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Invisible invasion: potential contamination of Yellowstone hot springs by human activity

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Discovery of life at high temperature (Brock 1978) led to advances in biotechnology made possible by *Thermus aquaticus* (Taq), which was isolated in Yellowstone National Park (YNP; Brock and Freeze 1969). Since Taq polymerase chain reaction (PCR) became a patented process for in vitro amplification of DNA, PCR has generated significant scientific advances in biotechnology and substantial revenues (Brock 1994) for the patent holder. The commercial success of *T. aquaticus* led to an increase in collection activity and research in the Yellowstone geothermal ecosystem. Scientists seeking other utilitarian, heat-stable enzymes in the protected, mostly unexplored hot spring habitats generated dozens of research permits in the 1990s, allowing microbial exploration in YNP.

Only a small number of organisms from the hot springs in YNP have been isolated (Lindstrom 1997). As a result, there is no existing microbial inventory to evaluate whether investigators who visit a series of springs inadvertently introduce exotic microbes via sampling equipment or mud carried from one spring to another. Hence, without a historic baseline inventory, we cannot determine whether a newly isolated species is an exotic microbe. Such an issue arose in the acid sulfate habitat of *Sulfolobus acidocaldarius* (Brock et al. 1972). This organism was the first species known to grow in this habitat. Then several new species were reported, *Metallosphaera sedula*, *Sulfolobus metallicus*, *S. solfataricus*, *Acidianus brierleyi*, *A. infernus*, *Acidobacillus fibrocaldarius*, *Lobobacillus acidocaldarius* (Zillig et al. 1980, Segerer et al. 1986, Huber et al. 1989, Huber and Stetter 1991, Weiss Bizzoco 1999). It was not known whether they were newcomers to the acid sulfate habitat or were always present, but due to advances in isolation technology, only recently identified and reported.

One approach to this question was pursued by Susan Barns and Norman Pace (Barns et al. 1994), who developed a complete inventory of a mineral rich hot spring known as Jim’s Black Pool using PCR-based DNA fingerprinting of the small subunit rRNA. This classic study produced a grouping of the archaeal domain into 2 kingdoms, *Crenarchaeota* and *Korarchaeota*. This would not have been possible using traditional enrichment culture techniques, since most of the microbes cannot be cultured.

Key words: hot spring, sulfur, acid, contamination, microorganism, microbe, Yellowstone National Park.
We report herein a baseline inventory of organisms in the acid sulfate habitat based on both historical and recent study. Then we present the opinions of prominent Yellowstone researchers on the possibility of introduction of exotic species of microbes from human (including human researcher) activity.

**Study Sites**

We examined 2 types of acid hot springs in YNP, flowing springs and mixing pools. These thermal habitats are further distinguished by their chemical and physical characteristics. Among flowing springs, the south end of Roaring Mountain is the hottest acid spring (pH 2.1) in YNP, with a site temperature at the origin of 93°C. This compares with the western end of the Amphitheater basin, springs 2 and 3 (pH 2.3), with an origin temperature of 81°C. However, these springs have heavy deposits of sulfur that begin a few centimeters away from the origin. Phenotypically interesting organisms are associated with sulfur deposited along outflow channels (Amphitheater Springs and Norris Junction). No such deposits occur at Roaring Mountain.

In 1971 Norris Junction and Amphitheater Springs were similar in that both sites had flowing springs with sulfur deposits in the outflow channels. Over the years since 1971 at Norris Junction, both temperature and water flow have decreased. The main spring at Norris Junction still has heavy deposits of elemental sulfur. However, the spring is now a mixing pool with a much lower temperature (70°C) than that measured in 1971 (80°C).

We examined several mixing pools in the Norris Geyser Basin: Congress, Vermillion, Locomotive, and Growler (defunct in 1975). We also visited several sites in the Sylvan Springs complex (Gibbon Geyser Basin). Another spring we studied, Frying Pan Spring, lies north of Norris Junction along the road to Mammoth. In the Mud Volcano area we studied Sulfur Caldron and Moose Pool. We examined a series of springs in Hayden Valley located in Crater Hills. These springs are mixing pools; one with a visibly high content of elemental sulfur is known as Great Sulfur Spring.

**Methods**

Organisms in flowing springs were examined by immersing cleaned, one end–frosted glass slides in a spring as described previously (Weiss 1973). The slides (RITE-ON, Clay Adams, NY) were cleaned with ethanol (ETOH), rinsed with water, air-dried, and placed in outflow channels of springs. Organisms were allowed to attach and grow for selected periods of time; the slides were then removed, placed in a screw-cap Coplin jar or a small plastic slide carrying case (Cell Path, Hemel Hempstead, UK) filled with spring water, and returned to the laboratory.

For DNA staining, a stock solution of 4′,6-diamidino-2-phenylindole, HCl (DAPI) stain (10 µg mL⁻¹) was prepared in 0.1 M 2-(N-morpholino)ethanesulfonic acid, MES buffer, pH 6.8, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid, (EGTA), 0.5 mM MgCl₂·6H₂O. Samples were mixed with an equal volume of stain on a slide to deliver a final concentration of 5 µg mL⁻¹ DAPI. In some experiments cells were concentrated before staining by centrifugation at 10,000 g for 15 minutes at 23°C.

