Journal of Industrial Microbiology (1996) 17, 214–218

Looking for diversity of Yellowstone extremophiles

J Combie and K Runnion

JK Research, 1740 East Baseline Rd, Belgrade, MT 59714, USA

Thermal waters in the Yellowstone Park, WY, USA, ecosystem have created a diverse array of extreme microbial habitats. Microbial ecology studies have begun to show the diversity of extremophiles present. Focusing attention on use of a variety of collection sites, sampled materials, approaches to preservation immediately after collection, pretreatments and, perhaps most importantly, media components, will ensure a greater range of organisms in a culture collection. The objective here is to review methods for cultivating extremophiles from Yellowstone geothermal habitats.

Keywords: extremophiles; microbial diversity; thermophiles; Yellowstone

Introduction

The Yellowstone Park, WY, USA, ecosystem holds the world's greatest diversity of accessible, extreme microbial habitats. Not only are there temperature and pH extremes, but also areas with high levels of sulfur, heavy metals, radionuclides, and UV radiation and a range of organic nutrients. Parts of certain thermal microbial mats are supersaturated with respect to oxygen; anaerobic conditions are common at higher temperatures. Some pools provide a year-round, warm microclimate while others are subject to a cyclic temperature pattern.

Recent advances in microbial ecology have revealed a tremendous diversity of microorganisms in thermal pools [7,23,31]. It has been suggested that more than 99% of the world's microorganisms remain to be discovered [29] and extreme habitats are certain to hold many of the more unusual organisms. Scores of extreme environmental parameters combine here to offer an unparalleled diversity of extremophiles.

Value of extremophile diversity

Physiologically diverse microorganisms are likely to produce diverse chemistry, increasing the chance of finding novel compounds [10,12,22,29]. There is a logical progression from biodiversity to advances in biotechnology. Whether searching for new pharmaceuticals, compounds to protect agricultural crops against disease, unusual degradative pathways for remediation of recalcitrant pollutants or products of use in the food industry, availability of a broad diversity of microorganisms will improve the chances of success. Perhaps the greatest value of microbial diversity is the opportunity for a harvest of truly novel ideas [12]. Each new organism found offers the possibility of opening a new field of study heretofore not even imagined. For example, when Thomas Brock and his colleagues isolated *Thermus aquaticus* from a Yellowstone pool nearly 30 years ago [9], there was no way to foresee that polymerase from this bacterium would lead to the myriad of current PCR uses.

Diversity of extremophiles in Yellowstone

Interest in novel microorganisms thriving under environmental extremes reflects a growing awareness of their value in biotechnological and industrial processes [14,17]. Not only are the enzymes and metabolites often more stable, but in some cases, novel enzymes have been found in extremophiles, enzymes not known in organisms from moderate habitats [2].

Recent 16S rRNA of studies in organisms from Yellowstone have indicated an even greater diversity of microorganisms than previously believed [7,23,31]. Over the years, numerous scientists have studied the microbial mat community of Octopus Spring (Figure 1) in Yellowstone. It was believed there was a good understanding of mat structure, with at least the major players identified [32]. As work on 16S rRNA began, it became apparent that much remained to be learned about cultivation of organisms in the laboratory. The Octopus Spring mat was thought to be formed by a single cyanobacterium, *Synechococcus lividus* and a filamentous green, non-sulfur bacterium, *Chloroflexus*

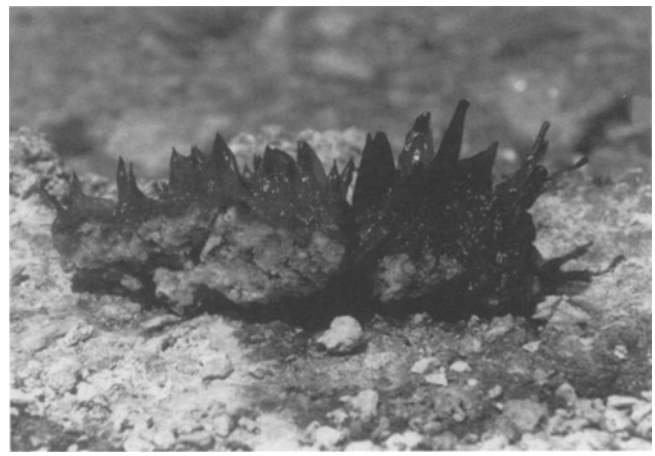

Figure 1 Section of microbial mat from Octopus Spring in Yellowstone Park. The mat is approximately 2 cm thick.

