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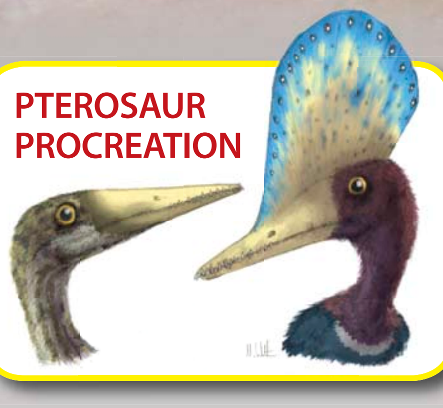

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Bacteria Back from the Brink

Thousand- and Million-Year-Old Microbes Found Living in Salt Crystals: Could they also exist on other planets?

Tim Lowenstein

In 1993, "Jurassic Park" thrilled the world with the idea that dinosaurs could be resurrected from bits of DNA preserved in mosquitoes trapped in ancient amber. In the 18 years since the movie came out, scientists have been finding that parts of this scenario are closer to reality than anyone ever imagined.

In 2000, microbiologist Russell Vreeland of West Chester University in Pennsylvania and his colleagues found a 250-million-year-old bacterium — still alive — inside a tiny droplet of water in a salt crystal, they wrote in Nature. Although the find was controversial, further studies have found evidence of other microorganisms called archaea surviving tens of thousands, if not millions, of years in salt crystals. And just two years after the Vreeland study, Steven Fish, a microbiologist at the University of Leicester in England, and colleagues reported in Nature that they extracted DNA from bacteria and another type of microbe called haloarchaea from halite samples up to 425 million years old.

The discovery of ancient life trapped in salt raises many exciting questions: Could these hibernating microbes be brought back to active life today? If so, what might they tell us about ancient life on Earth? And do they hold the secrets to finding life in outer space?

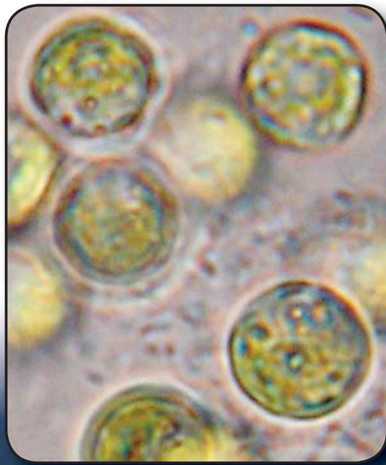
Researchers have found microbes living in salt crystals that have been buried beneath Earth's surface for tens of thousands of years.

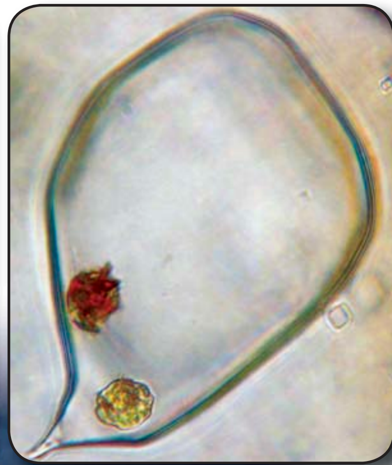
Cindy (Satterfield) Magruder collects water and salt samples from Saline Valley, Calif., during a microbial bloom in 2004 when the brine was pink. Salt crystals grew on old wooden piers as the lake water evaporated.

Both: Michael Timofeeff

THE FIRST SHOCKING FINDS

In the 1960s, the geobiology world got its first inkling that live prokaryotes (single-celled organisms of two domains — bacteria and archaea — that lack a nucleus and other membrane-bound specialized structures) might be found in ancient salt deposits. Heinz Dombrowski of Justus-Liebeg University in Germany and others found that living prokaryotes were preserved in salt hundreds of millions of years old. That work was startling, but most microbiologists thought the microbes were recent contaminants and dismissed the findings. Four decades later, Vreeland and his colleagues were more careful about avoiding contamination, and their report in Nature rocked the geobiology world.





Left inset: Halophile (salt-loving) microbial bloom from Saline Valley, Calif., showing living, green *Dunaliella*. Right inset: Fluid inclusion with algal cells (probably *Dunaliella*) in modern halite from Saline Valley.