Samples were observed and photographed with a Leitz Dialux 20 phase contrast epifluorescence (A filter block) microscope equipped with a Wild MPS 55 automatic camera. Images were recorded using Kodak T-MAX 400 film and T-MAX developer.

Electron microscope samples from flowing acid hot springs were taken in the channel center. Samples of sediment were collected with an inverted sterile 10-mL pipette with a tip broken to accommodate a pipet pump. Samples from pools were collected in a 1-L Nalgene beaker attached to a telescoping 8-foot paint roller pole. The samples were transferred into 140-mL plastic bottles.

For electron microscopy, we cooled environmental samples in the field to ambient temperature, ~25°C. Glutaraldehyde (4%) in 4 mM KPO4 buffer, pH 7.0, with 0.1 mM CaCl₂·2H₂O and 0.1 mM MgCl₂·7H₂O was added to an equal sample volume to obtain a final concentration of 2% V/V glutaraldehyde. After fixation for 2 hours at ~25°C, the cells were centrifuged at 8000 g for 15 minutes, washed in buffer, and fixed in 1% OsO₄ in 50 mM cacodylate-HCl buffer, pH 7, for 1 hour at 25°C, cooled on ice, and fixed for 4 hours longer. Fixed cells were dehydrated for 10 minutes each in 25, 50, 75, and 100% acetone and embedded in Spurr’s epoxy resin. Ultrathin
sections cut on a Reichert OMU 2 ultramicrotome with a diamond knife were stained with uranyl acetate and lead citrate. For negative staining, we placed a small drop of cells on a Formvar plastic-coated grid. Organisms were allowed to attach for 5 minutes. The cells were stained with 2% W/V aqueous uranyl acetate and dried with filter paper. Samples were examined with a Philips 400 transmission electron microscope.

Scanning electron microscope (SEM) samples were prepared on 13-mm-diameter cover glasses. Cells and sulfur attached to cover glasses coated with a thin layer of poly-l-lysine to increase binding of sulfur and cells. Cells were fixed for 1 hour or longer on the cover glass with 1% OsO₄ in 50 mM Na cacodylate, pH 7.2. After dehydration for 5 minutes each in 25, 50, 75, and 100% ETOH, samples were dried by the critical-point method using a semiautomatic Tousimis sandri 790 drier and then sputter-coated with Au-Pd on a Hummer VI. Cells were photographed in a Hitachi model 2700 SEM equipped with a Princeton Gamma Tech IMIX X-ray microanalyzer. Sulfur was identified in the SEM on Au-Pd–coated samples attached to aluminum stubs.

Sampling of YNP Thermobiologists

An abstract was prepared for a conference talk to be presented at the 5th Biennial Scientific Conference on the Greater Yellowstone Ecosystem entitled, “Exotic organisms in greater Yellowstone: native biodiversity under siege.” The abstract on potential contamination of YNP thermal springs by human activity was sent (by e-mail) to a selected list of researchers known to be experts in their field and active in YNP microbial research. The e-mail requested comments and opinion on the question for inclusion in the present paper.

Information on sampling methods from one investigator, Karl O. Stetter, was adapted from a video tape (Films for the Humanities and Science 1993) with the approval of the investigator.

RESULTS

Microbial Inventory

HISTORICAL STUDIES.—In the 1970s, Sulfolobus was the predominant organism at temperatures above 70°C at Norris Junction and Amphitheater Springs 1 and 2. It could be isolated easily from most sites in both areas either on sulfur as an autotroph or on yeast extract as a heterotroph. It usually took about a week to isolate new strains, sometimes slightly longer, from most sites. At our Roaring Mountain sites, it was the only organism we saw. Here, we define Sulfolobus as Sulfolobus-like, meaning that the organism was a lobed sphere. It is important to note that at this time we had not yet described it as a genus. We were just beginning to write up the work, which was initially submitted in July 1971 (Brock et al. 1972).

On all of our slide immersion studies, whether at Roaring Mountain, Norris Junction, or Amphitheater Springs, we found roughly equal numbers of attached rods and Sulfolobus spheres around 70–75°C. Above 75°C the main organism was Sulfolobus; few if any rods were seen. Below 70°C rod-shaped cells began to appear in very high numbers at sites along thermal gradients at Norris and Amphitheater, but not at Roaring Mountain, where we saw principally Sulfolobus. Investigators Carl Fliermans and David Smith found metabolic activity in the soils of Roaring Mountain due to both Sulfolobus and rods (perhaps Thiobacillus). The highest temperature at which they observed rods was about 60°C.

