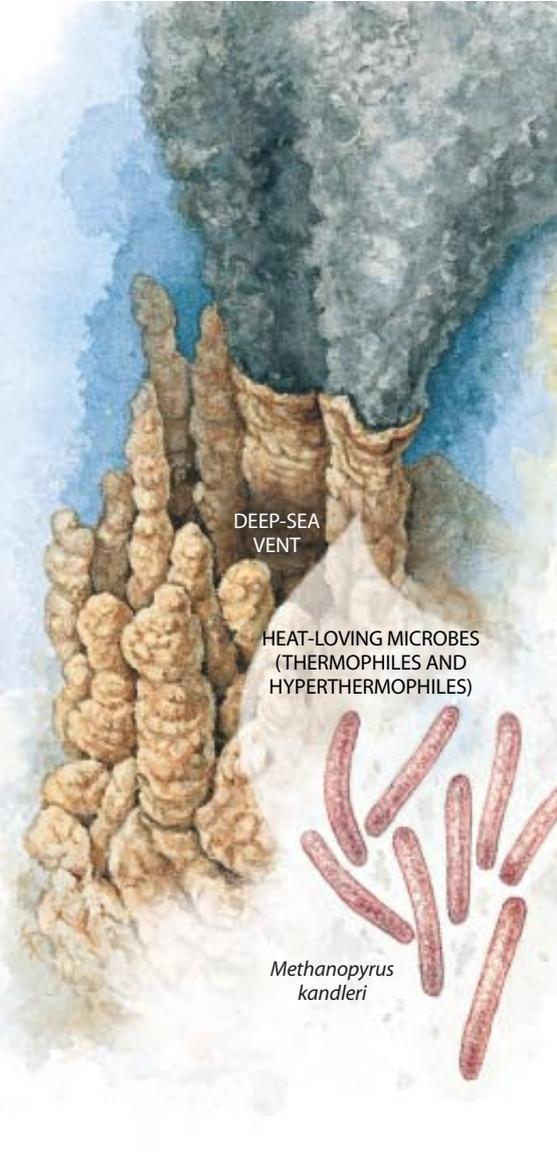


# Extremophiles

*These microbes thrive under conditions that would kill other creatures. The molecules that enable extremophiles to prosper are becoming useful to industry*

by Michael T. Madigan and Barry L. Marrs



Imagine diving into a refreshingly cool swimming pool. Now, think instead of plowing into water that is boiling or near freezing. Or consider jumping into vinegar, household ammonia or concentrated brine. The leap would be disastrous for a person. Yet many microorganisms make their home in such forbidding environments. These microbes are called extremophiles because they thrive under conditions that, from the human vantage, are clearly extreme. Amazingly, the organisms do not merely tolerate their lot; they do best in their punishing habitats and, in many cases, require one or more extremes in order to reproduce at all.

Some extremophiles have been known for more than 40 years. But the search for them has intensified recently, as scientists have recognized that places once assumed to be sterile abound with microbial life. The hunt has also been fueled in the past several years by industry's realization that the "survival kits" possessed by extremophiles can poten-