Layered halite (salt) with pink and green coloration from trapped microorganisms, collected from a modern salt pan in Saline Valley.

All: Michael Timofeeff

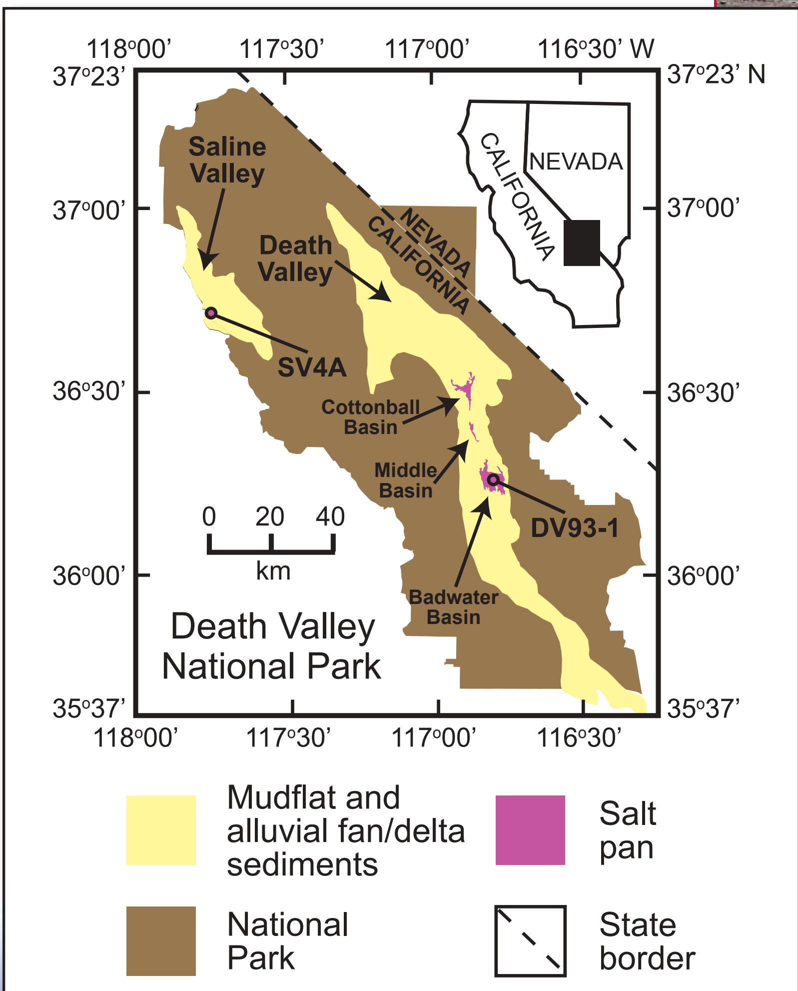
Vreeland's team described how they drilled into a salty fluid inclusion, 9 cubic millimeters in size, in a halite sample that they had collected from a depth of 564 meters along a mine shaft through the marine salt deposits of the Permian-aged Salado Formation near Carlsbad, N.M. The team sampled the liquid with a syringe and released the sample into a nutrient-rich but sterile liquid medium. Soon a single species of bacterium grew in the liquid medium, providing proof that the organism had been living inside the salt crystal since the Permian period, which ended about 250 million years ago.

This was the first study of its kind to be accepted by many in the scientific community. Geobiologist John Parkes of the University of Bristol in England, for example, hailed the work in a statement in Nature in 2000 as "the best evidence yet for the extremely long-term survival of microorganisms."

The excitement was not universal. Questions quickly arose regarding the veracity of the team's methods and the conclusions. For example, some researchers said that the samples Vreeland and his colleagues studied were not the primary crystals that formed layered salts in the Permian seas, but came from a later-formed halite cement crystal that filled a dissolution cavity. Without primary growth features in the halite crystals, they said, the age of the crystals, and thus also their fluids, is questionable. Meanwhile, other researchers noted that the Permian bacterium Vreeland and his colleagues found was suspiciously similar to a known bacterium cultured from the Dead Sea. So perhaps the Permian bacterium is not authentic, but is the product of sample-based or laboratory-based contamination. Similar questions were raised about the study by Steven Fish.