In 1997–98 we surprisingly found mainly rod-shaped cells in the samples from Amphitheater Spring 2. We found many more types than the few we had observed earlier in our 1970–1973 thin-section and negative-stain electron microscopy. In the early samples we saw only 2 different rods at Amphitheater. The first had a Sulfolobus-like subunit cell wall, and the second, a long filament, had a layered, bacterial-like cell wall. With so much diversity apparent, it became difficult for us to characterize all the different types of organisms (rod shaped) that were present. When the samples at Amphitheater Springs were prepared and examined by electron microscopy, some Sulfolobus-like spheres appeared. But the striking difference was the ratio of rods to spheres at temperatures up to 75°C. Whereas previously there had been about a 1:1 ratio, now the ratio was on the order of 10:1 rods to spheres. We have not yet found a suitable explanation for these dynamics, but the sites have undergone many changes over the years.
We also did slide immersion studies in the same spring in 1997–98 and found no colonies of Sulfolobus-like cells at temperatures of 70–80°C but significant attachment of rod-shaped cells. The slides immersed in springs had many elongated, thin filaments (0.4 × 20 µm), rods of medium thickness and length (~0.6 × 6 µm), and smaller rods (~0.5 × 2 µm). Here again, this differed significantly from our earlier observation that Sulfolobus-like and rod-shaped cells were present in about equal numbers, but above 75°C Sulfolobus-like cells usually outgrew rods on immersed slides and developed into colonies. The slide immersion studies usually correlate well with phase contrast microscopic observations of samples taken from the same habitat.

Recent studies.—Two other features of these early observations are worth noting. (1) Sulfolobus, when it did occur within the deposits of sulfur at Amphitheater Springs 1 and 2, was present in large numbers in microhabitats. (2) It was not seen at every sample site. This compares with rods that were the dominant type in our recent observations. Amphitheater Spring 3 (summer 1997), with unusual dark deposits at the origin and elemental sulfur, had almost totally Sulfolobus-like cells and only a few rods. The deposits (perhaps iron) were bound tightly to the siliceous bottom of the spring. Sulfur deposits begin at the origin, and extensive deposits occur further down the thermal gradient.

This difference (rods vs. spheres) is particularly evident when cells are examined by DAPI staining (Fig. 1). Samples taken at 78°C at Amphitheater Spring 2 compared with those from nearby Spring 3, 75–78°C (Fig. 2), emphasize the difference in colonization of sulfurrich habitats by rods and Sulfolobus-like cells. The sulfur crystal structure (c) is particularly evident as are the differences in morphology of the microbes attached to sulfur. The individual rod and spherical shapes can readily be seen by DAPI staining and phase contrast microscopy.

More detail can be observed when cells attached to sulfur are processed for negative staining (Fig. 3). Here, the 2 different cell types and their relative sizes are evident. The Sulfolobus-like cell is a lobed sphere ~1 µm in diameter with several thin filaments, termed pili, extending from the cell surface. The thin filaments possibly play a role in attachment of cells to sulfur. The wall structure is formed from subunits arranged in a 2-dimensional array. The rod-shaped cell has a somewhat different cell wall, with small, circular units arranged in rows that form a regular 2-dimensional array. The cell is about 0.45 × 2.6 µm, uniform in width, and has rounded ends. It has no thin filaments or other obvious surface structures. We made an interesting observation on other cells: flagella bearing wings of unknown function. Commonly, 2 of these are arranged opposite each other (180° apart) along the flagellum. They extend from the surface of the flagellum as featherlike rows that run as a helix along the full length of the flagellum (Weiss Bizzoco et al. 2000).

Samples from Amphitheater Spring 2 taken at 70°C, 74°C, and 78°C sites were examined by SEM. The high-temperature (78°C) sample is shown in Figure 4. Here, rod-shaped cells (R) and spherical, Sulfolobus-like cells (S) appear in about equal numbers. Both cell types are attached to the surface of crystals. Because there are many different minerals present in hot springs, some of which are crystalline, we examined individual crystals with attached cells. X-ray microanalysis reveals that sulfur (S) is a major component of the crystal to which microbes are attached (Fig. 5). At temperatures of 70°C and 74°C, rod-shaped cells appear by the thousands, while Sulfolobus-like cells are seen less frequently in samples examined in the SEM.

Frying Pan Spring is a large pool, pH 2.4, with a temperature of 75°C (vs. 77°C in 1971). In 1971 at Frying Pan Spring, a mixing pool, we saw only rod-shaped microorganisms. One was a long, slender filament just visible by phase contrast microscopy, and the other was a short, sausage-shaped rod. In 1997 when we examined control cells from this spring without the addition of DAPI using fluorescence microscopy, we found no autofluorescence. DAPI staining identifies DNA in several morphological kinds of rod-shaped cells (Fig. 6). The observed DAPI fluorescence coincides with the DNA of cells visualized in phase contrast images of cells. Thin filaments and sausage-shaped cells exhibit DAPI fluorescence with different intensities (Fig. 6). Some cells showed small, intensely fluorescent sites, suggesting they are undergoing cell division.
In contrast, other cells had low levels of fluorescence, suggesting the cells are in the stationary or death phase of the growth cycle. We also fixed cells, prepared thin sections, and examined the resulting cells by electron microscopy. We found thin, microbial filaments and sausage-shaped cells resembling those seen in the light microscope (Fig. 7, cf. Fig. 6). Examination of these by negative staining revealed several different types of flagellated cells (data not shown). Some flagella on these cells lack any obvious substructure, while others are unique in comprising units with a linear substructure. In this sense they are reminiscent of the winged flagella at Amphitheater Spring 2 that also have a linear substructure.

