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Deep-sea smokers: Windows to a subsurface biosphere?*

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Abstract—Since the discovery of hyperthermophilic microbial activity in hydrothermal fluids recovered from "smoker" vents on the East Pacific Rise, the widely accepted upper temperature limit for life (based on pure culture data) has risen from below the boiling point of water at atmospheric pressure to approximately 115°C. Many microbiologists seem willing to speculate that the maximum may be closer to 150°C. We have postulated not only higher temperatures than these (under deep-sea hydrostatic pressures), but also the existence of a biosphere subsurface to accessible seafloor vents. New geochemical information from the Endeavour Segment of the Juan de Fuca Ridge indicative of subsurface organic material caused us to re-examine both the literature on hyperthermophilic microorganisms cultured from deep-sea smoker environments and recent results of microbial sampling efforts at actively discharging smokers on the Endeavour Segment. Here we offer the case for a subsurface biosphere based on an interdisciplinary view of microbial and geochemical analyses of Endeavour smoker fluids, a case in keeping with rapidly evolving geophysical understanding of organic stability under deep-sea hydrothermal conditions.

INTRODUCTION

THE DISCOVERY OF DEEP (>2000 m) hydrothermal vents along seafloor spreading zones in the East Pacific (CORLISS et al., 1979) provided new intellectual and sampling territory for exploring questions about the origins and limits of life on this planet and elsewhere (CORLISS et al., 1981; BAROSS et al., 1982, 1984; BAROSS and DEMING, 1983; BAROSS and HOFFMAN, 1985). Reports of microbial activity at 100°C and atmospheric pressure from several samplings of smoker fluids on the East Pacific Rise (BAROSS et al., 1982; LILLEY et al., 1983) focused attention on the possibility that microorganisms modulated the chemistry of vent fluids even at temperatures conventionally viewed as prohibitory. The contentious issue of microbial existence at smoker temperatures of 150°C and higher (see disputes of BAROSS and DEMING, 1983, by TRENT et al., 1984; WHITE, 1984; BERNHARDT et al., 1984; JANNASCH and MOTTL, 1985; and MILLER and BADA, 1988; and counter-arguments by BAROSS and DEMING, 1984, 1985, 1993; BAROSS et al., 1984; DEMING, 1984, 1987; NICKERSON, 1984; and YANAGAWA and KOJIMA, 1985) quickly subsumed the related hypothesis that the presence of such microorganisms in smoker fluids reflected a subsurface biosphere of potentially vast proportions underlying vent fields. Some investigators were sufficiently curious to conduct in situ tests of this idea using the submersible *Alvin*. However, failing to obtain evidence in support of it, they concluded that any microorganisms detected in smoker fluids must be "contaminants" entrained from environments immediately peripheral to the smoker sampling point (KARL et al., 1984, 1988; JANNASCH and MOTTL, 1985).

A decade later, the concept of a subsurface biosphere underlying seafloor spreading zones is being revisited from a number of perspectives. Microbiologists are taking a closer look at the effects of hydrostatic pressures greater than those encountered at the seafloor on a new group of hyperthermophilic (growth at >90°C) microorganisms isolated directly from deep-sea smokers (REYSENBACH and DEMING, 1991; PLEDGER, 1992; PLEDGER et al., 1993; PRIEUR et al., 1992; ERAUSO et al., 1993). Biochemists are finding record-setting thermal stabilities for enzymes (AONO et al., 1989; BRAGGER et al., 1989; BRYANT and ADAMS, 1989; CONSTANTINO et al., 1990; EGGEN et al., 1990; KOCH et al., 1990; SCHULIGER et al., 1993) and lipids (HEDRICK et al., 1991, 1992) derived from these organisms and the environments they inhabit (BAROSS et al., 1989; BAROSS and DEMING, 1993). Geochemists and geophysicists are examining organic stability under subsurface hydrothermal conditions reproduced more insightfully than before both in theory and in the laboratory (e.g., SHOCK and HELGESEN, 1990; SHOCK, 1990a,b, 1992; HENNETT et al., 1992; ENGEL et al., 1993). Chemical oceanographers have identified smoker fluid characteristics indicative of organic material underlying the Endeavour Segment of the Juan de Fuca Ridge (LILLEY et al., 1993). Planetismal and petrological rationales for reconsidering the hypothesis have also been put forward (GOLD, 1992). It therefore seemed appropriate to re-examine some of our most recent work in light of these many new perspectives. In particular, we present recent trends in the upper temperature and pressure limits of hyperthermophilic smoker microorganisms studied in pure culture and an interdisciplinary version of the "Endeavour Model" (BAROSS and DEMING, 1993) which predicts a subsurface biosphere.

