbial genome cannot be identified under the best of circumstances."

Even as some microbiologists seem aligned with pure culture techniques, others have used metagenomics as a guide in raising obstinate microbes. Jill Banfield of the University of California, Berkeley, and her colleagues used 'shotgun' sequencing methods to characterize five species of iron-oxidizing bacteria and archaea in biofilms from an acid mine-drainage system in Iron Mountain, California, and discovered that only one carried the genes that code for enzymes to fix nitrogen. "We got a pretty complete inventory of the pathways in each organism that's present," Banfield says. "Because we had near-complete genomes we could say, 'This organism — ha ha! — has the ability to also fix nitrogen, whereas the others don't.'" By raising them in a sulphuric-acid bath supplemented with iron, with no nitrogen except the gaseous form, the team was able to isolate the nitrogen-fixing bacterium, *Leptospirillum ferrodiazotrophum*.

As metagenomic efforts ramp up, more of these extreme-culture techniques are likely to follow because the two approaches inform each other. "You get totally different bits of information," says microbial ecologist Anna-Louise Reysenbach of Portland State University in Oregon, who for the first time has cultured an acid-loving, thermophilic, sulphur-reducing species of archaea called *Aciduliprofundum boonei* from deep-sea vents at Valu Fa Ridge between Tonga and Fiji. "I love being able to have the organism in culture, but also knowing what its distribution is like. I definitely think that both have great value. There shouldn't be one without the other." ■

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