Though diversity is limited, hypersaline environments can be incredibly productive, with more than 10,000 *Dunaliella* and 100 million smaller prokaryotes living in one milliliter of water! Such vast numbers of microorganisms turn clear water into tomato soup.

Map of Saline Valley and Death Valley, showing locations of the cores (SV4A and DV93-1) drilled by Tim Lowenstein and others.



These are valid concerns, and something anyone who undertakes this kind of study needs to overcome. But they are addressable.

Over the past 10 years, my colleagues at the State University of New York at Binghamton and I have been looking at new ways to study microorganisms and DNA in salt. We have examined some of the same salt samples Vreeland, Fish and others have studied, and we have found our own. For example, in 2002, Binghamton graduate student Cindy (Satterfield) Magruder analyzed the fluid inclusions from the same halite cement crystals used in the Vreeland study and found that they contained evaporated Permian seawater. Those results, published in Geology in 2005, supported the 250-million-year-old age of the halite and fluid

A salt lake in Saline Valley, looking east.

Bottom to top: Michael Timofeeff; modified from Lowenstein et al., GSA Today, 2011; Michael Timofeeff

Michael Timofeeff at Saline Valley, with the Inyo Mountains in the background.

inclusions in the Vreeland study. But of course, the results could not address the potentially more serious contamination questions.

To answer that question and to further validate the question of age, we have to learn more about microorganisms in modern hypersaline environments — how they get trapped inside salt crystals and how they can stay alive for so long in buried salt deposits.

THE PRESENT IS THE KEY TO THE PAST

Hypersaline environments — such as the Great Salt Lake in Utah, the Dead Sea in Jordan and Israel, and Death Valley in California — contain brines loaded with dissolved salts, well above the 3.5 percent salinity of seawater. At roughly 10 times their concentration in seawater, sodium and chloride levels are high enough for halite to crystallize. It is in these types of harsh brines that halophilic organisms live.

Such salt-loving organisms are mostly microscopic plankton — one genus of algae (the primary producer *Dunaliella*) and more than 20 genera of heterotrophic prokaryotes (archaea and a few bacteria). The first battle these organisms face is water loss: If a cell contains fresher water than the surrounding environment, it will give up water to achieve osmotic equilibrium and die. Microorganisms living in shallow brines also need protection from intense sunlight; therefore, many produce red organic pigments, called carotenoids, such as beta-carotene. Though diversity is limited, hypersaline environments can be incredibly productive, with more than 10,000 *Dunaliella* and 100 million smaller prokaryotes living in one milliliter of water! Such vast numbers of microorganisms turn clear water into tomato soup.

The first breakthrough in our studies came in March 2004 on a field trip to Saline Valley, a remote closed basin in Death Valley National Park. There, we found a thriving microbial community in a bright red brine pool, and salt was crystallizing at the bottom. We discovered that, under the right conditions, the entire community of halophilic microbes can get encapsulated inside the crystallizing salt.

Even more interesting was that the microorganisms were trapped not in the solid salt, but in the numerous fluid inclusions that were incorporated within the forming crystals. The microbial community inside the fluid inclusions — including *Dunaliella*, a variety of prokaryotes and associated organic and mineral debris that were all suspended in the water column — appeared identical to that in the Saline Valley brine lake.

Thus it became apparent that the fluid inclusions sealed inside the salt crystals are magnificent time capsules.

THE DARK SIDE OF THE BRINE

What happens to the community of microorganisms when the salts get buried is another question. The best way to assess the preservation of halophilic communities in buried salt is through meticulous layer-by-layer examination. We had two good samples to work with: borehole cores drilled 90 meters deep in Death Valley in 1993 by our group at Binghamton and cores drilled to 93 meters in Saline Valley in 1978 by Jack Crowley of the U.S. Geological Survey in Reston, Va. Colleagues at the University of Southern California dated the salt cores to 100,000 years ago in Death Valley and 150,000 years ago in Saline Valley. We are now studying a similar salt core, from nearby Searles Lake in California, drilled into deeper and older salts, with samples from greater than 400 meters and ages older than 2 million years.

