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THERMOPHILIC BACTERIA ASSOCIATED WITH BLACK SMOKERS ALONG THE EAST PACIFIC RISE

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ABSTRACT - At depths of about 2 600 m along the East Pacific Rise, sulfide chimneys or «black smokers» are emitting superheated fluids at temperatures of about 350°C into cold seawater. Dissolved in these fluids are a suite of gases, including CH_4, H_2, CO, and N_2O. Baross and colleagues reported the production and utilization of some of these gases at 100°C and atmospheric pressure by bacterial communities cultured from black-smoker fluids collected at 21°N. Subsequently, we obtained evidence for the growth of these bacteria at 265 atm pressure and temperatures of at least 250°C, and for their possible production of CH_4 at 300°C. In this paper, the evidence for (and against) bacterial growth at high temperature and pressure is reviewed and results of new laboratory and field studies at 13°N and the Juan de Fuca Ridge are presented.

Key words : thermophilic bacteria, black smokers, pressure.

RÉSUMÉ - A des profondeurs de 2 600 m, le long de la dorsale Est-Pacifique, des cheminées de sulfures ou «fumeurs noirs» émettent des fluides à une température de 350°C dans des eaux froides. Plusieurs gaz dont CH_4, H_2, CO et N_2O sont dissous dans ces fluides. Baross et ses collègues ont montré la production et l'utilisation de plusieurs de ces gaz par des communautés bactériennes, prélevées au niveau des fluides des «fumeurs noirs» à une latitude de 21°N, et cultivées à 100°C sous pression atmosphérique. Par la suite nous avons eu la preuve que ces bactéries peuvent se développer sous des pressions de 260 atm. et à des températures d'au moins 250°C, ainsi que celle de leur capacité à produire du CH_4 à 300°C. Dans cette publication, les arguments en faveur (ou contre) le développement bactérien à hautes température et pression sont réexaminés et les résultats de nouvelles études au laboratoire et sur le terrain, entre 13°N et la ride de Juan de Fuca sont présentées.

Mots clés : bactéries thermophiles, fumeurs noirs, pression.

INTRODUCTION

A recent series of submersible expeditions at depths of about 2 600 m along the East Pacific Rise have revealed sulfide chimneys or «black smokers», 3-17 m high, scattered along axes of seafloor spreading centers (Corliss *et al.* 1979 ; Spiess *et al.* 1980 ; Edmond *et al.* 1982). Spewing from these submarine vents into the surrounding, cold seawater are jets of hydrothermal fluid at temperatures sometimes exceeding 350°C (hydrostatic pressures at the depths of emerging vents keep seawater liquid to 460°C ; Chen 1981). The venting fluids contain supersaturated levels of reduced gases and metals (Corliss *et al.* 1979 ; Welhan and Craig 1979 ; Lilley *et al.*, 1982 ; Edmond *et al.* 1982), potential sources of inorganic energy for chemosynthetic bacteria. Early microbiological studies at the vents established that such bacteria abound at relatively mild temperatures (2 - 40°C) on surfaces and in seawater surrounding the vents, and serve as the primary producers in the vent food chain (Corliss *et al.* 1979 ; Karl, Wirsen, and Jannasch 1980).

The high temperature fluids emerging from black smokers were sampled microbiologically by Baross during the Alvin Rise Expedition to 21°N in 1979. He and his colleagues reported the presence of viable, extremely thermophilic microorganisms in samples of black-smoker effluent with temperatures as high as 306°C (Baross, Lilley, and Gordon, 1982). These bacteria grew rapidly at 100°C and atmospheric pressure, using inorganic sources of carbon, nitrogen, and energy to produce biomass and a number of gases, including methane (Baross, Lilley, and Gordon 1982). The full impact of this discovery was realized when we obtained evidence that one of these cultures could also be grown under controlled laboratory conditions at the hydrostatic vent pressure of 265 atm and temperatures of at least 250°C (Baross and Deming, 1983; Baross, Deming, and Becker 1984). These results indicated the upper temperature limit for microbial growth to be at least 140°C higher than previously recorded (Stetter 1982) and raised new questions on the origin of life (Nickerson 1984; Baross and Hoffman 1985; Yanagawa and Kojima 1985), prokaryotic evolution (Baross and Hoffman 1985), marine geochemistry, exobiology, and industrial microbiology (Sonnleitner and Fiechter 1983; Deming 1986). They also met with considerable septicism (Trent, Chastain, and Yayanos 1984; White 1984), as discussed further below.