In mixing pools we found 2 distinct populations of cells. In 2 sites there was nearly a pure culture of *Sulfolobus*-like cells. This occurred at Moose Pool and Great Sulfur Spring. Sulfur Caldron, Locomotive, Growler, and several of the springs in the Sylvan

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**Fig. 1.** DAPI-stained *Sulfolobus*-like control sample. Sulfur crystal (c) with attached spherical cells was collected from a flowing spring. A, Phase contrast; B, DAPI stain. DNA is seen as uniform DAPI fluorescence. Amphitheater Spring 3: 75°C, pH 2.3. X1300.

**Fig. 2.** DAPI-stained, rod-shaped cells associated with sulfur crystals (c) in a flowing spring. Cells attach to crystal (c) and extend from the surface. A, Phase contrast; B, DAPI stain. DNA appears as uniform DAPI fluorescence. Amphitheater Spring 2: 74°C, pH 2.3. X1300.
Springs area had a mixture of rods and Sulfolobus-like cells. In contrast, only rod-shaped cells were observed at Frying Pan Spring, although it is known to contain Thermoplasma (Segerer et al. 1988), a spherical organism. From 1971 to 1999 there was a significant change in 2 springs, Evening Primrose and Growler. In 1995 Evening Primrose (Sylvan Springs, see Fig. 6.7b, Brock 1978) caved in. When we visited it in 1997, the temperature had dropped from 89°C (1971) to 33°C. Whereas previously it had been a Sulfolobus habitat, it now contained mainly algae and no Sulfolobus-like cells. The second spring, Growler, had collapsed earlier (1975), and above (northeast) it a new spring, Sulfur Spring, appeared. From 1997 to 1998 Sulfur Spring changed in temperature from 88°C to 74°C. Despite such changes, the microbial populations of most mixing pools remained relatively stable over the period from 1971 to 1999. Our most recent microbial survey of these springs is presented in Table 1.

Human-vectored Contamination: Perspectives of Yellowstone Microbiologists

**Thomas D. Brock**
E.B. Fred Professor Emeritus, Department of Bacteriology, University of Wisconsin, Madison. Area of study: Thermophiles

Although human cross-contamination could occur, it seems to me that this process would occur much more often via wild animals in the area, or via airborne contamination. Sorting out human from non-human contamination experimentally would be difficult.

**Craig J. Oberg,**
Department of Microbiology, Weber State University, Ogden, UT. Area of study: Metabolism and genetics of thermophiles

Lower temperature acidic and photosynthetic environments are continually inoculated by environmental biota and are thereby probably immune to xenic species contamination. Hyperthermophilic habitats, although refractory to most species, are
not often visited by natural vectors and may be inoculated only by contaminated human probes.

Richard W. Castenholz

Department of Biology, University of Oregon, Eugene. Area of study: Yellowstone photosynthetic thermophiles

With present practices I do not believe there is a real danger of introducing exotic microorganisms into Yellowstone hot springs. First of all, most thermophilic microorganisms from far distant hot spring clusters (which are by their nature disjunct) would be rarely transported in sufficient inoculum size to become established in a hot spring community (usually of different chemistry). It is a “principle” of ecology that established native communities rarely become displaced by exotics unless the recipient community is disturbed. This may also apply to hot springs communities. Sufficient inoculum may come frequently enough from hot springs in the same cluster (e.g., the springs of Yellowstone), by insects for example, but this would be expected and constitute natural dispersal. Although thermobiologists are probably the most likely vectors of viable cells from one geothermal area to another (e.g., Oregon to Yellowstone), the amount of inoculum would be essentially inconsequential, i.e., some old mud on boots, unwashed collecting equipment, etc., unless of course there is a purposeful mass inoculation. Nevertheless, I have always made it a practice to use new materials or sterilized old. Scrubbing boots and/or steam sterilizing them or simply using a new pair is a good idea if collectors have been in a distant hot spring area. I am sure that transferring boots from one lake or temperate stream to another constitutes a much greater risk (well documented) of introducing exotic species than in the refractory habitat of hot springs. The natural photosynthetic populations of alkaline and acid hot springs and their patterns in Yellowstone have not changed in any apparent way during the past 33 years of my studies, but of course this does not include the more recent studies using molecular methods. As for the shift in dominant species in various springs, there are well-documented changes in the chemistry, temperature, and flow in many springs in Yellowstone, especially in unstable areas such as Mammoth and Norris. I have observed many pH and temperature changes in springs over these years, and these changes correlated with some changes in microorganisms. As for the “possible” shift from *Sulfolobus* to *Acidianus* in some acid springs, the latter genus was unknown earlier and would not have been recognized at that time in the 1970s when *Sulfolobus* was discovered. Even if such a shift has taken place, in order to understand it, studies of correlative changes in temperature, pH, and chemistry should have been made in order to find possible causes. The introduction of *Acidianus* by collectors or human vectors of any type seems (by orders of magnitude) the least likely scenario.

As far as other impacts of hot spring collectors are concerned, taking an occasional thimbleful or even 100 mL of water and mat/sediment is not going to affect in any way the integrity of the hot spring community of microorganisms. However, trampling of soil and plants surrounding certain heavily used hot springs (e.g., Octopus Spring) should be discouraged as much as possible. There are many springs similar to Octopus that could be used by collectors.