All of our salt cores come from the subsurface of closed basins. With progressive burial, the salts and trapped microbes are removed from damaging solar radiation and enter a completely dark environment. Temperatures during burial are never higher than the original environments in which the salts formed. Fluid inclusions in this salt are completely sealed from their surroundings — no oxygen is available to degrade organic compounds and destroy DNA. Salty fluid inclusions are therefore an excellent setting for preserving organic materials.

Binghamton graduate student Brian Schubert, post-doctoral researcher Michael Timofeeff and I examined hundreds of thin sections of salt searching for microbes in fluid inclusions. Many came from depths at which the halite formed under normally dry salt pan conditions (like Death Valley today); as a result, they had few microbes in fluid inclusions. Some layers of salt, however, formed in ancient saline lakes, and they had fluid inclusions loaded with algae and prokaryotes. Schubert found that the number of prokaryotes in those fluid inclusions was about the same as in modern productive hypersaline lakes. That study, published in Astrobiology in 2009, and Schubert's work on the algae in fluid inclusions from the Death Valley

core, published in Geomicrobiology Journal in 2010, showed the diversity of microorganisms in fluid inclusions — full communities, if not entire halophilic ecosystems.

We also observed *Dunaliella*-like algae, with greenish yellow and reddish coloration and fluorescent properties, which suggested that chlorophyll, beta-carotene and other biomolecules were still preserved in samples more than 100,000 years old. The microbial community in fluid inclusions looks exactly like that seen in surface brine lakes, with one notable exception — prokaryotes in fluid inclusions were always tiny and spherical, quite different from the variety of larger rod-like and spherical shapes of modern halophilic prokaryotes.

Both: Michael Timofeeff

SUNY Binghamton students in Saline Valley.

We discovered that, under the right conditions, the entire community of halophilic microbes can get encapsulated inside the crystallizing salt.

Right: Brian Schubert (left) and Tim Lowenstein collect brine samples in Saline Valley. Below: Lowenstein (left) and Schubert in Death Valley.

Both: Michael Timofeeff

It's rather strange, but not unprecedented. Bacterial cells in natural environments such as soils and the oceans become rounded and dwarfed when they experience nutrient deprivation and other unfavorable conditions. That is apparently what happened to the prokaryotes inside the fluid inclusions — they became miniaturized over time as a survival strategy.

CULTURING DONE RIGHT

The next question to address is whether any of these hundred-thousand-year-old microorganisms are still alive.

One way to test this is through culturing experiments. First, the surfaces of a salt crystal are sterilized to destroy any microorganisms that might be living on the exterior. Next, the salt is dissolved in a liquid medium that contains sodium, chloride, inorganic nutrients and a carbon source. Any prokaryotes trapped inside fluid inclusions are released into the medium where they may grow and multiply.

It's not easy: Out of almost 900 experimental attempts on the Death Valley core, Schubert only cultured five organisms, all archaea. Timofeeff, on the other hand, came up empty-handed after completing 500 experiments on the Saline Valley core. Together, less than 1 percent of all tested samples between 10,000 and 100,000 years old yielded live microbes.

Still, getting any cultures was quite exciting. To confirm our results, however, we had to reproduce them, which we did in our lab and another independent lab. First, the identical species was grown twice from two different samples of 34,000-year-old halite. Second, virtually identical strains of halophilic archaea from the genus *Natronobacterium* were cultured separately at Binghamton and at West Chester University. This experimental reproducibility pushes the veracity of the results to a new level of certainty. In addition, Melanie Mormile, a microbiologist at Missouri University of Science and Technology in Rolla, and colleagues independently cultured yet another halophilic archaea from the Death Valley salt core, from a 100,000-year-old crystal. That study, published in Environmental Microbiology in 2003, used a different technique — drilling through the salt crystal, extracting the brine from an inclusion and introducing it to a growth medium — and got the same result.

