

## Solid Medium for Culturing Black Smoker Bacteria at Temperatures to 120°C

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**A solid, highly thermostable medium, based on the new gelling agent GELRITE, was devised to facilitate the culturing of extremely thermophilic microorganisms from submarine hydrothermal vents. The medium remained solid at temperatures to 120°C at vapor pressures and hydrostatic pressures to 265 atm. It proved useful to its maximum tested limits in isolating colonies of black smoker bacteria from hydrothermal fluids recently collected at the Juan de Fuca Ridge in the Pacific Ocean.**

Extremely thermophilic bacteria are those microorganisms with a maximal growth temperature of 70°C or higher (17). To date, they have been isolated, purified, and characterized at the higher temperatures almost exclusively through the use of liquid media (4-6, 10, 14, 21, 24). Under those circumstances, observations of unusual growth behavior, such as inefficient substrate utilization, population instability, pigment loss, culture collapse, and high death rate after subcultivation, have not been uncommon (5, 6, 10, 21).

To facilitate subculturing efforts, purification, and growth studies of black smoker bacteria, those extremely thermophilic microorganisms derived from superheated (>100°C) hydrothermal fluids along the East Pacific Rise (1-3; J. A. Baross and J. W. Deming, *Bull. Biol. Soc. Washington*, in press; J. W. Deming, *Proceedings of the Second International Colloquium of Marine Bacteriology*, in press) and, as reported in this study, from the Juan de Fuca Ridge in the Pacific Ocean, we devised a highly thermostable, solid culturing medium using the new gelling agent GELRITE (Kelco Div. of Merck & Co., Inc., San Diego, Calif.). GELRITE is an agarlike bacterial polysaccharide that forms an irreversibly solid gel, after heating and cooling, with the aid of a cation such as magnesium or calcium (15). Previously reported media in which GELRITE was used were judged to be superior to agar-based media in terms of thermal properties and clarity, permitting cultivation of thermophilic bacteria at temperatures to 70°C (16). Here we report the composition and preparation of a new GELRITE medium that allowed colony formation of black smoker bacteria at temperatures to 120°C at vapor pressures and hydrostatic pressures to 265 atm.

### MATERIALS AND METHODS

**Media composition and preparation.** A solid GELRITE medium with thermal properties superior to those reported previously (15, 16) was determined empirically by varying the concentration of cations (gelling aids) present in liquid media used in earlier studies of black smoker bacteria (2). The resulting medium consisted of the following (per liter of distilled water): 25 g of NaCl, 2 g of MgSO<sub>4</sub>, 0.8 g of KCl, 0.5 g of Na<sub>2</sub>HPO<sub>4</sub>, 0.01 g of FeSO<sub>4</sub>, 3.0 ml of a trace elements solution (19), 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 g of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 5 g of sodium

acetate. The pH was adjusted to 5.5 with 4 N HCl, followed by the addition of 8 g of GELRITE. The medium was then autoclaved without delay at 121°C and 18 lb/in<sup>2</sup> for 30 min.

In this study, the medium was prepared routinely in 100-ml volumes in 250-ml Erlenmeyer flasks. Immediately after the flasks were autoclaved, they were transferred to an oil bath (100°C), in which the medium could be held in liquid form for about 30 min (beyond 30 min, wisps of solid gel formed at the liquid-air interface). Volumes (10 ml) of the medium were distributed with a sterile glass pipet, preheated to 80°C, into 30-ml-volume serum tubes (Bellco Biotechnology, Vineland, N.J.) which were preinoculated with 0.03 to 0.5 ml of the desired sample. The serum tubes were also preheated or held at room temperature, depending on the temperature of the inoculum. Rapid distribution of the medium with preheated pipets was necessary to ensure homogeneous mixing of the inoculum before gelation, which occurred within seconds of cooling below 80°C. Tubes were then capped with sterile butyl rubber stoppers and aluminum seals (Bellco) and used for incubation studies at atmospheric and vapor pressures.