Correspondence: J Combie and K Runnion, JK Research, 1740 East Baseline Rd, Belgrade, MT 59714, USA
Received 8 February 1996; accepted 28 May 1996

auranticus [32]. Now it is known that at least seven different cyanobacteria are present. Altogether, sixteen species had been known in the Octopus Spring mat on the basis of culture work. Molecular biology techniques suggested 21 new species. Most striking was the fact that none of the rRNAs of previously cultivated species were recovered [31].

rRNA sequences obtained by polymerase chain reaction amplification of mixed population DNA extracted directly from sediment of another Yellowstone hot spring, Obsidian Pool (formerly Jim's Black Pool), have been phylogenetically characterized. The great diversity of archaeal rDNA clones recovered was unexpected and without precedent. But the 98 clones inspected are believed to still not exhaust the community diversity. Although some gene sequences indicate the presence of evolutionary relatives of cultivated species, others are without known close relatives [7].

Range of techniques to recover diverse organisms

Current schemes of bacterial classification are based on variations in the sequences of conservative macromolecules, mainly members of the rRNA complex. Construction of probes based on known sequences has resulted in discovery of related organisms, but the power to uncover further diversity may be limited [22]. On the other hand, application of the classical approach to sampling and recovery of microorganisms from the environment, often produces the same organisms over and over. Only certain microorganisms will be recovered using one isolation method. More attention must be paid to diversifying the approach to organism recovery if novel microbes are to be found.

The concept of unculturable microorganisms is a popular explanation for our inability to successfully bring more than a small fraction of the world's microorganisms into the laboratory. Although many will undoubtedly remain unculturable, experience in the authors' laboratory indicates that use of several different approaches can improve the recovery rate. The need for numerous media and trying several techniques at each sampling location certainly hampers one's ability to collect from every pool in sight. However, such approaches may decrease the number of repeats in a collection.

Diversity of collection sites

In Yellowstone, there are 10 000 thermal features including pools, geysers and mudpots. Thermal vents, in some ways analogous to deep ocean black smokers, are located on the floor of Lake Yellowstone [16]. In high temperature areas where there is insufficient water to fill a pool, the small amount of water available is turned into steam forming a fumarole.

Of critical value is the fact that Yellowstone's thermal features have been subjected to minimal disturbance by man. This may have been especially important to preservation of mat communities. Microbial mats, found in some thermal springs, are believed to be modern analogues of ancient stromatolites, dated to 3.5 billion years old [4]. Mats are microbial communities of numerous species that form a laminated multilayer of biofilms.

Geologists were among the earliest scientists studying Yellowstone thermal waters. As a result, a lot of chemical data were collected for some thermal features. Reference to this information allows selection of sampling sites that may visually appear similar but actually have significantly differing chemistries. Sulfur in all its oxidation states is common in these waters [28]. Other waters are saturated with calcium carbonate [28]; metal ions are abundant in certain pools [28]. The pH of these waters has been measured from under 1 to over 10 (unpublished).

Temperature also contributes to the formation of unique microbial habitats. Water temperatures range from ice-cold streams to superheated springs [28]. For many features, water heated deep within the earth allows maintenance of a nearly constant temperature, regardless of the season. Geysers create habitats with cycling temperatures. Microorganisms living in outflow channels or on geyser walls are subjected to near boiling temperatures, followed by rapid cooling to near ambient temperatures (which can be –40°C in winter), followed by another slug of near boiling water, all within as little as a few minutes.