The possibility that bacteria or their enzymes could function at temperatures above 100°C, if pressure was applied to prevent a phase change from liquid to vapor, had been considered earlier (Zobell 1958; Morita and Haight 1962; Morita and Mathemeier 1964; Brock 1978; Heinen and Lauwers 1981, and Stetter 1982), but not tested above 110°C. Prior to the Rise Expedition of 1979, there was no known marine habitat where seawater reached extreme temperatures but remained liquid due to hydrostatic pressure and, thus, little incentive to do more than propose the possibility of life in seawater at temperatures above 100°C. Even after the discovery of hydrothermal vents, black smokers, and liquid seawater at superheated temperatures, most microbiologists with access to vent samples focused their studies on surfaces, animal tissues, and water samples at temperatures of 40°C or lower (Karl, Wirsen, and Jannasch 1980). After all, marine environments have been viewed traditionally as habitats for psychrophilic and mesophilic, but not thermophilic, bacteria. Furthermore, temperatures above 100°C at vapor pressure have been known to degrade biomolecules (DNA, protein, etc.) in aqueous solution and are used regularly in autoclaves to kill most bacteria.

Our experiments (Baross and Deming 1983) were novel, not so much because we tested extreme temperatures, but because we used a deep-sea hydrostatic pressure of 265 atm (and not vapor pressure) and an inoculum of potentially new microbial forms of life, cultured from a hot, pressurized deep-sea environment and already known to grow at 100°C at 1 atm (Baross, Lilley, and Gordon 1982). The thermostability of previously characterized, extremely thermophilic bacteria or their biomolecules under deep-sea hydrostatic pressures is virtually unknown (Zobell 1958; Morita and Haight 1962; and Morita and Mathemeier 1964). It is well known, however, that the characteristics of naked DNA, free amino acids, or other biomolecules in aqueous solution differ from their characteristics as bound components in a complex, living organism uniquely adapted to its environment (Heden 1964). Well before our findings were published, others had documented the existence of thermophilic bacteria in shallow terrestrial and submarine hot springs that were capable of growth 10-20°C above the melting point of naked DNA (Brock 1978; Heinen and Lauwers 1981, and Stetter 1982). Although the effects of elevated hydrostatic pressure on such organisms were unexplored, slight gas pressures had been used to achieve growth at 105°C (Heinen and Lauwers 1981) and at 110°C (Stetter 1982), and even to keep some eukaryotic forms of life such as crustacean eggs (Carlisle 1968) and fly larva (Hinton 1960) viable at 103°C. At this writing, there appears

to be no experimental data in the literature that can be used *a priori* to refute the existence of highly adapted forms of microbial life in seawater at 250°C and 265 atm pressure (Yanagawa and Kojima 1985). In fact, evidence is accumulating in support of our findings, as discussed below.

Nevertheless, the conclusion that bacteria grew at temperatures of at least 250°C so thoroughly questioned «conventional wisdom» (Walsby 1983) that some rejected our work outright. Trent, Chastain, and Yayanos (1984), working with our culturing medium but without vent samples or thermophilic bacteria, concluded that the evidence we presented for bacterial growth at 250°C and 265 atm was based on «artifacts produced in the medium and contaminants introduced during sample processing» and, therefore, should not be «seriously considered supportive evidence for the existence of black smoker bacteria». Their claims to have obtained results «nearly identical» to ours, using the same experimental and analytical procedures but no bacterial inoculum, are misleading. Both their methods and results differed from ours. For example, we stained samples with the DNA-specific stain DAPI for 5 min, according to standard microbiological procedures, while they stained for 1-3 h. We measured a linear increase in DAPI-staining «particles», while they reported a step-function increase immediately upon heating and erratic numbers thereafter. Our starting medium, freshly prepared and autoclaved, contained $< 1\ \mu g$ total bound amino acids ml^{-1}(Deming 1984), while theirs contained $23 \mu g\ ml^{-1}$, primarily in the form of serine, glutamic acid, glycine, and aspartic acid, the four classic contaminants in amino acids chromatography. At 250°C, we measured a linear increase ($n=4$; $r = .991$), representing a doubling time of one hour, in the total amino acid content of particulate protein (as well as in concentrations of each of the standardly-detected 15 amino acids and 5 unknown chromatographic peaks) from 1.6 to 145 $\mu g\ ml^{-1}$ (Baross, Deming, and Becker 1984), while they measured the aformentioned 23 $\mu g\ ml^{-1}$ before heating and 14 $\mu g\ ml^{-1}$ of the same 4 contaminant amino acids after heating. We photographed by transmission electron microscopy hundreds of intact bacteria in a sample incubated 6 h at 250°C and 265 atm, many of which were unique in their resemblance to known thermophiles (Stetter 1982), while Trent, Chastain, and Yayanos detected isolated bacteria present as contaminants in their TEM embedding agar.