For incubation studies at elevated hydrostatic pressures, 5- or 10-cm<sup>3</sup> sterile glass syringes (Becton Dickinson and Co., Paramus, N.J.) were used instead of serum tubes. Syringes in the fully open position were inoculated through the needle end and filled to capacity with the hot liquid medium, using preheated Pasteur pipets. Syringes were sealed with Luer-lok plastic tips, which were made from the bases of Luer-lok needles (Becton Dickinson) by removing the needle and melting shut the remaining hole in the plastic base.

**Sample incubation.** Serum tubes were incubated at room temperature or at elevated temperatures of 70 to 120°C in standard laboratory ovens (±1.5°C) or silicon oil baths (±0.5°C). In some cases, tubes were incubated in a sand bath in a temperature gradient of 102 to 110°C. At temperatures above 100°C, the sealed tubes were allowed to reach vapor pressure without degassing.

Stainless steel pressure vessels, custom built by Tem-Pre Div. of Leco Corp. (Bellefonte, Pa.), were used for incubation studies at elevated hydrostatic pressures. Sealed, gel-containing glass syringes were loaded into preheated (90°C) vessels which were filled to capacity with hydraulic fluid (distilled water) at 90°C. Each vessel was closed, positioned in a large drying oven at 90°C, and plumbed with high-pressure capillary tubing (High Pressure Equipment Co., Inc.,

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TABLE 1. Incidence of bacteria in samples of hydrothermal fluid

Fluid temp (°C)		Total no. of bacteria ml <sup>-1</sup> <sup>c</sup>	Colonies ml <sup>-1</sup> in acetate gels at:		
At point of collection <sup>a</sup>	In sampler <sup>b</sup>		25°C	70°C	102–110°C <sup>d</sup>
15	15	4.5 × 10 <sup>6</sup>	+ <sup>e</sup>	— <sup>f</sup>	—
113	4–5	3.8 × 10 <sup>5</sup>	+	3	—
60	56	4.5 × 10 <sup>5</sup>	—	+	2
250	220	>10 <sup>6g</sup>	—	—	>1,000
315	270	>10 <sup>6g</sup>	—	—	>10 <sup>h</sup>
345	345	7.1 × 10 <sup>6</sup>	—	—	>500

<sup>a</sup> Measured in situ by thermistor probe aboard *ALVIN*.

<sup>b</sup> Determined by chemical analysis (Mg correction) of retrieved sample (see text and reference 3 for details).

<sup>c</sup> Determined by epifluorescence microscopy (see text for details).

<sup>d</sup> Temperature gradient achieved in sand bath; colonies per milliliter were estimated from duplicate 10-ml gels inoculated with 0.1 and 0.5 ml of sample fluid.

<sup>e</sup> +, Confluent growth.

<sup>f</sup> —, No discrete colonies, bands, or growth in any form was detected.

<sup>g</sup> Estimates only, because bacteria appeared primarily in clumps.

<sup>h</sup> Growth observed as a narrow band mid-gel, in addition to isolated colonies (see Fig. 1B for examples of colony formation at 100°C).

Erie, Pa.) through an oven vent hole to a pressure gauge and hydraulic hand pump (Enerpac, Butler, Wis.) mounted on an adjacent lab bench. Samples were pressurized hydrostatically to 70 atm (a relatively low pressure that could be monitored accurately) or 265 atm, which is the approximate pressure at the depth of hydrothermal vents in the East Pacific.

After pressurization, the oven setting was increased to the desired temperature (115 or 120°C in this study). Equilibration of the temperature of the hydraulic fluid in the pressure vessels with the increased air temperature of the oven required about 3 h. A relatively constant pressure was maintained on the samples during this heating period by leaving the vessels open to the gauge and hand pump and by releasing pressure manually as it increased above the desired level. Once the pressure was stabilized, indicating that the internal vessel temperature had reached equilibrium with the oven temperature, the vessels were isolated from the gauge and pump and allowed to incubate for 2 to 5 days. Before the vessels were decompressed to examine gels for colony formation, the vessels were cooled to <90°C, during which time the pressure was kept constant, in a reverse manner, by applying pressure hydrostatically as it fell below the desired level. These procedures were followed to prevent boiling in the pressure vessels at temperatures above 100°C.