Since oxygen solubility in water decreases as the temperature increases, anaerobes are common at the highest temperatures [9]. Splashing or fast flowing water can impart significant aeration. Photosynthetic organisms in some mat communities produce superoxic conditions. Most thermal areas are not shaded and are at 7–8000 foot elevation in Yellowstone. Therefore many organisms have adaptations for dealing with superoxic conditions and/or intense UV radiation [20]. Some organisms have high levels of enzymes of the oxygen defense system, while others produce large amounts of carotenoids (unpublished).

Material collected

The 10 000 thermal features create a diversity of extreme microbial habitats in Yellowstone. Not only can samples be collected from the water column, but sediment or scrapings from rock walls of a pool can reveal additional organisms. Where microbial populations are low, pumping several liters of water through a filter concentrates organisms, improving chances of recovery. Other types of samples include very small pieces of microbial mats, animal dung found in thermal features, and organisms found in association with thermophilic plants [26]. The fly *Ephydra thermophila* carries out its complete life cycle on acid algal mats [9] and could be expected to harbor interesting microorganisms.

Soil biodiversity is a field of study in itself, for which specialized techniques have been developed [15]. Collection of soil samples is only mentioned here in reference to the thermal feature called a fumarole. Superheated steam warms soil in the immediate vicinity, provides moisture and is a constant source of certain elements.

One variation on use of chemoattractants, is to place substrate directly in a pool for a period of time to allow microbial colonization. For example, Kelly placed coal samples in a pool for several days, in an attempt to recover organisms metabolizing coal components (RM Kelly, North

Carolina State Univ, Raleigh, NC, USA, personal communication). Brock suspended slides in a spring to allow microscopic examination of microbes, even before techniques were developed for their cultivation [9].

Transport to laboratory

A common approach to collecting in Yellowstone involves filling tubes with water and culturing organisms that survive the trip to the collectors' laboratory. Although some organisms live many months between removal from their natural habitat and transfer to laboratory media, a large percentage are lost. Recovery of many microorganisms requires that samples be processed quickly.

Attention to transport conditions will increase the diversity of recovered microbes. A thermos, filled with water from a collection site, will hold tubes near the habitat temperature for several hours. Another option is to carry suitable equipment to the collection site and process the sample in the field, using a portable incubator to maintain habitat temperature.

Alternatively, dry ice is taken to the field, and samples are frozen immediately upon collection, Daily addition of dry ice to a well-insulated container has proven to be quite successful for organism preservation. Working with deep subsoil samples, Amy *et al* [6] found that 4°C refrigeration produced higher microbial counts as a result of resuscitation, but diversity was markedly reduced. She, too, found that freezing was the most satisfactory method for maintaining microbial diversity in samples that could not be processed immediately.

Treatments

One of the most common techniques to ensure recovery of thermophiles has been to pasteurize samples, generally for a few minutes at temperatures well above those of the collection sites, before using the samples as inoculum. At sufficiently high temperatures, the procedure will tend to select for hyperthermophiles and spore-forming bacteria.

When searching for some groups of extremophiles, antimicrobials can be useful. Broad spectrum antifungal agents are utilized for isolation of bacteria, while antibacterial compounds including penicillin, and streptomycin aid in isolation of fungi [17]. Some extremophiles are unaffected by these antibiotics and the antibiotics become less stable at high temperatures.

Treatments may be designed for recovery of organisms with desired characteristics. For example, while searching for carotenoid-producing organisms, the authors utilized oxygen radical-generating chemicals. It was reasoned that organisms protected by carotenoids would be more likely to survive the presence of toxic oxygen species, while unprotected species would be killed. Of course, organisms protected by high levels of enzymes from the oxygen defense system (superoxide dismutase, catalase) were also recovered, but with some undesired organisms removed, the task of visually selecting pigmented organisms was simplified.