White (1984) rejected the existence of black smoker bacteria at 250°C and 265 atm on the basis of his experiments showing the thermolability of various biomolecules from non-thermophilic organisms in glass testubes at 250°C and 39 atm vapor pressure. As discussed by Yanagawa and Kojima (1985), such experiments are irrelevant to studies of novel, extremely thermophilic bacteria from the deep sea at their *in situ* hydrostatic pressure of 265 atm. Furthermore, White misinterpreted the micrographs published by Trent, Chastain, and Yayanos (1984), and misused those of Fox (1965), to argue that bacteria in our micrographs were non-living materials condensed from soluble proteins and nucleic acids. The levels of protein and nucleic acids used by Fox (and White) to generate bacteria-like structures at high temperature were not present in our medium and the similar structures of Trent, Chastain, and Yayanos (1984), by their own analysis, were bacterial contaminants unrelated to experimentation at high temperature and pressure.

The issue of bacterial growth at 250°C will not be settled until we or others reproduce the original growth studies (to this end, a modified version of the hydrothermal system used in those experiments has recently been installed at the Chesapeake Bay Institute), but the stability of some amino acids and peptide bonds at 250°C and 265 atm pressure has recently been confirmed (Yanagawa and Kojima 1985), as well as the existence of extremely thermophilic bacteria at smoker sites. While Trent, Chastain, Yayanos, and White were developing what they published as refutations of our data, other investigators

returned to the vents and collected black-smoker samples to examine them firsthand for the presence or absence of extremely thermophilic bacteria. In each case, evidence was obtained for the existence of extremely thermophilic bacteria in association with smoker environments. Karl (1983) returned to 21°N on the Oasis Expedition, collected particles in the plume of a 250-300°C smoker, and extracted from them 100 ng ATP per gram. This represents a living biomass several times greater than that found in overlying surface waters and 10-100 times greater than surrounding, ambient deep-sea water. Karl *et al.* (1984) reported rapid microbial activity in this same smoker sample at 90°C and 1 atm, the most extreme conditions tested. From another sample of smoker fluid (unknown temperature), collected by Jannasch on the same expedition, Jones *et al* (1983) isolated the most thermophilic methanogen yet discovered, *Methanococcus jannaschii.* From venting fluids (unknown temperature) collected on a more recent ALVIN expedition, Jannasch (1985) and Belkin and Jannasch (1985) also isolated extremely thermophilic, sulfur-respiring archaebacteria.

In March 1984, I joined the Byocyarise Expedition, organized by Daniel Desbruyères (IFREMER, Brest, France), to 13°N along the East Pacific Rise. Using the submersible "Cyana", two samples of black fluid exiting from one of the multiple conduits at the apex of a 17-m high smoker were collected in titanium water samplers pre-rinsed with acid and .2 μm-filtered distilled water. Difficulties in positioning the entry pipe of the sampler into the exiting conduit of this unusually tall smoker resulted in the collection of hydrothermal fluid mixed with ambient seawater. The second sampling effort was similarly plagued but, visually, appeared to have collected a purer sample of the black fluid (although a pH of 5 was measured for both samples after retrieval). Similar difficulties with manipulations of the temperature probe gave maximum recordings of 138°C in the plume, where temperatures up to 280°C had been measured at the same point during earlier dives. After water collection, a 15 cm^3 rock that included an exiting conduit for the black fluid was removed from the smoker, using the pincer of the "Cyana" arm. Finally, a third titanium water sampler was filled several hundred meters from the smoker as an ambient seawater control.