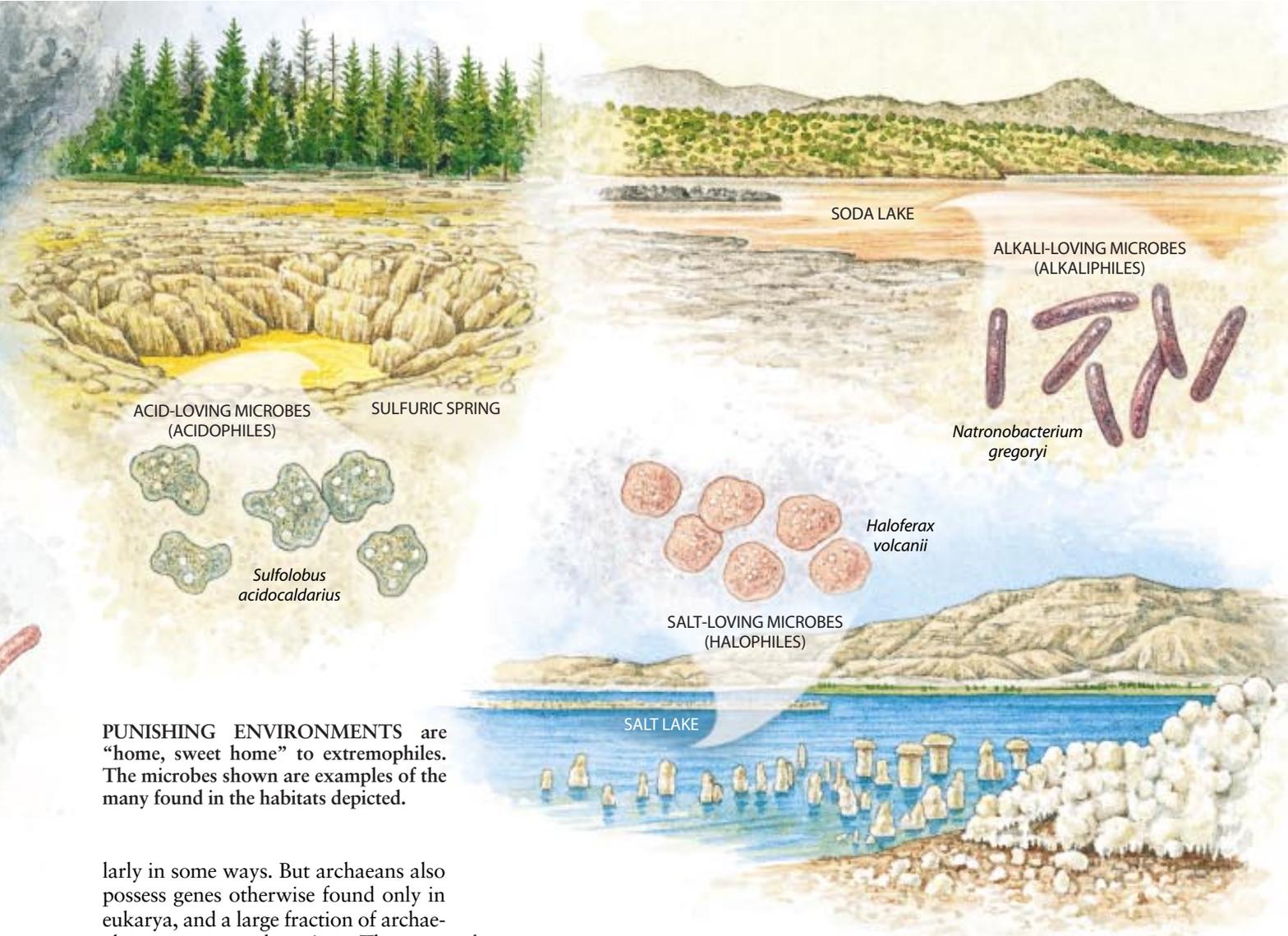
tially serve in an array of applications.

Of particular interest are the enzymes (biological catalysts) that help extremophiles to function in brutal circumstances. Like synthetic catalysts, enzymes, which are proteins, speed up chemical reactions without being altered themselves. Last year the biomedical field and other industries worldwide spent more than \$2.5 billion on enzymes for applications ranging from the production of sweeteners and "stonewashed" jeans to the genetic identification of criminals and the diagnosis of infectious and genetic diseases. Yet standard enzymes stop working when exposed to heat or other extremes, and so manufacturers that rely on them must often take special steps to protect the proteins during reactions or storage. By remaining active when other enzymes would fail, enzymes from extremophiles—dubbed "extremozymes"—can potentially eliminate the need for those added steps,

thereby increasing efficiency and reducing costs. They can also form the basis of entirely new enzyme-based processes.

Perhaps 20 research groups in the U.S., Japan, Germany and elsewhere are now actively searching for extremophiles and their enzymes. Although only a few extremozymes have made their way into use thus far, others are sure to follow. As is true of standard enzymes, transforming a newly isolated extremozyme into a viable product for industry can take several years.