Where populations are large as in mats, isolating slow growing organisms from the fast growing population, is a problem. Serial dilution of inoculum has proven to be useful for the authors and others [17,21]. The work of Ward and colleagues using 16s rRNA reinforced this line of thinking when it was found that many more-readily recovered species were not dominant in terms of absolute numbers [21,31].

Media

The nature of the medium is perhaps the easiest factor to manipulate with the objectives of a given search in mind, but many prefer to rely on a few standard formulations. It is not possible to cultivate a broad diversity of microbes with only a couple of culture media. A useful starting point is to take into account environmental parameters associated with each habitat sampled. Designing media matching sample water chemistry is ideal, but it is time consuming to search out published values and expensive to analyze the chemistry of every collection site. Even then, critical organic compounds would remain largely unknown. Nevertheless, the extra time and cost of using a range of media and additives, is worthwhile.

Minimal medium

With the exception of those with mat communities, many thermal waters are quite low in organic compounds. For thermophiles, Castenholz's Medium D and modifications thereof [11] have been used extensively, and perhaps most successfully, because it is low in organic compounds while salts and trace elements are within the ranges found in many thermal waters.

Selective media

Providing nutrients that either favor organisms of interest or selectively inhibit competitors is useful when microbial metabolism includes specialized pathways. For example, hydrocarbon-utilizing bacteria such as *Thermoleophilum* were successfully enriched in a mineral salts medium supplemented with *n*-heptadecane [17]. When the authors were searching for microorganisms to remove polyurethane paint, collected water samples were incubated with minimal salts and polyurethane paint. Only organisms able to use the paint as a source of carbon could grow. This eliminated an initial, costly screening step, leaving only organisms with a reasonable possibility of having some activity on paint [24]. A similar approach has been taken by others searching for cellulose decomposers.

A variation on utilizing selective media, is use of what might be called selective habitat. Some more extreme habitats apparently have less biodiversity. Brock discovered that *Sulfolobus* was the only organism that would grow in spring water supplemented with a low level of yeast extract when samples came from strongly acidic habitats at temperatures above 65°C [9].

Growth 'factors'

One reason some microorganisms do not grow under laboratory conditions may be that they require 'factors' from other organisms found in their natural habitat. Excreted products of one organism serve as a nutrient source for another community member. Nutritional interdependence between

Thermoanaerobacter thermohydrosulfuricus and *Clostridium thermocellum* is one example. The symbiotic relationship was satisfactorily replaced by a mixture of nine vitamins [19]. Suzuki *et al* were unable to identify the nutritional factor required by *Symbiobacterium thermophilum* that was provided by only one *Bacillus* sp [27]. In the laboratory complex substrates such as yeast extract and peptone provide a range of vitamins, amino acids and trace elements.

Starting isolations with a plentiful portion of native water, such as 50% sample water and 50% medium [11] initially supplies factors found in the environment. Die-off upon transfer alerts one to the possibility of factors missing from the medium. Mat homogenate has been used successfully to recover novel organisms from a well studied thermal mat community [21]. These generalized approaches circumvent the need to identify crucial 'factors' when performing initial screens.

Three specific factors are of particular relevance to work on extremophiles: PQQ, glycolate and tungsten.

PQQ

Pyrroloquinoline quinone (PQQ) is a co-factor for a number of enzymes. Peculiarly, many microorganisms have been found which synthesize non-functional apo-enzymes that lack PQQ, the required prosthetic group [1,18]. The mystery was solved when it was discovered that other organisms produced large quantities of PQQ and excreted it into the milieu [30]. In their natural habitat, many organisms have no need to produce PQQ, since it is readily available. The problem shows up when we try to grow these organisms on laboratory media that do not include PQQ.

One particularly intriguing point for those isolating thermophiles, is that PQQ is extraordinarily heat stable. Effects of PQQ on growth patterns were first discovered when a fermentation flask was not rinsed, before being refilled with fresh medium and sterilized. PQQ produced by the first culture survived the autoclave and caused a markedly shortened lag phase for the second culture [5]. Others have attributed growth-stimulating effects to PQQ [1,13]. Studies by the authors suggested the value of PQQ for recovery of certain extremophiles (unpublished).