**Sources of inocula.** Sources of inocula used in this study were samples of hydrothermal fluid that were taken from actively discharging sulfide mounds or chimneys (smokers) during dives by the submersible *ALVIN* at the Juan de Fuca Ridge off the coast of Washington State in August 1984. Each sample was collected in a titanium syringe sampler which was rinsed with acid and ethanol prior to deployment aboard *ALVIN*. Details of the design and operation of the sampler, which represents an improved (nonleaky) version of the one used in earlier studies (1–3, 13; Baross and Deming, in press), have been described elsewhere (24a).

Before the intake pipe of the sampler was inserted several centimeters into a smoker orifice and flushed with hydrothermal fluid, the temperature of the smoker effluent was determined in situ at the same point with a thermistor probe aboard *ALVIN* (Table 1). The temperature of the fluid then drawn into each sampler was calculated, as described previously (3), on the basis of its Mg content which was determined by chemical analysis (R. E. McDuff, unpublished data; Table 1).

Strict precautions were taken aboard the ship to remove fluids from the samplers under anaerobic conditions. Specially designed, sterile, argon-gassed receiving flasks were

attached directly to the main body of the sampler (the intake pipe was removed), and all manipulations were performed in an argon hood. Samples were stored in these anaerobic flasks at room temperature until they were inoculated into the gels, as described above.

**Microscopy.** Concentrations of bacteria in subsamples of the smoker fluids, fixed in 2% glutaraldehyde (Tousimis, electron microscopy grade) as soon as they were recovered on board the ship, were determined by epifluorescence microscopy after they were stained with acridine orange (11). The acridine orange counts, based on duplicate slide preparations, were confirmed by staining additional subsamples with the DNA-specific stain 4',6'-diamidino-2-phenylindole · 2HCl (18). In samples in which bacterial morphologies appeared in clumps or in association with angular particulates that also fluoresced after staining with acridine orange, bacterial concentrations could only be estimated with 4',6'-diamidino-2-phenylindole · 2HCl (Table 1).

Individual colonies that formed in acetate gels inoculated with the hotter smoker samples were removed from random depths in selected gels with sterile Pasteur pipets. Some of these gel plugs were extruded from the pipets onto glass slides, overlaid with cover slips, and examined by phase microscopy (without fixation or staining). Other gel plugs were immersed in a 2% solution of glutaraldehyde, which was prepared in filtered (pore size, 0.2 μm) artificial seawater, for subsequent examination by transmission electron microscopy. Samples for transmission electron microscopy were fixed with 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer (pH 7.3) for 1 h, washed three times in distilled water, prestained in 1% aqueous uranyl acetate for 1 h, dehydrated in a graded series of ethanol solutions, and embedded in Spurr medium (Polysciences, Inc., Warrington, Pa.). Thin sections were poststained with 0.1% lead citrate before they were viewed on an Hitachi HU-12 transmission electron microscope at 75 kV. Gel plugs removed from an uninoculated control incubated at 100°C were also examined by transmission electron microscopy.

**Gel photography.** After they were cooled to room temperature, gel-containing serum tubes and syringes were placed on a light box, examined at double magnification for colony formation, and photographed with high contrast black and white Polaroid POLAGRAPH<sub>RX</sub> film. The sealed syringes were photographed while they were immersed in pure silicon fluid, a procedure that eliminated refractive interference from scratches on their exterior surfaces which were incurred during loading and unloading of the pressure vessels.