Studies of extremophiles have also helped redraw the evolutionary tree of life. At one time, dogma held that living creatures could be grouped into two basic domains: bacteria, whose simple cells lack a nucleus, and eukarya, whose cells are more complex. The new work lends strong support to the once heretical proposal that yet a third group, the archaea, exists. Anatomically, archaeans lack a nucleus and closely resemble bacteria in other ways. And certain archaeal genes have similar counterparts in bacteria, a sign that the two groups function simi-



**PUNISHING ENVIRONMENTS** are “home, sweet home” to extremophiles. The microbes shown are examples of the many found in the habitats depicted.

larly in some ways. But archaeans also possess genes otherwise found only in eukarya, and a large fraction of archaeal genes appear to be unique. These unshared genes establish archaea’s separate identity. They may also provide new clues to the evolution of early life on the earth [see box on pages 86 and 87].

**Some Need It Hot**

**H**eat-loving microbes, or thermophiles, are among the best studied of the extremophiles. Thermophiles reproduce, or grow, readily in temperatures greater than 45 degrees Celsius (113 degrees Fahrenheit), and some of them, referred to as hyperthermophiles, favor temperatures above 80 degrees Celsius (176 degrees Fahrenheit). Some hyperthermophiles even thrive in environments hotter than 100 degrees Celsius (212 degrees Fahrenheit), the boiling point of water at sea level. In comparison, most garden-variety bacteria grow fastest in temperatures between 25 and 40 degrees Celsius (77 and 104 degrees Fahrenheit). Further, no multicellular animals or plants have been found to tolerate temperatures above about 50 degrees Celsius (122 degrees Fahrenheit), and no microbial eukarya yet discovered can tolerate

long-term exposure to temperatures higher than about 60 degrees Celsius (140 degrees Fahrenheit).

Thermophiles that are content at temperatures up to 60 degrees Celsius have been known for a long time, but true extremophiles—those able to flourish in greater heat—were first discovered only about 30 years ago. Thomas D. Brock, now retired from the University of Wisconsin–Madison, and his colleagues uncovered the earliest specimens during a long-term study of microbial life in hot springs and other waters of Yellowstone National Park in Wyoming.

The investigators found, to their astonishment, that even the hottest springs supported life. In the late 1960s they identified the first extremophile capable of growth at temperatures greater than 70 degrees Celsius. It was a bacterium, now called *Thermus aquaticus*, that would later make possible the widespread use of a revolutionary technology—the polymerase chain reaction (PCR). About this same time, the team found the first hyperthermophile in an extremely hot and acidic spring. This organism, the archae-

an *Sulfolobus acidocaldarius*, grows prolifically at temperatures as high as 85 degrees Celsius. They also showed that microbes can be present in boiling water.

Brock concluded from the collective studies that bacteria can function at higher temperatures than eukarya, and he predicted that microorganisms would likely be found wherever liquid water existed. Other work, including research that since the late 1970s has taken scientists to more hot springs and to environments around deep-sea hydrothermal vents, has lent strong support to these ideas. Hydrothermal vents, sometimes called smokers, are essentially natural undersea rock chimneys through which erupts superheated, mineral-rich fluid as hot as 350 degrees Celsius.

To date, more than 50 species of hyperthermophiles have been isolated, many by Karl O. Stetter and his colleagues at the University of Regensburg in Germany. The most heat-resistant of these microbes, *Pyrolobus fumarii*, grows in the walls of smokers. It repro-

ROBERTO OSTI

duces best in an environment of about 105 degrees C and can multiply in temperatures of up to 113 degrees C. Remarkably, it stops growing at temperatures below 90 degrees C (194 degrees F). It gets too cold! Another hyperthermophile that lives in deep-sea chimneys, the methane-producing archaean *Methanopyrus*, is now drawing much attention because it lies near the root in the tree of life; analysis of its genes and activities is expected to help clarify how the world's earliest cells survived.

counterparts in structure but appear to contain more of the ionic bonds and other internal forces that help to stabilize all enzymes.