Norman R. Pace

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder. Area of study: Biodiversity—PCR-based DNA fingerprinting

I’m in full concurrence with Brock and Castenholz about this. I’m not too concerned about contamination (of Octopus Spring or Obsidian Pool) because the mats are so rich in biomass. We always try to use aseptic technique and are less concerned about contaminating the site than the sample.

Carl R. Woese

Department of Microbiology, University of Illinois, Urbana. Area of study: Gene isolation techniques

Although the direct gene isolation method now fails us by not identifying the actual phenotype of the organism from which the gene has come, the approach more than compensates for this by (1) telling us that phenotypically characterized organisms are related to unisolated ones; (2) allowing us to
design probes and primers to aid in efforts to isolate the organism in question by enrichment culturing; (3) best of all, having the potential of a complete accounting of microbial species occurring in a niche, complementing enrichment culturing. Together, the 2 approaches give microbiologists the power to define, understand, and reveal the full richness of the microbial world.

Francisco F. Roberto  
Department of Biotechnology, Idaho National Engineering and Environmental Laboratory, U.S. Department of Energy, Idaho Falls, ID.  
Area of study: PCR-based Yellowstone microbial extremophile diversity

On *Acidianus brierleyi*, I don’t believe it’s an alien species, but actually it has probably been there all the time. It’s likely that it was in Brock’s original enrichments, as it’s virtually indistinguishable from *Sulfolobus acidocaldarius* under a microscope. I’m not surprised by Brock being cool to the idea. I saw the call for papers and also think that rather than introducing new microbial species, the biggest effect of man is altering the environment, leading to the succession and dominance of “non-native” species, which have probably always been around in small numbers, but not had the right environments to proliferate.

Karl O. Stetter  
Department of Microbiology, University of Regensburg, Regensburg, Germany. Area of study: Hyperthermophilic microbes

I define hyperthermophilic microbes as those growing fastest above 80°C. I isolate hyperthermophiles from many terrestrial, subterranean, and abyssal and shallow submarine areas all over the world. From Yellowstone (I have had a collecting permit for about 15 years), my co-workers and I have cultivated and described (1) *Thermoplasma volcanium*, the first *Thermoplasma* (wall-less microbe) in YNP; (2) *Thermosphaera aggregans*, from Obsidian Pool; (3) *Thermocrinis ruber*, Brock’s “pink filaments,” from Octopus Spring. For sampling and storage of samples, I always use glass bottles to avoid diffusion of oxygen into samples. This would occur with plastic bottles.

Like other Yellowstone investigators, Karl Stetter samples hot springs in a temperature range from 70°C to boiling water. In addition to Yellowstone, Solfatara Crater near Pozzuoli, Italy, is one of his choice hot habitats; it is laden with sulfur. Sampling here at extreme temperatures, he is most concerned with keeping the organisms happy. To sample an inaccessible spring, he uses a long, 2-piece pole with a small 250-mL stainless cup attached to one end. He fills up the cup by sampling in the...
middle of a spring and transfers the sample to a
glass sample bottle that he fills to the top without
introducing bubbles during pouring. In springs that
are easy to sample, he uses a 50-mL plastic syringe
to withdraw samples. Here again, he fills up a glass
bottle to the top to exclude oxygen. Then he may
add sodium dithionite to the sample. He notes,
“There is one important thing, there is practically
no oxygen here. With a powerful oxygen killer,
sodium dithionite, the organisms will be very
happy” (Films for the Humanities and Science
1993).

Robert F. Ramaley
Department of Biochemistry and Molecular
Biology, University of Nebraska Medical Cen-
ter, Omaha. Area of study: Thermus species

As of this point, I don’t know if we have any
hard data that “native microbial populations” in Yel-
lowstone have been displaced by either “native”
transfer (wildlife) or any evidence that investigators
themselves could be contributing to displacement
from any use of nonsterile collecting equipment.
This is a constant worry, especially when you see
investigators doing physical sampling of hot springs
for released gases, etc., much as I observed on 16
July 1999 at Octopus Spring by Gavin Chan and
other students from Washington University in St.
Louis during their detailed mapping of the outflow
channels of Octopus Spring (Web site http://
epsc.wustl.edu). I have been very careful to always
use sterile materials (wrapped sterile sampling
material) and suggest that approach for other indi-
viduals or investigators to avoid or minimize any
direct contamination problems.

Perry Walker Russell
Department of Biology, Cumberland College,
Williamsburg, KY. Area of study: Sampling
protocol

A sampling protocol needs to be established that
will preserve these sites as much as possible. Since
my background is in pathogenic bacteriology, I
have always as a habit used techniques that are as
close to “sterile technique” as possible. (1) I use a
telescoping golf ball retriever with a glass beaker
attached to the end for gathering my samples and a
thermometer hanging from the end of the retriever
shaft. (2) Before collecting a sample, I sterilize the
end of the retriever, the beaker, and the thermome-
ter with alcohol. (3) When actually collecting the
sample, I never stand in the water and, in fact, like
to stand back a little way. Of course, for really hot
or dangerously weak crustal areas, standing back is
a necessity, but I also like to stay back a couple of
feet from more easily accessible and cooler runoff
streams. After all, I don’t need to be stomping
around with my Kentucky microflora–laden boots
in Yellowstone thermal springs. (4) Upon retrieving
a sample in the beaker, I first check the tempera-
ture reading and then pour the sample into an indi-
vidually wrapped sterile polypropylene tube.
(maybe 8–10 mL). The residual in the beaker I pour out into the dirt or sand (not back into the water).