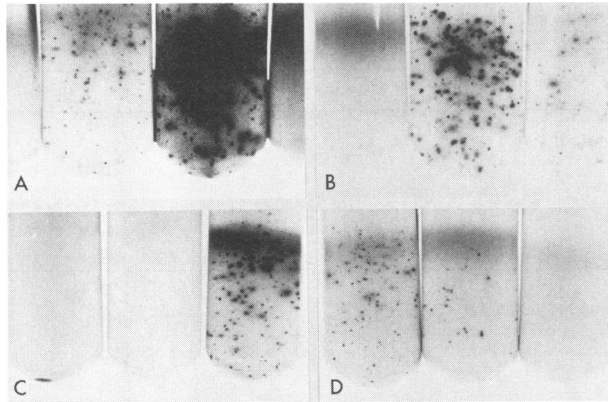


FIG. 1. Colony formation in inoculated acetate gels, along with colony-free control gels, in serum tubes incubated in a silicon oil bath at 100°C ( $\pm 0.5^\circ\text{C}$ ). Depth of tube immersion in the bath is indicated by the zone of colony formation and slight gel discoloration, which was frequently observed near the oil-air interface. (A) Effect of extended incubation on gel clarity and colony size, shown by serum tubes containing the following (left to right): uninoculated gel after incubation for 3 days (no colonies); gel inoculated with 220°C smoker fluid after 3 days; gel inoculated with 220°C smoker fluid after 9 weeks; uninoculated gel after 9 weeks (no colonies). (B) Well-defined and diffuse colony formation, shown by serum tubes containing the following (left to right): gel inoculated with sterile acetate broth (no colonies); gel inoculated with 220°C smoker fluid; gel inoculated with 270°C fluid. (C) Absence of colony formation in gel lacking added carbon source, shown by serum tubes containing the following (left to right): gel without acetate inoculated with 220°C smoker fluid (no colonies); uninoculated gel with acetate (no colonies); inoculated gel with acetate. (D) Effect of sample dilution, shown by serum tubes containing the following (left to right):  $10^{-2}$  dilution of 220°C fluid;  $3.3 \times 10^{-2}$  dilution of same; uninoculated gel (no colonies).

## RESULTS AND DISCUSSION

The GELRITE medium devised in this study remained solid at temperatures to 120°C, at vapor pressures and hydrostatic pressures to 265 atm, for tested incubation periods of 5 days at the upper end of the temperature range (115 to 120°C) and of at least 3 months at the lower end (80 to 110°C). Examples of gel solidity and clarity are shown in Fig. 1 and 2. Beyond a 3-day incubation period at the higher temperatures, or a 3-week incubation period at the lower temperatures, gel clarity often began to diminish because of the onset of gel caramelization (Fig. 1A and 2B). Supplementing or substituting alternate carbon sources (bicarbonate, formate, methanol, amino acids, lactate, urea) for ace-

tate did not alter the solidity or clarity of the medium at temperatures of 80 to 120°C. In a single test at 130°C and 120 atm of hydrostatic pressure, the acetate-GELRITE formulation completely liquefied and caramelized in less than 2 days.

Colony formation was observed at temperatures of 80 to 110°C in all acetate-GELRITE pour tubes that were inoculated with samples of smoker fluid for which original temperatures of 56°C or greater were determined by Mg correction (Table 1). No colonies were observed in replicate gels, which were inoculated with the same samples, when incubated at temperatures of 70°C or lower (Table 1). Conversely, no colonies formed in gels inoculated with the lower temperature ( $\leq 15^\circ\text{C}$  by Mg correction) samples (composed primarily of ambient seawater; see below) when incubated at temperatures above 80°C, although growth was apparent at the lower temperatures tested (Table 1). All of the smoker fluids contained in excess of  $3 \times 10^5$  bacteria  $\text{ml}^{-1}$  by epifluorescence microscopy (Table 1), with the highest concentration ( $7.1 \times 10^6$   $\text{ml}^{-1}$ ) being observed in the hottest fluid (345°C). Predominant bacterial morphologies in the fluids were cocci (1 to 1.5  $\mu\text{m}$  in diameter), irregular cocci (also 1 to 1.5  $\mu\text{m}$  in diameter), and rods (1.5 to 5  $\mu\text{m}$  in length).