Whatever the reason for their greater activity in extreme conditions, enzymes derived from thermophilic microbes have begun to make impressive inroads in industry. The most spectacular example is *Taq* polymerase, which derives from *T. aquaticus* and is employed widely in PCR. Invented in the mid-1980s by Kary B. Mullis, then at Cetus Corporation, PCR is today the basis for the forensic "DNA fingerprinting" that received so much attention during

merase). Its high tolerance for heat led to the development of totally automated PCR technology. More recently, some users of PCR have replaced the *Taq* polymerase with *Pfu* polymerase. This enzyme, isolated from the hyperthermophile *Pyrococcus furiosus* ("flaming fireball"), works best at 100 degrees C.

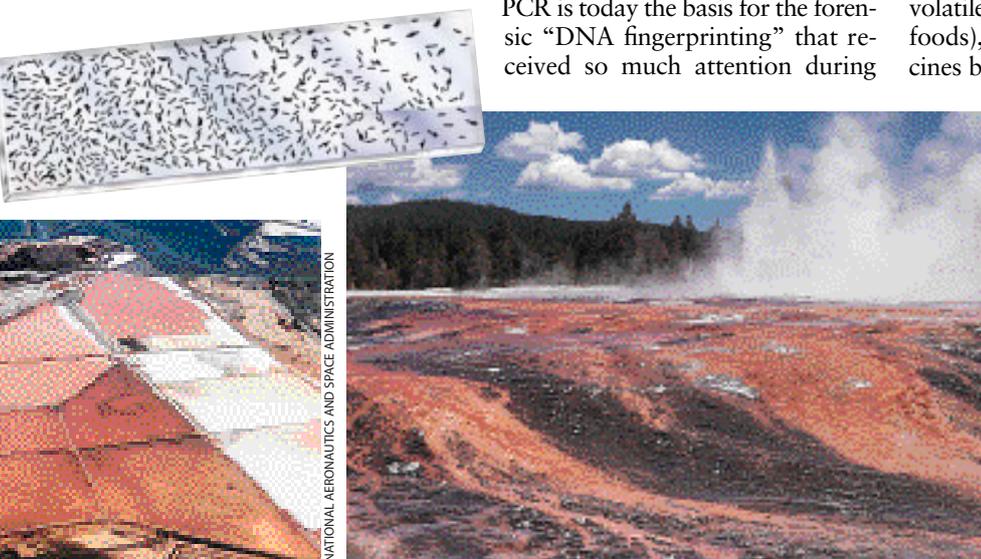
A different heat-loving extremozyme in commercial use has increased the efficiency with which compounds called cyclodextrins are produced from cornstarch. Cyclodextrins help to stabilize volatile substances (such as flavorings in foods), to improve the uptake of medicines by the body, and to reduce bitterness and mask unpleasant odors in foods and medicines.

### Others Like It Cold, Acidic, Alkaline

Cold environments are actually more common than hot ones. The oceans, which maintain an average temperature of one to three degrees C (34 to 38 degrees F), make up over half the earth's surface. And vast land areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer. Surprisingly, the most frigid places, like the hottest, support life, this time in the form of psychrophiles (cold lovers).

James T. Staley and his colleagues at the University of Washington have shown, for example, that microbial communities populate Antarctic sea ice—ocean water that remains frozen for much of the year. These communities include photosynthetic eukarya, notably algae and diatoms, as well as a variety of bacteria. One bacterium obtained by Staley's group, *Polaromonas vacuolata*, is a prime representative of a psychrophile: its optimal temperature for growth is four degrees C, and it finds temperatures above 12 degrees C too warm for reproduction. Cold-loving organisms have started to interest manufacturers who need enzymes that work at refrigerator temperatures—such as food processors (whose products often require cold temperatures to avoid spoilage), makers of fragrances (which evaporate at high temperatures) and producers of cold-wash laundry detergents.