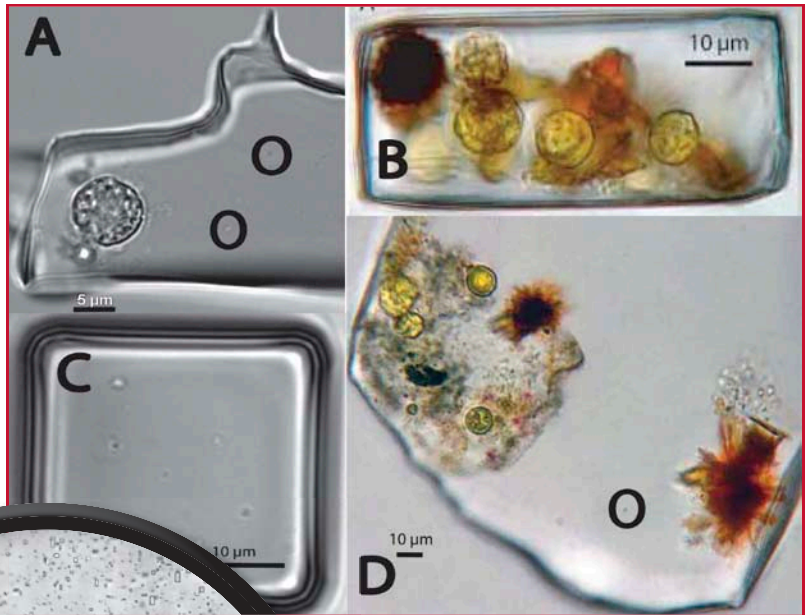
IDENTIFYING MICROBIAL COMMUNITIES TRAPPED IN SALT

Now that we've found that we can actually culture halophilic microbes living inside ancient fluid inclusions, we are turning toward studying ancient DNA that is also trapped in the fluid inclusions. Culturing studies, though appealing, are limited to those organisms that are alive and culturable, which is a minute fraction of the microbial community in the world today and certainly also in ancient fluid inclusions. Identifying ancient DNA in fluid inclusions, whether from within cells or as loose strands, in contrast, can give a more complete picture of entire fluid inclusion ecosystems — archaea, bacteria, algae and perhaps fungi and other eukaryotes (organisms with a cell nucleus and other membrane-bound structures).

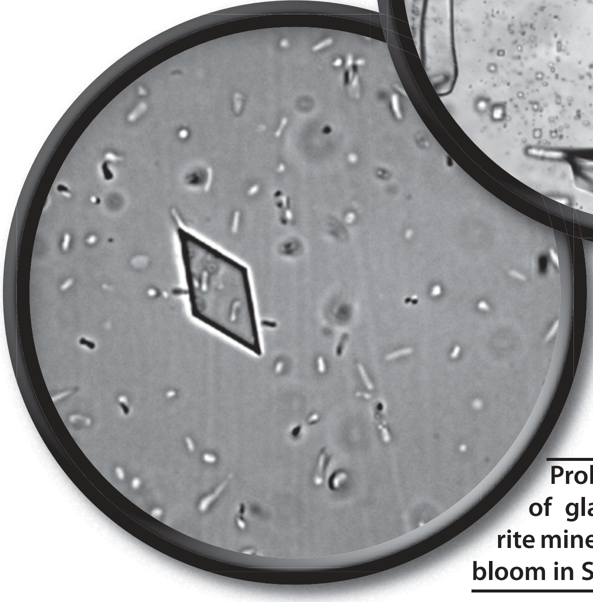
Such community DNA studies have been very successful in samples of permafrost, so there is no reason to doubt this approach can work in fluid inclusions. And indeed, some previous studies, including the 2002 Nature study by Fish, have found microbial DNA up to 425 million years old in halite. A second study by Jong Soo Park, a microbiologist at Dalhousie University in Nova Scotia, Canada, and colleagues found DNA from haloarchaea in halite samples 23 million, 121 million and 419 million years old. Both of those studies, however, are controversial and need further verification by microscopy and testing for reproducibility before the results will be widely accepted by the scientific community.