Glycolate

Waters which include microbial mats are rich in organic compounds produced by community members. *Synechoccus lividus*, a thermophilic phototroph inhabiting a cyanobacterial mat in Yellowstone's Mushroom Spring, creates superoxic and alkaline conditions which promote glycolate excretion. Glycolate is incorporated by filamentous heterotrophs, also present in the mat [8].

Tungsten

Adams and colleagues found that tungsten, an element seldom used in biological systems, greatly stimulated growth of the hyperthermophile *Pyrococcus furiosus* [3]. Before 1989, only one tungsten-containing enzyme was known. Since then Adams *et al* found other tungsten-containing enzymes and they now believe these may play key roles in primary metabolic pathways of some heterotrophic hyperthermophiles [3].

Media form

Microorganisms from aqueous habitats do not always thrive on gelled media. Now and then, this can be attributed to the specific gelling agent [17], but getting good growth on gelled media is sometimes more difficult as the temperature increases. At high temperatures, agar is of limited usefulness, being hydrolyzed at pHs outside the neutral range above 65°C. Substitutes include Gelrite (clarified gellan gum), κ-carrageenan, starch and silica gel [17].

Other incubation parameters

Generally, habitat temperature and pH should be matched as closely as possible in the laboratory. However, many organisms have been found under non-optimum growth conditions so adjustments may be made. An extreme example is Stetter's discovery of hyperthermophiles in cold sea water [25].

Oxygen must be removed and various gas mixtures may be required for recovery of anaerobes. On the other hand, for aerobes, oxygen is frequently growth rate-limiting because it is poorly soluble at high temperatures. Gases should be humidified to avoid loss of volume.

For initial isolation, some organisms seem to require extended periods of time for growth. Prolonged incubation of highly diluted samples, may pick up slow growing microorganisms. Plates with gelled media should be inverted for incubation to control spreading due to surface condensation. Plates should be sealed with tape or, more conveniently, placed in closed bags to prevent gel drying.

Focusing on use of many different techniques will result in recovery of more diverse microorganisms.

Acknowledgements

We gratefully acknowledge the support of the SBIR program funded under the Department of Defense, DOE and EPA.

References

1 Adamowicz M, T Conway and KW Nickerson. 1991. Nutritional complementation of oxidative glucose metabolism in *Escherichia coli* via pyrroloquinoline quinone-dependent glucose dehydrogenase and the Entner–Doudoroff pathway. Appl Environ Microbiol 57: 2012–2015.
2 Adams M and R Kelly. 1995. Enzymes from microorganisms in extreme environments. Chem Eng News 73: 32–42.
3 Adams M. 1993. Enzymes and proteins from organisms that grow near and above 100°C. Ann Rev Microbiol 47: 627–658.
4 Ahern H. 1994. Does a molecular clock tick for bacteria? ASM News 60: 600–603.
5 Ameyama M, E Shinagawa, K Matsushita and O Adachi. 1984. Growth stimulation of microorganisms by pyrroloquinoline quinone. Agric Biol Chem 48: 2909–2911.
6 Amy P. 1995. Seminar presented at Mont. State Univ.
7 Barns SM, RE Fundyga, MW Jeffries and NR Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci 91: 1609–1613.
8 Bateson MM and DM Ward. 1988. Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat. Appl Environ Microbiol 54: 1738–1743.
9 Brock TD. 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag, NY.