Examples of colony formation are shown in Fig. 1 and 2. In general, colonies appeared within a 1- to 5-day incubation period and increased in size with an extension of the incubation period (Fig. 1A). When tested, colonies in 80°C gels increased in size after they were shifted to 100°C for further incubation. Most colonies were off white to beige in color. They appear dark in Fig. 1 and 2 because of the lighting that was used during gel photography. Colonies were never observed in any of the control gels (incubated alongside the inoculated acetate gels), which routinely included uninoculated gels (Fig. 1A through D and Fig. 2A through C), gels inoculated with sterile acetate broth (Fig. 1B), and gels inoculated with sample but prepared without the addition of the acetate (Fig. 1C).

Many of the colonies that were examined by phase microscopy, especially those cultured from the 220°C smoker sample, were comprised of highly refractile, micron-sized cocci. Mixed morphologies were observed in other colonies, with there being irregular cocci and rods toward the periphery and tightly packed filaments in the center. Examination of colonies (from the gels shown in Fig. 1D) by transmission electron microscopy revealed microorganisms with unusually thick cell walls and internal membranes (Fig. 3). However, conclusions regarding ultrastructural features and culture purity cannot be drawn until additional studies are completed.

Occasionally, bacterial growth in the acetate gels was observed not as macroscopically well-defined colonies but as

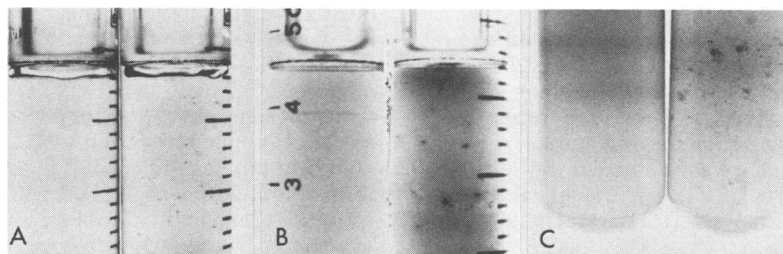


FIG. 2. (A) Colony formation at 115°C and 70 atm of hydrostatic pressure (right); uninoculated gel (left). (B) Colony formation at 120°C and 265 atm hydrostatic pressure (right); uninoculated gel (left). (C) Diffuse colony formation at 100°C (right) on subcultivation of colonies in a 120°C syringe gel, an undisturbed duplicate of which is shown in the serum tube on the right in panel B; uninoculated gel (left).

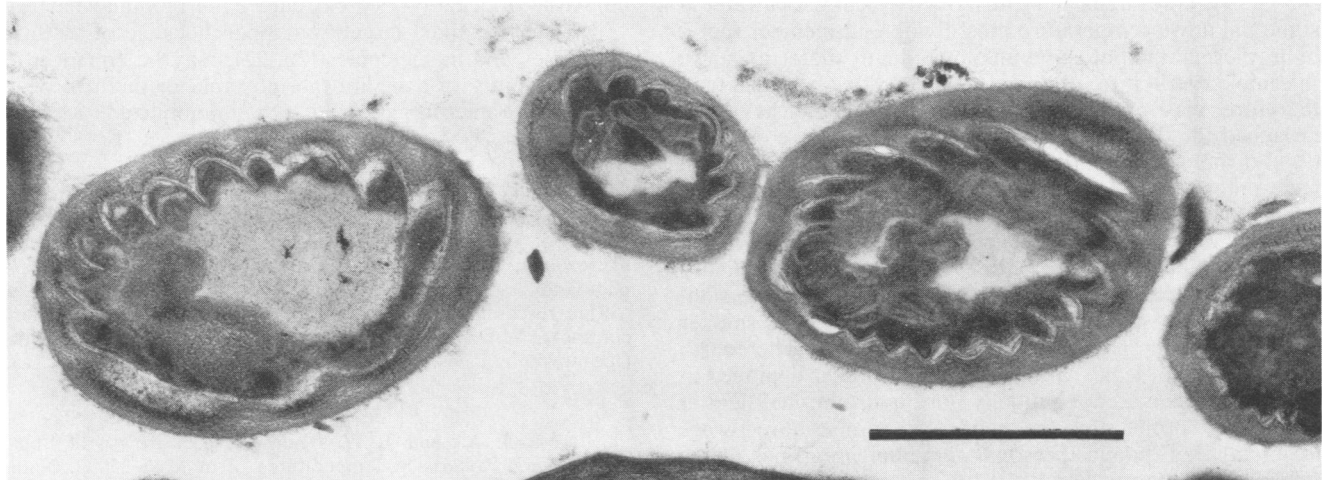


FIG. 3. Transmission electron micrograph of cells in a colony formed at 100°C in an acetate gel inoculated with 220°C smoker fluid (left tube shown in Fig. 1D). Bar, 1  $\mu\text{m}$ .