We are working with Binghamton molecular anthropologist Koji Lum and doctoral student Krithivasan Sankaranarayanan to identify ancient

Fluid inclusions with microbes in ancient halite from Saline Valley and Death Valley cores. A: Tiny prokaryotes (circled) and an algal cell, collected from a depth of 93 meters below the surface in Saline Valley. These prokaryotes and algal cell are 150,000 years old. B: Orange and yellow-green algal cells suggest preservation of beta-carotene and chlorophyll in a 34,000-year-old fluid inclusion in halite taken from 17.8 meters below the surface in Death Valley. C: Miniaturized 31,000-year-old prokaryotes from 16.5 meters below the surface in Death Valley. D: Large fluid inclusion with algal cells, some green and red in color, and tiny prokaryotes (one circled) from a 29,000-year-old halite sample from 16 meters below the surface in Death Valley.



Cubic, rectangular prism and large, irregular-shaped fluid inclusions in a modern halite crystal from Saline Valley.

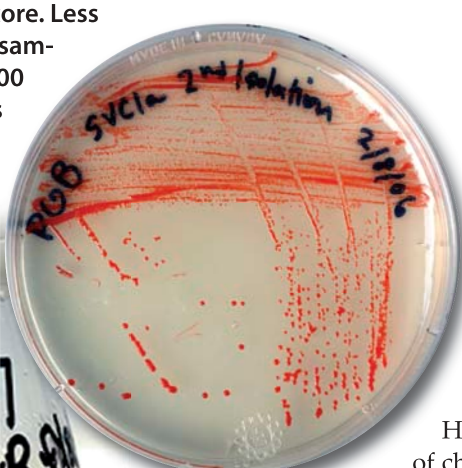


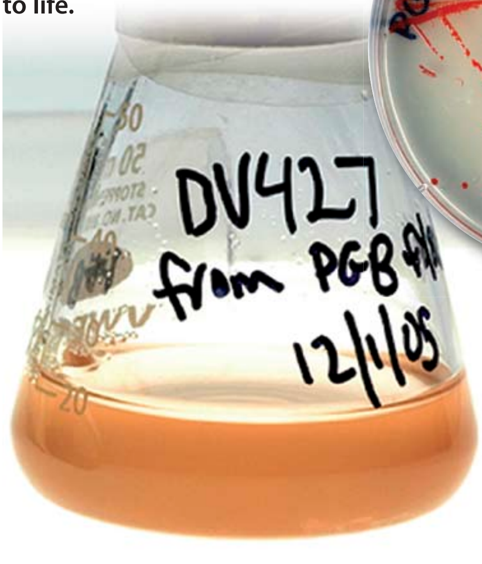
Prokaryotes and crystal of glauberite (an evaporite mineral) from a halophile bloom in Saline Valley.

Bottom to top: From Schubert et al., Astrobiology, 2009; courtesy of Tim Lowenstein; modified from Lowenstein et al., GSA Today, 2011

Schubert collects samples in Saline Valley.

Right: An agar plate (petri dish with a growth medium used to culture microorganisms) with halophilic archaea from Saline Valley halophile bloom. Below: A flask showing cultured ancient archaea from the Death Valley core. Less than 1 percent of all tested samples between 10,000 and 100,000 years old yielded live microbes — but that means that five microbes were brought back to life.