Among the other extremophiles now under increasing scrutiny are those that



EXTREME ENVIRONMENTS can also be extremely colorful. Halophiles account for the redness in salt collection ponds near San Francisco Bay in California (left), and thermophiles brighten a hot spring in Yellowstone National Park in Wyoming (right). A glass slide dipped directly into one of Yellowstone's boiling springs soon revealed the presence of abundant microbial life (top).

What is the upper temperature limit for life? Do "super-hyperthermophiles" capable of growth at 200 or 300 degrees C exist? No one knows, although current understanding suggests the limit will be about 150 degrees C. Above this temperature, probably no life-forms could prevent dissolution of the chemical bonds that maintain the integrity of DNA and other essential molecules.

### Not Too Hot to Handle

Researchers interested in how the structure of a molecule influences its activity are trying to understand how molecules in heat-loving microbes and other extremophiles remain functional under conditions that destroy related molecules in organisms adapted to more temperate climes. That work is still under way, although it seems that the structural differences need not be dramatic. For instance, several heat-loving extremozymes resemble their heat-intolerant

the recent O. J. Simpson trials. It is also used extensively in modern biological research, in medical diagnosis (such as for HIV infection) and, increasingly, in screening for genetic susceptibility to various diseases, including specific forms of cancer.

In PCR, an enzyme known as a DNA polymerase copies repeatedly a snippet of DNA, producing an enormous supply. The process requires the reaction mixture to be alternately cycled between low and high temperatures. When Mullis first invented the technique, the polymerases came from microbes that were not thermophilic and so stopped working in the hot part of the procedure. Technicians had to replenish the enzymes manually after each cycle.

To solve the problem, in the late 1980s scientists at Cetus plucked *T. aquaticus* from a clearinghouse where Brock had deposited samples roughly 20 years earlier. The investigators then isolated the microbe's DNA polymerase (*Taq* poly-

prefer highly acidic or basic conditions (acidophiles and alkaliphiles). Most natural environments on the earth are essentially neutral, having pH values between five and nine. Acidophiles thrive in the rare habitats having a pH below five, and alkaliphiles favor habitats with a pH above nine.

Highly acidic environments can result naturally from geochemical activities (such as the production of sulfurous gases in hydrothermal vents and some hot springs) and from the metabolic activities of certain acidophiles themselves. Acidophiles are also found in the debris left over from coal mining. Interestingly, acid-loving extremophiles cannot tolerate great acidity inside their cells, where it would destroy such important molecules as DNA. They survive by keeping the acid out. But the defensive molecules that provide this protection, as well as others that come into contact with the environment, must be able to operate in extreme acidity. Indeed, extremozymes that are able to work at a pH below one—more acidic than even vinegar or stomach fluids—have been isolated from the cell wall and underlying cell membrane of some acidophiles.

Potential applications of acid-tolerant extremozymes range from catalysts for the synthesis of compounds in acidic solution to additives for animal feed, which are intended to work in the stomachs of animals. The use of enzymes in feed is already quite popular. The enzymes that are selected are ones that microbes normally secrete into the environment to break food into pieces suitable for ingestion. When added to feed, the enzymes improve the digestibility of inexpensive grains, thereby avoiding the need for more expensive food.

Alkaliphiles live in soils laden with carbonate and in so-called soda lakes, such as those found in Egypt, the Rift Valley of Africa and the western U.S. Above a pH of eight or so, certain molecules, notably those made of RNA, break down. Consequently, alkaliphiles, like acidophiles, maintain neutrality in their interior, and their extremozymes are located on or near the cell surface and in external secretions. Detergent makers in the U.S. and abroad are particu-

larly excited by alkaliphilic enzymes. In Japan, where industry has embraced extremozymes with enthusiasm, much of the research into alkaliphilic extremozymes has been spearheaded by Koki Horikoshi of the Japan Marine Science and Technology Center in Yokosuka.

To work effectively, detergents must be able to cope with stains from food and other sources of grease—jobs best accomplished by such enzymes as proteases (protein degraders) and lipases (grease degraders). Yet laundry detergents tend to be highly alkaline and thus destructive to standard proteases and lipases. Alkaliphilic versions of those en-

**EXTREMOPHILE PROSPECTORS** Karl O. Stetter (*left*) of the University of Regensburg in Germany and James T. Staley (*right*) of the University of Washington brave the elements to find extremophiles in a Yellowstone hot spring and in Antarctic sea ice, respectively. The psychrophiles in one ice core are evident as a dark band in the inset.