(5) I can now take a pH reading of my sample by quickly putting a drop or two from my sample tube onto pH paper. (I tend to prefer this method over sticking a probe end into the spring or the sample because of the potential for contamination plus I have always found my pH test strips to be accurate enough for my purposes.) (6) Before moving to the next site, I record my site data (temperature, pH, location, and elevation) and sterilize the end of the retriever, the beaker, and the thermometer with alcohol again.

Hopefully by using this protocol, I am not contaminating any sampling site with bugs from Kentucky or cross-contaminating springs with other thermophiles. My only worry lies in the fact that while alcohol should destroy any bacterial contaminants, it may not eliminate all bacteriophage contaminants.

**DISCUSSION**

Several kinds of experimental approaches provide a microbial inventory of hot spring ecosystems. These include (1) PCR-based methods to amplify rRNA gene sequences (ribosomal DNAs), (2) DNA staining with DAPI to distinguish microbes from minerals, and (3) electron microscopic analysis to identify microbes and their phenotype. Small subunit rRNA sequence-based analysis provides the most complete inventory of microbial populations. While this method does not assess the microbial growth phase or phenotype of unknown microbes, as do DAPI staining and electron microscopy, it provides an elegant method to gain information on microbes that can be seen microscopically, but not cultured. Such approaches are strengthened by microbial culture and an analysis of phenotypes to reveal the presence of new characteristics such as the “winged” flagellum (Weiss Bizzoco et al. 2000).

Our observations of *Sulfolobus*-like cells in pools ranging in temperature from 68°C to 88°C and in a flowing spring at Amphitheater (79°C) indicate that in many acid habitats this...
is the dominant morphological type of organism. It is also clear from our results that population dynamics in mixing pools have not changed substantially since 1971 when cells of environmental samples were first observed by means of electron microscopy. It seems clear from our results of the past 3 years that a morphologically diverse collection of rod-shaped microbes exists at 70–89°C, pH 2–2.4. This temperature range is substantially higher than that reported for most rod-shaped thermophiles in comparable habitats below pH 3. The exceptions perhaps are *Thermofilum* and *Thermoproteus*, which grow anaerobically (70–97°C, pH 2.5–6.5) and share *Sulfolobus* habitats (Stetter 1986).

Our studies show that in one flowing spring at Amphitheater a substantial change has taken place. Whereas our earlier study (Weiss 1973) showed that at temperatures above 75°C *Sulfolobus*-like cells were the predominant cell type, we have demonstrated here that rod-shaped microbes significantly outnumber *Sulfolobus*-like cells at all temperatures above 65°C in Amphitheater Spring 2. Our light and electron micrographs indicate that in this spring there may be significant interactions among phenotypically diverse rod-shaped cells as well as between the rod-shaped cells and spherical *Sulfolobus*-like cells. Such cell-cell interactions may allow survival of the associated cell types and appear to involve direct attachment by cell walls or short surface pili. One feature of considerable interest is that whether cells attach to sulfur directly, as with rod-shaped organisms, or by pili, as with *Sulfolobus*-like cells, the majority of the attached cluster remains separated from the sulfur crystal surface. *Sulfolobus*-like cells accomplish this by means of long pili, whereas rods attach to each other, forming elongated cell groups that extend away from the sulfur crystal. The significance of this separation distance for the oxidation of sulfur and the long-term survival of the microbial community remains to be determined.

The finding that *Sulfolobus*-like cells no longer represent the dominant organism at Amphitheater Spring 2 indicates that a change in population dynamics has occurred. We note with interest that it is still the most abundant microbe in nearby spring 3 in the Amphitheater group. At another flowing spring, Roaring Mountain, changes are not as obvious because of lower cell numbers and the absence of sulfur. This type of microbial community change has been considered by Yellowstone microbiologists who generally believe that other organisms have always been present along with *Sulfolobus*, and that a shift in the dominant species does not result from research activities but instead represents natural succession within native microbial communities. In keeping with that opinion, it seems unlikely that the change represents an “invasion” or exotic microbes. It is also unlikely that this habitat has been intentionally disturbed by research or other human activity. The pH and temperature of this site have been rather stable over the years. However, changes such as flow rate, chemistry of the spring, or nutrients entering from the algal mat above the spring may all be factors contributing to microbial changes.

**Human-vectored Contamination**

This study shows that microbial community changes have occurred over a period of time in the acid hot spring habitat. An important question is whether such changes might be expected to occur naturally over time within an established community or whether they might be the result of human research activities. We will consider below 5 points related to this question, i.e., human-vectored contamination.

1. The argument is given that human and animal contamination would be hard to separate.

While this is most likely correct, animal cross-contamination might be more easily dismissed. Although animals such as moose and bison range in the area of acidic hot springs and pools and might step into a spring, transfer of acquired microbes seems more likely limited to nearby springs which themselves have already been exposed, possibly through abiotic processes, to these same organisms. With springs that are more distant, say miles apart, it could take many hours for the animal to travel to the next spring, and the microbes might die or be removed during transit time from the first to the second spring.