Aboard the Research Vessel "Nadir", the water samples were expressed from the syringe-like samplers into sterile containers. A portion of each sample was split into 4.5-ml aliquots, each of which was then spiked with 0.5 μCi ^{3}H-thymidine (1 Ci/mMol ; CEA, France) and loaded into a sterile glass syringe. The syringes were returned to vent pressure (265 atm) in pressure vessels (Tem-Pres Division of Leco Corporation, P.O. Box 390, Blanchard St. Extension, Bellefonte, PA 16823 USA) and incubated 10 h at 2, 55, and 98°C (the hottest temperature that could be reached in the available shipboard oven). After decompression, samples were extracted in cold 5% tricarboxylic acid (TCA), vacuum-filtered onto .2 um filters, and rinsed with twice the volume of TCA. Radioactivity in the particulate fraction was assayed by scintillation counting (counting efficiency was 80.4 %)) and compared to duplicate samples extracted and filtered immediately after addition of label. Dpm ml^- were corrected against killed controls using $HgCl_2$. The results of this experiment indicated thermophilic microbial activity associated with black-smoker effluent but not with ambient seawater. Although no increases in dpm ml^- were measured in the first hot water sample under any of the test conditions, dpm ml^- in the second one doubled in 10 h at 55 and 98°C with no increase measured at 2°C. Dpm ml^- in the ambient seawater control increased 6-fold at 2°C, but decreased about 50 % at 55 and 98°C.

In addition to thymidine uptake studies, a variety of different media compositions, selective for chemolithotrophic bacteria or devised for other known thermophiles, were inoculated with portions of the hot water samples and sealed in serum bottles or tubes.

These were incubated anaerobically at 85°C and 1 atm, 110°C and slight gas (H_2CO_2) pressure, or in glass syringes in pressure vessels at 98°C and 265 atm hydrostatic pressure. Additional sources of inocula included external scrapings and gut contents of a polychaete worm (*Alvinella* sp.) found buried within its organic tube in the smoker rock 1-2 cm from an exit conduit, macerated bits of the worm tube peeled away from the rock after breaking it open, and scrapings from the underlying rock surfaces after both the worm and its tube had been removed. Visual differences (changes in indicator dyes, yellow sulfur crystals, or turbidity) and differences in gas production (increased gas pressure, the smell of H_2S) between samples and sterile controls indicated potential microbial activity in 17 of 91 inoculated samples. Of these, the most frequent source of inoculum was the black-smoker effluent itself or scrapings from the smoker rock. A few positive reactions were detected for *Alvinella* tube samples and external scrapings, but not for gut samples. The most common medium showing positive indications of activity was a sulfur-based medium, formulated according to Baross, Deming, and Becker (1984), or a medium prepared shipboard using .2μm-filtered black smoker effluent supplemented with 0.03 % $(NH_4)_2SO_4$, 0.01 % $NaHCO_4$, and 0.5 % flowers of sulfur. Activity was apparent under the most favorable incubation conditions (anaerobic, 110°C, slight gas H_2/CO_2 pressure) 18-72 h after inoculation.

After the cruise, enrichments were transported back to the lab in a Dewar flask containing hot sand and boiling water, which measured 60°C upon arrival. Transfers of the presumptive cultures into fresh media resulted in positive activity for 6 of the 17 samples. Of these 6, only 2 transferred a second time. Further attempts to revive these remaining cultures have not been successful.

Field samples fixed in 2 % glutaraldehyde immediately after collection were also examined by acridine orange staining and epifluorescence microscopy. The hot water samples, which required $> 10^{-3}$ dilution to minimize interfering orange fluorescence from angular particles, contained about 10^5 yellow or green- fluorescing bacteria-like structures ml^{-1}. Surrounding ambient and low temperature (<40°C) vent waters, which did not require dilution and could be counted more accurately, contained 6.3 x 10^4 to 1.3 x 10^6 bacteria ml^{-1}. An incredible biomass of widely diverse bacterial morphologies, including 8-x 100-μm filaments, was observed on the internal surfaces of the tube produced by the *Alvinella* worm. A similar filamentous microorganism was detected microscopically in one of the enrichment cultures, but failed to grow upon transfer. In spite of difficulties in obtaining cultures of bacteria from these various samples, the microscopic work indicated the presence of significant bacterial populations in every smoker-related sample.