MICHAEL MILSTEIN



COURTESY OF JAMES T. STALEY, University of Washington (photograph and inset)

zymes can solve the problem, and several that can operate efficiently in heat or cold are now in use or being developed. Alkaliphilic extremozymes are also poised to replace standard enzymes wielded to produce the stonewashed look in denim fabric. As if they were rocks pounding on denim, certain enzymes soften and fade fabric by degrading cellulose and releasing dyes.

### A Briny Existence

The list of extremophiles does not end there. Another remarkable group—the halophiles—makes its home in intensely saline environments, especially natural salt lakes and solar salt evaporation ponds. The latter are human-made pools where seawater collects and evaporates, leaving behind dense concentrations of salt that can be harvested for such purposes as melting ice. Some saline environments are also extremely alkaline because weathering of sodium

carbonate and certain other salts can release ions that produce alkalinity. Not surprisingly, microbes in those environments are adapted to both high alkalinity and high salinity.

Halophiles are able to live in salty conditions through a fascinating adaptation. Because water tends to flow from areas of high solute concentration to areas of lower concentration, a cell suspended in a very salty solution will lose water and become dehydrated unless its cytoplasm contains a higher concentration of salt (or some other solute) than its environment. Halophiles contend with this problem by producing large

amounts of an internal solute or by retaining a solute extracted from outside. For instance, an archaean known as *Halobacterium salinarum* concentrates potassium chloride in its interior. As might be expected, the enzymes in its cytoplasm will function only if a high concentration of potassium chloride is present. But proteins in *H. salinarum* cell structures that are in contact with the environment require a high concentration of sodium chloride.

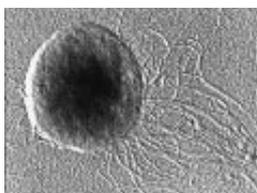
The potential applications for salt-tolerant enzymes do not leap to mind as readily as those for certain other extremozymes. Nevertheless, at least one intriguing application is under consideration. Investigators are exploring incorporating halophilic extremozymes into procedures used to increase the amount of crude extracted from oil wells.

To create passages through which trapped oil can flow into an active well, workers pump a mixture of viscous guar gum and sand down the well hole.

## Archaea Makes Three

In the summer of 1996 a large collaboration of scientists deciphered the full sequence of units, or nucleotides, in every gene of *Methanococcus jannaschii*—a methane-producing extremophile that thrives at temperatures near 85 degrees Celsius. The results strikingly confirmed the once ridiculed proposal that life consists of three major evolutionary lineages, not the two that have been routinely described in textbooks.

The recognized lineages were the bacteria (with their simple cells that lack a true nucleus) and the eukarya (plants, humans and other animals having cells that contain a nucleus). By comparing molecules known as ribosomal RNA in many different organisms, Carl R. Woese and his collaborators at the University of Illinois had concluded in 1977 that a group of microbes once classified as bacteria and called archaeobacteria belonged, in fact, to a separate lineage: the archaea. *M. jan-*



*ALVIN* (top), a scientific submarine, reaches out an arm (middle) to snag material around a deep-sea vent. *Methanococcus jannaschii*, a spherical cell with many flagella, was among its finds in 1982. The diagram on the opposite page depicts the three major evolutionary lineages of life.

*naschii* is the first of the archaeans to have had its genes sequenced in full.

The sequencing made it possible to compare *M. jannaschii*'s total complement of genes with the many genes that have so far been sequenced in other organisms. Forty-four percent of *M. jannaschii*'s genes resemble those in bacteria or eukarya, or both. And consistent with Woese's scheme, fully 56 percent are completely different from any genes yet described.