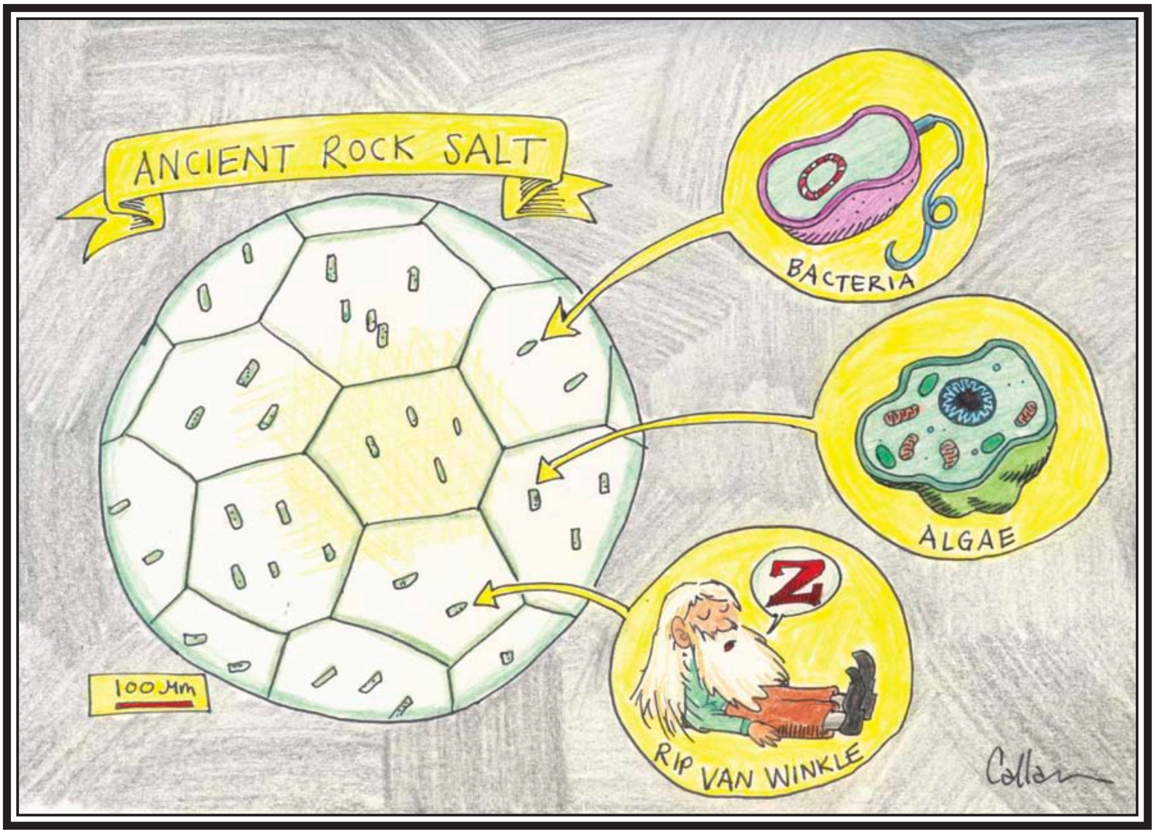


Bottom two: courtesy of David Tuttle; top: Michael Timofeeff

> Community DNA studies have been very successful in samples of permafrost, so there is no reason to doubt this approach can work in fluid inclusions.

DNA preserved in fluid inclusions in our core samples. Sankaranarayanan has developed methods that allow DNA from fluid inclusions to be reliably extracted, purified, copied using the standard DNA-replication technique called polymerase chain reaction, or PCR, and sequenced. Because of potential contamination, especially when PCR is involved, Sankaranarayanan has gone to great lengths to decontaminate the surfaces of samples with strong acid and bleach to destroy any DNA stuck on crystal surfaces. He has also taken the extra step of checking his methods by deliberately contaminating halite crystal surfaces with human DNA. Surface sterilization effectively removed all human DNA.

So far the results are promising: Sankaranarayanan has identified and sequenced DNA from many genera of archaea and algae in samples from the Saline Valley core that are 36,000, 64,000 and 150,000 years old. Further work will more completely describe the diverse microbial community from the subsurface halites of Death Valley, Saline Valley and even older samples millions to hundreds of millions of years old. If we get lucky, we may even find DNA from the same species found at both the top and bottom of a core. Genetic changes found in such a species over time could be used to calculate rates of molecular evolution.



There's no telling what researchers might find in a grain of salt.

WHAT MAKES A 30,000-YEAR-OLD MICROBE TICK?