10 Bull AT, M Goodfellow and JH Slater. 1992. Biodiversity as a source of innovation in biotechnology. Ann Rev Microbiol 46: 219–252.
11 Castenholz RW. 1981. Isolation and cultivation of thermophilic cyanobacteria. In: The Prokaryotes, Vol 1 (Starr M, Stolp H, Truper H, Balows A and Schlegel H, eds), pp 236–246, Springer-Verlag, Berlin.
12 Davies J. 1994. Microbial molecular diversity: past and present. J Ind Microbiol 13: 208–211.
13 Duine JA, J Frank Jzn and JA Jongejan. 1986. PQQ and quinoprotein enzymes in microbial oxidations. FEMS Microbiol Rev 32: 165–178.
14 Herbert RA and RJ Sharp (eds). 1992. Molecular Biology and Biotechnology of Extremophiles. Chapman & Hall, NY.
15 Iwai Y and Y Takahashi. 1992. Selection of microbial sources of bioactive compounds. In: The Search for Bioactive Compounds from Microorganisms (Omura S, ed), pp 281–302, Springer-Verlag, NY.
16 Klump JV, CC Remsen and JL Kaster. 1988. The presence and potential impact of geothermal activity on the chemistry and biology of Yellowstone Lake, Wyoming. NOAA-National Undersea Research Program, Vol 6.
17 Lacey J. 1990. Isolation of thermophilic microorganisms. In: Isolation of Biotechnological Organisms from Nature (Labeda D, ed), pp 141–181, McGraw-Hill, NY.
18 Linton JD, S Woodard and DG Gouldney. 1987. The consequence of stimulating glucose dehydrogenase activity by the addition of PQQ on metabolite production by *Agrobacterium radiobacter* NCIB 11883. Appl Microbiol Biotechnol 25: 357–361.
19 Mori Y. 1995. Nutritional interdependence between *Thermoanaerobacter thermohydrosulfuricus* and *Clostridium thermocellum*. Arch Microbiol 164: 152–154.
20 Nelis HJ and AP DeLeenheer. 1991. Microbial sources of carotenoid pigments used in foods and feeds. J Appl Bacteriol 70: 181–191.
21 Nold S and DM Ward. 1995. Diverse *Thermus* species inhabit a single hot spring microbial mat. Syst Appl Microbiol 18: 274–278.
22 Palleroni N. 1994. Some reflections on bacterial diversity. ASM News 60: 537–540.
23 Reysenbach A-L, GS Wickham and NR Pace. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 60: 2113–2119.
24 Runnion K and J Combie. 1993. Microorganisms from extreme environments as source of thermally stable enzymes for removal of polyurethane aircraft coatings. Final report contract #N60921-92-C-0170 for Naval Surface Warfare Center, Silver Spring, MD.
25 Stetter K. 1995. Microbial life in hyperthermal environments. ASM News 61: 285–290.
26 Stout RG and T Kerstter. 1996. Thermotolerant flowering plants of Yellowstone National Park. Am J Botany (submitted for publication).
27 Suzuki S, S Horinouchi and T Beppu. 1988. Growth of a tryptophanase-producing thermophile, *Symbiobacterium thermophilum* gen nov, sp nov is dependent on co-culture with a *Bacillus* sp. J Gen Microbiol 134: 2353–2362.
28 Thompson JM. 1979. Chemical analyses of waters from geysers, hot springs and pools in Yellowstone National Park, Wyoming from 1974 to 1978. USGS Open file report 79-704.
29 Tiedje JM. 1994. Microbial diversity: of value to whom? ASM News 60: 524–525.
30 van Kleef MAG and JA Duine. 1989. Factors relevant in bacterial pyrroloquinoline quinone production. Appl Environ Microbiol 55: 1209–1213.
31 Ward D, M Ferris, S Nold, M Bateson, E Kopczynski and A Ruff-Roberts. 1994. Species diversity in hot spring microbial mats as revealed by both molecular and enrichment culture approaches—relationship between biodiversity and community structure. In: NATO ASI Series, Vol G 35, Microbial Mats (Stal L and Caumette P, eds), pp 33–44, Springer-Verlag, Berlin.
32 Ward DM, R Weller, J Shiea, RW Castenholz and Y Cohen. 1989. Hot spring microbial mats: anoxygenic and oxygenic mats of possible evolutionary significance. In: Microbial Mats (Cohen Y and Rosenberg E, eds), pp 3–15, ASM, Washington, DC.