The time interval for springs several miles apart would be so great that such cross-contamination is less likely. Insect-mediated contamination may have a shorter interval for the
longer distances, but insects for the most part would likely be limited to the cooler surfaces of algal mats below 55°C. Furthermore, microbial communities in these mats have long been exposed to insect foraging activities, particularly ephydrid flies that carry out their life cycle year-round in algal mats. Possible dispersal of microbes in these lower temperature ranges seems to be part of the biotic activities to which springs and microbial communities have already been exposed. At the lower temperature ranges, there is a complex community structure. Algae constitute a food source for these herbivores (Brock 1978), but at higher temperatures insects do not seem to visit the springs to any great extent.

On the other hand, investigators travel by automobile from one site many miles to the next, within minutes. The general mobility of investigators who can travel rapidly from site to site and the presence of inexperienced investigators such as graduate students or assistants represent unknowns that could impact hot spring habitats. While researchers are well aware of and concerned about exotic species, the current awareness was not a major concern with microbes one or more decades ago.

We should note that even if mud from an investigator’s boot were to enter a new system accidentally, most of it would be carried downstream in a flowing spring like Amphitheater, leaving only a small and likely inconsequential amount of inoculum. In pools this could vary a bit where there might not be an outflow channel. Most pools are sampled with some type of extension device so that investigators stay further away from the edge of a pool than they would a flowing spring. Despite the lack of past attention to possible contamination, microbial investigators have always been careful to try to minimize input in hot springs and pools and in most cases use sampling methods and devices that would avoid any but the smallest possible input into a system. The fairly sizeable flow rate of spring water in pools and the continuous turnover of the contained water (Brock 1978) would likely remove the introduced inoculum.

(2) Microbial researchers are very careful to avoid contamination of their samples, but in the past they may not have been thinking about mud or soil that was on their feet.

Moreover, investigators taking hot spring samples to analyze physical or chemical properties may not have been as careful as microbiologists. So, both seem to be likely potential vectors.

Although this concern introduces an unknown, it is diminished somewhat because acid hot springs provide a hostile environment, perhaps even for the resident microbes. This is seen by our DAPI staining results where most cells in the springs are in a stationary phase and are not growing and rapidly dividing. Very few organisms present have been isolated in culture. This is usually consistent with stringent nutrient (or oxygen) requirements. As pointed out by Stetter (Films for the Humanities and Science 1993), many acid hot spring organisms utilize hydrogen and are inhibited or killed by oxygen. This implies that exotic organisms have complex growth requirements that likely will not be met in most new habitats.

(3) Castenholz argues that exotic species tend to establish only in disturbed habitats, and since these hot spring habitats are thought to be undisturbed, it is unlikely that any of the established species are introduced.

This is said despite the fact that some changes in communities have been observed over the years. While it seems clear that disturbance facilitates establishment of exotics, this system of hot springs is much more like a series of islands than vast tracts of continental land. In islands dispersal is a rare event, and unique and interesting species arise through adaptive radiation due to the large number of unfilled niches. Islands are prone to exotic species damage even when very undisturbed. When exotic species, e.g., rats, enter a Pacific island, they rapidly establish and decimate the local species, both competitors and local flora. It is easy to imagine that acid hot springs can have very unusual and unique species, but that an introduced bacterium from another pond will not have the bacteriophage load and may actually be better adapted. There might be underadapted species that are susceptible to competitive exclusion by introduced species. Islands can possess underadapted species because of the absence of introduced competitors and predators.

If bacteriophage in cells of the native community were lysogenic, their presence proba-
bly would not alter the ability of native cells to compete effectively with an introduced species. On the other hand, if bacteriophage are thought of as a means for keeping microbial populations in check, then a phage-free introduced organism could gain an advantage. If bacteriophage kill off the dominant species (by lysis), this might allow the introduced microbe to gain a competitive advantage and become established in the new ecosystem, assuming all other things are equal.

Although the existence of bacteriophage introduces some uncertainty, it is less of a concern than it might seem. So far, only 2 genera have been recognized as having bacteriophage or viruslike particles (Stetter and Zillig 1985). While this might seem to be a low number of organisms with bacteriophage, recall that we have examined a very large number of springs by electron microscopy of thin sections and whole mounts and find this to be consistent with our results. As for the 2 genera we know about, each has elements that decrease the possible problem. The bacteriophage of *Thermoproteus* cause cell lysis when the sulfur supply is exhausted; clearly, this might be cause for concern, but this organism is a strict anaerobe. This considerably reduces the concern because anaerobic contaminants are not likely to survive transfer via a human vector. Viruslike particles in *Sulfolobus* do not appear to affect active growth of this organism. The crystalline particle arrays seen in *Sulfolobus* are not known to cause cell lysis without an induction mechanism such as UV irradiation (Stetter and Zillig 1985); this required stimulus would not likely be encountered in the natural habitat.

Certainly a case can be made that underadapted species exist within acid hot springs because there is electron microscopic evidence for the existence of a rich diversity, and yet many cells are present in low number. It is easy to imagine that an exotic species might be better adapted than the underadapted species. This introduces some uncertainty, but the niche of the underadapted species would likely be small since they are few in number. So, the introduced species, even if successful, would likely be insignificant.