That *M. jannaschii* has characteristics of bacteria and eukarya but also has marked differences suggests that archaea and the other two lineages have a common distant ancestor. Partly because many archaea and some bacteria are adapted to the conditions widely believed to have existed on the early earth—especially high heat and little or no oxygen—a majority of investigators suspect that those two groups appeared first, diverging from a common ancestor relatively soon after life began. Later, the eukarya split off from the ar-

Then they set off an explosive to fracture surrounding rock and to force the mixture into the newly formed crevices. The guar facilitates the sand's dispersion into the cracks, and the sand props open the crevices. Before the oil can pass through the crevices, however, the gum must be eliminated. If an enzyme that degrades guar gum is added just before the mixture is injected into the well-head, the guar retains its viscosity long enough to carry the sand into the crevices but is then broken down.

At least, that is what happens in the ideal case. But oil wells are hot and often salty places, and so ordinary enzymes often stop working prematurely. An extremozyme that functioned optimally in high heat and salt would presumably remain inactive at the relatively cool, relatively salt-free surface of the well. It would then become active gradually as it traveled down the hole, where temperature rises steadily with increasing depth. The delayed activity would provide more time for the sand mixture to spread through the oil-bearing strata, and the tolerance of heat and salt would enable the enzyme to function longer for breaking down the guar. Preliminary laboratory tests of this prospect, by Robert M. Kelly of North Carolina State University, have been encouraging.

If the only sources of extremozymes were large-scale cultures of extremophiles, widespread industrial applications of these proteins would be imprac-

tical. Scientists rarely find large quantities of a single species of microbe in nature. A desired organism must be purified, usually by isolating single cells, and then grown in laboratory culture. For organisms with extreme lifestyles, isolation and large-scale production can prove both difficult and expensive.

### Harvesting Extremozymes

Fortunately, extremozymes can be produced through recombinant DNA technology without massive culturing of the source extremophiles. Genes, which consist of DNA, specify the composition of the enzymes and other proteins made by cells; these proteins carry out most cellular activities. As long as microbial prospectors can obtain sample genes from extremophiles in nature or from small laboratory cultures, they can generally clone those genes and use them to make the corresponding proteins. That is, by using the recombinant DNA technologies, they can insert the genes into ordinary, or "domesticated," microbes, which will often use the genes to produce unlimited, pure supplies of the enzymes.

Two approaches have been exploited to identify potentially valuable extremozymes. The more traditional route requires scientists to grow at least small cultures of an extremophile obtained from an interesting environment. If the scientists are looking for, say, protein-

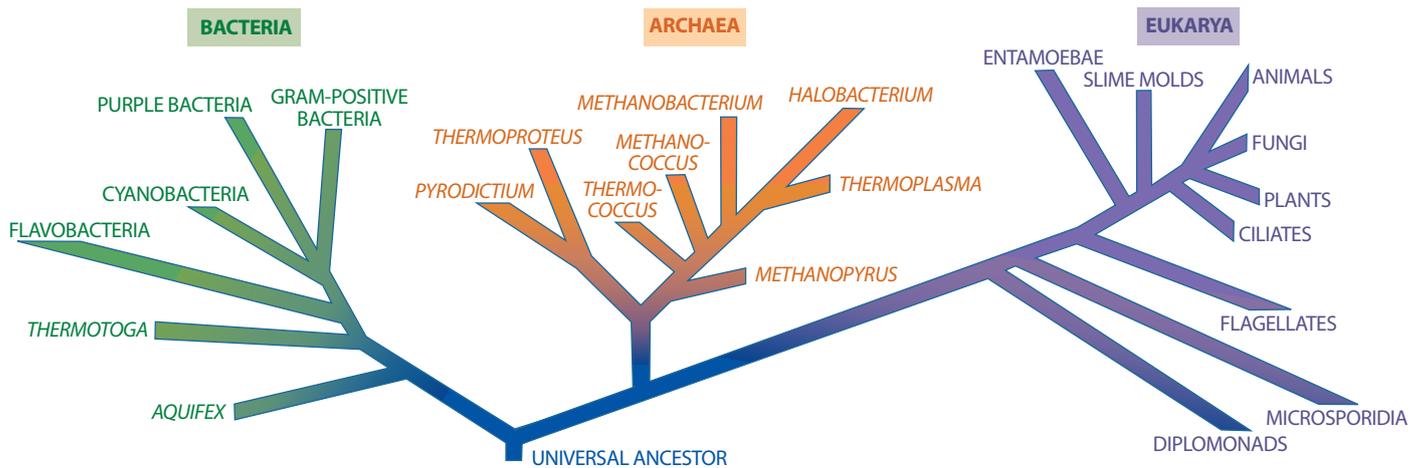
degrading enzymes, they test to see whether extracts of the cultured cells break down selected proteins. If such activity is detected, the researchers turn to standard biochemical methods to isolate the enzymes responsible for the activity and to isolate the genes encoding the enzymes. They then must hope that the genes can be induced to give rise to their corresponding proteins in a domesticated host.