diffuse or poorly formed colonies. This appeared to be related to specific sources of inocula (Fig. 1B), to subculturing from a higher temperature to a lower one (Fig. 2C), and to incubation in a temperature gradient (Table 1). When gels were inoculated with the 270°C sample of smoker fluid and incubated in a temperature gradient of 102 to 110°C, growth was observed not only as colonies but also as a well-defined band, in which small, 1- to 2- $\mu\text{m}$  rod-shaped bacteria were apparent by phase microscopy, at mid-depth in the gel (Table 1). In cases in which the inoculum was not adequately diluted, confluent growth rather than distinct colonies was observed (Table 1).

Because bacterial growth in gels inoculated with the 220°C sample occurred reproducibly as well-defined colonies that could be obtained in predictable numbers by sample dilution (Fig. 1D) and subcultured in new gels after resuspension in acetate broth (Fig. 2C), acetate gels of this sample were prepared for incubation at higher temperatures and pressures. Pinpoint colonies developed in these gels after an incubation period of 2 days at 115°C and vapor pressure, as well as hydrostatic pressures of 70 and 265 atm. Examples of the colonies in a gel incubated at 115°C and 70 atm are shown in Fig. 2A.

Colonies also formed after an incubation period of 3 days at 120°C and the same three pressures. At this higher temperature, the colony size was related directly to increased hydrostatic pressure. The largest colonies (approximately 0.5 mm in diameter) formed at 265 atm (Fig. 2B), while comparable numbers of colonies barely visible without magnification formed at vapor pressure (data not shown). These results suggest that bacteria from the 220°C Juan de Fuca sample may be barophilic at 120°C, requiring elevated hydrostatic pressure for optimal growth at that temperature. This interpretation is consistent with results of earlier studies of black smoker bacteria from latitude 21°N in liquid culture (1, 2) and also with results of other studies in which relative colony size in pressurized versus nonpressurized silica gel pour tubes was evaluated as an index of barophilic bacterial growth (7, 8).

An anaerobic mode of existence can also be inferred for these, as well as the other bacteria, that were successfully cultured in this study because (i) all original sources of inocula leading to colony formation were maintained under

anaerobic conditions in sealed containers (colonies were not obtained from selected subsamples that had been stored aerobically) and (ii) conditions in the gels during colony formation were anaerobic, as indicated by the occasional use of resazurin in the medium as an  $E_h$  indicator (and as would be expected at the elevated incubation temperatures that were used). However, we cannot conclude that these black smoker bacteria are strict anaerobes, because they survived brief exposure to air at room temperature during the period (typically <1 min) between the time a serum tube or syringe was inoculated and filled with the reduced liquid medium. Also, if bacteria unusually sensitive to oxygen were present in the original inocula, the culturing procedures we used may have selected against them. Indeed the procedures described above may have selected against a variety of microorganisms, e.g., any that may have been stressed initially or during the sampling and recovery process; inhibited by the incubation conditions tested; or dependent on carbon, nitrogen, or energy sources not provided in the acetate-GELRITE formulation. Nevertheless, estimated plating efficiencies (colonies per milliliter of smoker fluid divided by the total number of bacteria per milliliter times 100) achieved by the procedures we used (0.001 to 0.1%) were not unlike those achieved by standard plate count studies of environmental water samples (12).