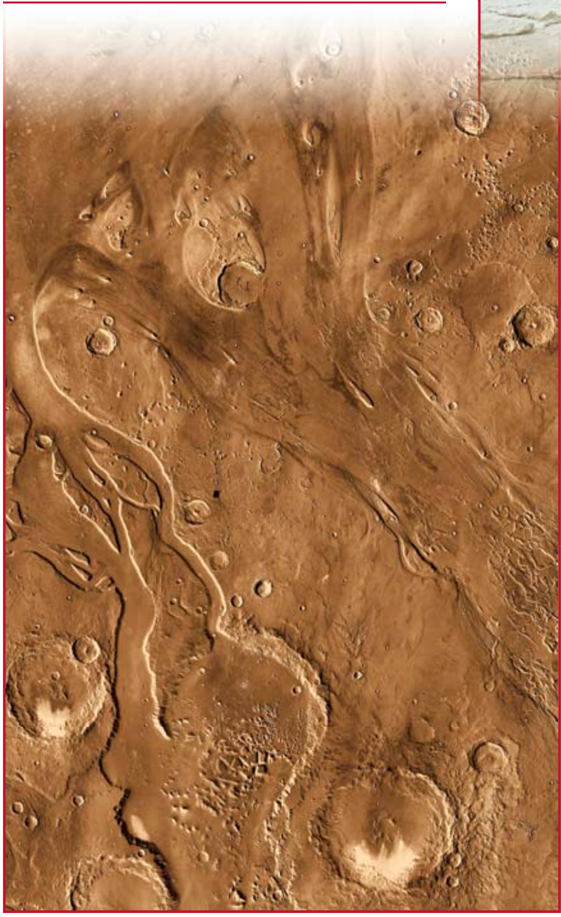
The next step, which we admit we still know very little about, is to figure out how halophilic archaea survive for prolonged periods inside fluid inclusions. But we are beginning to make a few connections.

Ancient archaea in fluid inclusions are miniaturized, presumably in some state of prokaryote hibernation — starvation survival. It is well-known that the macromolecules that make up cells, such as proteins, nucleic acids and lipids, are unstable over time and break down to simpler compounds in the absence of organism repair. Miniaturized halophilic archaea that survive for thousands of years in fluid inclusions must be able to repair damage to their macromolecules. For this repair, some low-level metabolism is required — and one of the microbes in these inclusions provides the perfect food to fuel such metabolism. *Dunaliella* makes its own sugar alcohol, glycerol, to attain osmotic equilibrium against the high salt content of the surrounding brines. In modern hypersaline lake communities, this carbon source is food for other microorganisms, so why wouldn't this be the case in fluid inclusions?

Schubert (left) and Lowenstein in Death Valley.

Bottom: Michael Timofeeff; top: Callan Bentley

All salt deposits on Earth have fluid inclusions, so it is almost guaranteed that the salt deposits of Mars also contain inclusions that may yield clues about ancient surface conditions — and maybe even life.



Above: Salt flats in Death Valley. Left: New research on fluid inclusions in halite on Earth suggests that halite on Mars might also host ancient microorganisms.

Bottom: NASA/JPL-Caltech; top: Michael Timofeeff

Indeed, we noted in a 2009 publication in Geology that the glycerol from one *Dunaliella* cell could supply enough carbon for one miniaturized archaea cell to repair macromolecular damage for 12 million years. That's pretty amazing.

A STEP IN THE RIGHT DIRECTION

It is exciting to know that any form of life can subsist for thousands to millions of years, but we still need more data on the distribution, survival and diversity of microorganism communities in buried salt. We need to know about the suite of microorganisms that existed at the time the salt was deposited; we also need to know about the associated biomaterials, such as DNA, chlorophyll and carotenoids, and the inorganic materials, such as major elements and nutrients. We want to better understand how communities of microorganisms in fluid inclusions have evolved over time, and how prokaryotes survived and obtained energy to perform necessary functions such as DNA repair.

Such knowledge is vital as we further explore the evolution of microbial communities over geological time and the preservation of life within Earth's crust — and elsewhere in the solar system, where materials that potentially shelter microorganisms are millions and even billions of years old. Biological communities trapped in fluid inclusions are particularly relevant in the search for signs of life in the sulfate and chloride salts on the surface of Mars, which may have preserved samples of ancient Martian surface brines. All salt deposits on Earth have fluid inclusions, so it is almost guaranteed that the salt deposits of Mars also contain inclusions that may yield clues about ancient surface conditions — and maybe even life.

Lowenstein is a professor of geology at the State University of New York at Binghamton. Michael Timofeeff and Brian Schubert (now at the University of Hawaii) were the major collaborators in this research, with help from Russell Vreeland (West Chester University) and Matthew Parker, Koji Lum, Krithivasan Sankaranarayanan and Yaicha Winters (all at Binghamton). **Funding is from the U.S. National Science Foundation, Earth Sciences Division.**