In August of 1984, six samples of hydrothermal fluid, ranging in temperature from 15°C to 345°C, were collected in goldlined titanium samplers by Alvin along the Juan de Fuca Ridge off the coast of Oregon and Washington. Fixed and unfixed portions of these samples, which had been removed from the samplers and handled under strictly anaerobic conditions, were made available to me by Baross. Concentrations of bacteria in these samples, determined by epifluorescence microscopy (using acridine orange and DAPI stains), ranged from 3.8 x 10^5 to 7.1 x $10^6 ml^{-1}$ with the highest level detected in the hottest (345°C) sample (Deming and Baross, 1986).

More success was obtained in culturing bacteria from these samples than from samples collected at 13°N. We devised a new solid medium, based on the highly thermostable solidifying agent GELRITE (Kelco, San Diego, CA), that contained 0.5 % NaAcetate and 0.3 % thiosulfate in addition to various sea salts and trace elements. It was inoculated in liquid pour tubes at 95°C, solidified by slight cooling to about 80°C, and incubated in

solid form at temperatures up to 120°C, with or without slight gas pressures (Deming and Baross, 1986). Colony-forming units were observed in solid Gelrite pour tubes, incubated 2 d at a temperature range of 85-120°C, for the hottest Juan de Fuca water samples (>250°C), but not the cooler ones (< 60°C). Colonies were also obtained in similar fashion from the original culture of black smoker bacteria from 21°N, used in our high temperature/pressure experiments (Baross and Deming 1983), and from the fluid remaining in the titanium syringe at the completion of our experiment at 250°C and 265 atm (Baross and Deming 1983). Examples of these colonies are shown in Figure 1.

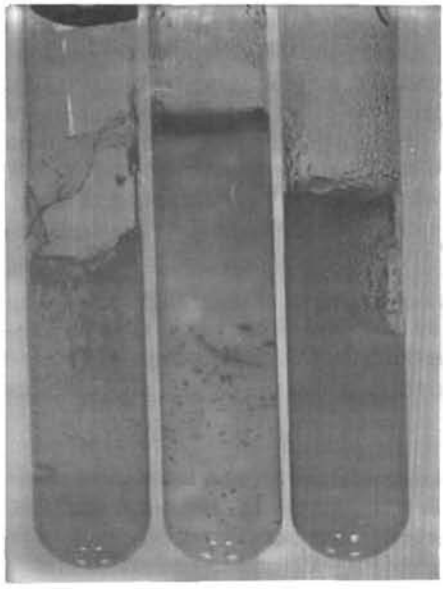

Figure 1 : Colony-forming units in a new medium, based on the highly thermostable solidifying agent GELRITE. The left tube was inoculated with the original culture of black smoker bacteria from 21°N, after exposure to 250°C and 265 atm (Baross and Deming, 1983). It was incubated in a temperature gradient of 85°C (top) to 110°C (bottom). The middle tube was inoculated with 250°C hydrothermal fluid from the Juan de Fuca Ridge and immersed halfway into a 95°C oil bath. The right tube is an uninoculated control, similarly incubated at 95°C.

With the development of this new medium and a modified version of the hydrothermal system used in the original experiments, we anticipate analyses of ultrastructure, optimal growth conditions, and nucleic acids (especially 5S rRNA sequences) for black smoker bacteria in the near future. Information will also be forthcoming from many other laboratories, now actively engaged in studies of extreme thermophiles from smoker environments and elsewhere. Data obtained very recently at the Johns Hopkins University demonstrated that pressures of 50-153 atm enhanced the survival and growth of the well-known thermophilic archaebacterium, *Sulfolobus acidocaldarius,* at temperatures above its maximum growth temperature at atmospheric pressure (Hurwitz 1985).

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