Although all three must be closely linked, the parameter in this study that was more critical than either pressure or oxygen availability to obtaining colonies from smoker samples was temperature. The exact temperature(s) of origin and natural habitat of the extremely thermophilic bacteria, successfully cultured only from the hotter of the titanium syringe samples in this study, is not known. However, some important temperatures that allow constraints to be placed on the various possibilities are known. These include (i) the temperature of the hydrothermal fluid emerging from each smoker (*ALVIN* probe measurements, Table 1); (ii) the temperature to which a given smoker effluent was reduced due to entry of ambient (<2°C) seawater into the titanium syringe during sampling (Mg-corrected temperatures, Table 1); and (iii) the temperatures at which colony formation occurred (or did not occur) in acetate gels (Table 1).

Of the smokers sampled in this study, four were emitting fluids at superheated temperatures of 113, 250, 315, and

345°C. The first three can be described as leaky. Seawater at some unknown temperature mixed with end-member (pure) hydrothermal fluid at some unknown depth subterranean to the smoker structure; otherwise, the fluid that emerged (and therefore was available for collection) would have approached the theoretical end member temperature of 350°C (9). Furthermore, some additional seawater at <2°C must have entered the titanium syringes during the sampling of these three smokers (compare Mg-corrected temperatures with probe measurements in Table 1), an event that occurs if the intake pipe of the syringe does not remain in the primary (undiluted) stream of smoker effluent throughout the sampling procedures. The sample collected at the 113°C smoker is the best example of this kind of problem. The fourth smoker was not leaky, and the syringe sampler deployed in its effluent recovered essentially pure hydrothermal fluid at 345°C (the probe and the calculated temperatures were identical). J. Edmond (personal communication) has determined, using heat strips, that during the sampling of such near end member fluids, the entire body of a titanium syringe (and therefore its contents) is heated to 180°C before it eventually cools to ambient seawater temperature (<2°C).

Each of the three successfully collected superheated smoker fluid samples used in this study (220, 270, and 345°C), regardless of the presence or absence of ambient seawater, contained more than  $10^6$  bacteria  $\text{ml}^{-1}$  by direct count. Not surprisingly, none of these bacteria could be cultivated in acetate gels at temperatures of 70°C or lower. Mesophilic bacteria would not be expected to survive the superheated temperatures (well in excess of 100°C) to which all bacteria in these samples were exposed during sample collection. Indeed, colonies were obtained from these samples only in gels incubated at 80°C or higher. Although we have no experimental evidence to indicate that temperatures as high as 250 to 345°C characterize the natural habitats of these extremely thermophilic bacteria, they were tolerant (recoverable as colonies) of such temperatures at the in situ pressure of 265 atm. To the extent that available cardinal growth temperatures and pressures can be used as indicators of environmental habitats, the black smoker bacteria reported here must proliferate in their pressurized environments at temperatures (the maximum has not yet been determined) to at least 120°C.

With few exceptions (24), previous attempts to culture thermophilic bacteria as colonies supported by a solid matrix have been restricted to maximum temperatures of about 70°C by the absence of a medium that remains stable at significantly higher temperatures (16). The acetate-GELRITE medium described here remained clear and solid for useful periods of time at temperatures to 120°C and hydrostatic pressures to 265 atm. It proved to be a suitable medium for isolating colonies of extremely thermophilic bacteria from samples of black smoker fluids that were originally under pressure at superheated temperatures. Because components in the medium not involved in polymerization of the GELRITE can be altered without apparent adverse effects on the thermostability of the final gel, it should be possible to develop specific chemolithotrophic and heterotrophic formulas for studies of other extremely thermophilic microorganisms described previously (4-6, 10, 14, 17, 21, 23, 24) or of those awaiting discovery. Indeed, variations in the medium have enabled new thermophiles to be cultured from more recent collections of smoker fluids, as well as from the surfaces of smoker rock, the gut contents of the smoker polychaete *Alvinella*, and the thermal springs on Mt. St. Helens (unpublished data). Finally, the potential for

industrial applications of extremely thermophilic bacteria, which has remained largely untapped because of culturing difficulties and inefficiencies (20, 22), may be enhanced by the availability of a highly thermostable medium in which such unique microorganisms can be immobilized.

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