(4) One researcher stated that alcohol cleaning would not eliminate bacteriophage contaminants.

Introduced bacteriophage could be a major disturbance in these systems. Bacteriophage are strongly resistant, perhaps even to attempts at sterilization by ETOH. Bacteriophage in the acid sulfate habitat likely follow the general rule that those with a wide range of species are more rare than those with species or strain specificity. Phage would have to make specific contact with sensitive cells. This might be difficult in a flowing spring with continuously moving water. In a mixing pool bacteriophage could be removed by nonspecific adsorption to a surface such as a mineral particle. Even direct contact with a suitable host would not assure a productive infection. We mentioned that most organisms in these habitats are not actively growing and dividing. Thus, while these considerations (a narrow host range and a quiescent microbial host population) certainly do not eliminate the potential for bacteriophage to disturb the system, the problem is diminished to some extent from a conceptual point of view because the more abundant the bacteriophage might be, the less likely they are to interact effectively with the existing microbial population. In keeping with this idea, viruslike structures in thermoacidophilic cells from natural samples or cultures were seen only rarely (Weiss Bizzoco 1999) in many hundreds of samples examined by electron microscopy. Thus, even if bacteriophage present in these springs are vectored by humans, their activity is likely to be at a low level.

(5) Although we have stated that these systems do not vary, several springs show minor temperature changes or major changes induced by cave-ins.

Castenholz mentions documented natural changes in many springs in YNP, especially in unstable areas such as Mammoth and Norris. These natural changes could be the source of disturbance that would enhance the chance for success of introduced exotic microbes. While solfatara basins such as Norris or Amphitheater Springs seem fundamentally unchanged and stable, individual springs can and do undergo alterations. Far from being unusual, changes of this type, both minor and major, are normal events in solfatara areas. Such natural changes as water flow, temperature, pH, and chemistry all conceivably could enhance or reduce chances for success of
human-introduced species. Several arguments should be considered in evaluating which possibility is more likely. First, with present microbiological methods, numbers of any introduced organisms would be small. The likelihood of their becoming established seems low because they would have to undergo an abrupt, and not necessarily favorable, change in chemistry compared to their native habitat. One possible result is that introduced organisms would be washed away or die from these changes. Second, organisms may be introduced to a habitat, but, if not optimally adapted, their numbers will remain low or insignificant. The chances that a few introduced microbes will land in an acid hot spring may be great, but the likelihood of landing in a spring, whether disturbed or not, to which they are optimally adapted in temperature, pH, and flow or chemistry seems minimal. Third, the physiological state of these organisms is an important consideration that is usually overlooked. Most cells in springs are in the stationary growth phase and potentially quiescent. As a result, they are not necessarily going to grow, even if exposed to an appropriate and favorable environment. Fourth, organisms in acid hot springs are exposed to extremes of temperature, pH, and redox potential. Low redox potentials (anaerobic habitats) exist in these springs. Because of the low solubility of oxygen at high temperature and the presence of reducing gases like hydrogen sulfide, most of the organisms are anaerobes. Oxygen is toxic to these exotic organisms, particularly during transfer between springs. This toxicity would likely reduce their ability to displace native microbes or even survive. Fifth, many respondents in this paper are long-time thermobiologists, some with decades of experience. Over the years of attempting to grow organisms seen microscopically in samples, experienced investigators know it may be extremely difficult to duplicate conditions required for growth. This suggests that the organisms have complex nutritional requirements or interactions in their natural habitats. The fastidious nature of these microbes is not particularly evident in native communities where these organisms coexist in large numbers. Introduced organisms with unique nutritional requirements might survive in a new habitat for a prolonged period of time, but it is much less likely that they would displace native residents, even in the face of natural changes or disturbances in a spring. Taken together, these 5 points on human-vectored contamination introduce some unknowns that may be cause for concern. Although some human-vectored species might survive, if introduced into springs, our rebuttal arguments favor the view that exotic species are likely to have a low probability of displacing native microbial communities, even with the present level of research activities in the acid hot springs of YNP. Because unknowns exist, the use of sound microbiological technique in sampling acid thermal habitats seems absolutely essential to provide the most protection for the unique native microbes.

Conclusions

Results of this study suggest that the microbial flora in YNP has changed in some cases, and some things not seen in the 1970s may now be present in the system. Whether this is a result of biotic or abiotic processes, including successional change, or introduction of new exotic species (human-vectored contamination) cannot be determined from the results presented here. While the consensus of prominent YNP microbiologists is that research activities have not produced human-vectored contamination, the question has not been studied in detail (using PCR-based analysis), and uncertainty on the issue remains. It will serve the long-term stability of YNP hot springs as well as other similar resources if all investigators (both beginning and experienced) are aware of the possible introduction of exotic species into the springs and thermal sites that are being studied. With care and consideration on the part of investigators, undisturbed hot spring microbial populations will have the best chance to exist for the benefit of future generations. Significant contributions already made include the discovery of life at high temperatures, the invention of PCR (Saiki et al. 1988), and establishing the Archaea as one of the primary lines of evolutionary descent (Woese et al. 1990). That YNP has fostered these contributions suggests that microbial research represents an important activity.

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