In the other approach, investigators bypass the need to grow any cultures of extremophiles. They isolate the DNA from all living things in a sample of water, soil or other material from an extreme environment. Then, using recombinant DNA technology once again, they deliver random stretches of DNA into a domesticated host—ideally one insert per host cell—without knowing the identities of the genes in those fragments. Finally, they screen the colonies that grow out, looking for evidence of activity by novel enzymes. If they find such evidence, they know that an inserted gene is responsible and that it will work in the domesticated host. This method thus avoids many bottlenecks in the traditional process. It turns up only enzymes that can be manufactured readily in tried-and-true hosts. And investigators can mine the genes for the enzymes from mixed populations of microbes without needing to culture extremophiles that might have trouble growing outside their native milieu.

chaea. Further support for this scenario can be seen in the evolutionary tree itself: hyperthermophilic, oxygen-sensitive organisms, such as *Methanopyrus* (archaea) and *Aquifex* (bacteria), branch off close to the root.

Scientists are eager to learn the nature of the genes that are unique to archaea. Genes are the blueprints from which en-

zymes and other proteins important to cell structure and function are made. Many of the unique archaeal genes undoubtedly encode novel proteins that may provide insights into how ancient cells survived. And certain of these unusual proteins can probably be enlisted for developing innovative medicines or to perform new tricks in industry. —M.T.M. and B.L.M.



Although the microbes of the world are incredibly diverse, scientists rarely find in them the perfect enzyme for a given task. Therefore, microbiologists at the cutting edge of industrial enzyme technology have begun to modify extremozymes, tailoring them to meet specific demands. For instance, after finding an extremozyme that degrades proteins fairly efficiently at high temperatures, investigators might alter the enzyme so that it functions across a broader range of acidity and salinity.

Biologists today generally achieve such modifications in either of two ways. Practitioners of the “rational design” approach first discern the structural basis of the property of interest. Next, they alter an enzyme’s gene to guarantee that the resulting catalytic protein will

gain that property. Devotees of the other approach, known as directed evolution, make more or less random variations in the gene encoding a selected enzyme and, from those genes, generate thousands of different versions of the enzyme. Then they screen the collection to see whether any of the variations has gained the hoped-for feature. This last strategy is also said to be Edisonian, because when Thomas Edison sought a material to serve as a filament for the lightbulb, he tried everything available, from bamboo splints to silk threads, and chose the one that worked best.

So far most extremozymes in commercial use are little altered from their original state. But rational design and Edisonian approaches promise to enhance extremozymes. They may also

help to convert enzymes from ordinary microbes into artificial extremozymes.

Discovery of extremophiles opens new opportunities for the development of enzymes having extraordinary catalytic capabilities. Yet for any new enzyme to gain commercial acceptance, its makers will have to keep down the costs of production, for example, by ensuring that the domesticated microbes used as the extremozyme-producing factory will reliably generate large quantities of the protein. The difficulties of perfecting manufacturing techniques, and the reluctance of industries to change systems that already work reasonably well, could slow the entry of new extremozymes into commerce. It seems inevitable, however, that their many advantages will eventually prove irresistible. SA

### The Authors

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### Further Reading

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