LABORATORY CULTURE WORK

The first extremely thermophilic microorganism (defined as capable of growth above 80°C) isolated in pure culture from a deep-sea smoker environment was the methanogen, *Methanococcus jannaschii* (JONES et al., 1983; Table 1). The hyperthermophilic (capable of growth above 90°C)

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Table 1. Hyperthermophilic microorganisms isolated from hot deep-sea vent environments.

Isolate	Sample source	Temp(°C) growth range	References
Staphylothermus marinus	11°N smoker fluid	65-98	Zillig et al., 1982; Fiala et al., 1986
Methanococcus jannaschii	21°N smoker wall	50-86	Jones et al., 1983
Methanococcus strain AG86	Guaymas sediment	48-92	Zhao et al., 1988
Desulfurococcus strain S	11°N smoker wall	50-94	Jannasch et al., 1988
Desulfurococcus strain SY	11°N smoker wall	50-96	Jannasch et al., 1988
Thermococcales strain ES1	Endeavour polychaete	50-91	Pledger & Baross, 1989
Methanococcus strain CS1	Guaymas sediment	48-94	Jones et al., 1989
Methanopyrus strain AV19	Guaymas sediment	84-110	Huber et al., 1989
Archaeoglobus profundus	Guaymas sediment	65-90	Burggraf et al., 1990
Pyrodictium abyssi	(Guaymas sediment?)	80-110	Stetter et al., 1990; Pley et al., 1991
Thermococcales strain ES4	Endeavour flange solids	66-110	Pledger & Baross, 1991
Thermococcus strain AL1	Endeavour smoker fluid	55-94	Reysenbach & Deming, 1991; Reysenbach et al., 1993
Thermococcus strain AL2	Endeavour flange solids	60-108	Reysenbach & Deming, 1991; Reysenbach et al., 1993
Pyrococcus strain GBD	Guaymas smoker wall	65-103	Jannasch et al., 1992
Unidentified strain GB4	Guaymas sediment	55-92	Jannasch et al., 1992
Thermococcales strain GE5	North Fiji Basin smoker fluid	67-102	Prieur et al., 1992; Erauso et al., 1993

chemoorganotroph, *Staphylothermus marinus*, was already known from a shallow marine environment at that time (ZILLIG et al., 1982) but was not discovered at deep-sea vents until later (FIALA et al., 1986). In Table 1, the reports on *M. jannaschii* and *S. marinus* are placed in chronological order with other reports of hyperthermophiles isolated from hot deep-sea vent environments for historical context. In spite of the diversity of investigators now seeking such isolates from smoker environments and their rationales behind choice of culturing media and thermal conditions (now more biotechnological than ecological), an upward trend in the upper temperature limit for growth is apparent (Table 1). This seems particularly true for the methanogens, the upper temperature limit having increased from 86 to 110°C during a six-year period of investigation. Some of the upper temperature limits indicated in Table 1 are, in fact, conservative values in that the strains actually grow to several degrees above the limits reported, although with less reproducibility and with signs of heat-shock response (PLEDGER and BAROSS, 1991; HOLDEN and BAROSS, 1993). Apart from us (we believe 150°C is a conservative prediction), most of the key investigators studying hyperthermophiles believe that the ultimate temperature limit may lie somewhere in the vicinity of 150°C (BROCK, 1985; KARL et al., 1988; STETTER et al., 1990). Based on recent research directions, all of us appear to recognize that tests for biological activity above 115°C must incorporate elevated pressure as an essential factor in the experimental design.

To this end, a number of pressurized microbial culturing systems tolerant of very high temperatures have been developed (reviewed by KELLY and DEMING, 1988, and BAROSS and DEMING, 1993). Some of these systems were designed for the application of hydrostatic (liquid) pressure (YAYANOS et al., 1983; DEMING and BAROSS, 1986; JANNASCH et al., 1988; BAROSS and DEMING, 1993; PLEDGER et al., 1993), while others were intended for hyperbaric (gas) pressure (BERNHARDT et al., 1987; MILLER et al., 1988; NELSON et al., 1991). Following the prediction that use of hydrostatic pressure would be the more fruitful avenue of ecological research for microorganisms indigenous to the deep sea, even though hyperbaric pressures might be more amenable to large-scale chemical engineering designs (DEMING, 1986), the first study comparing hydrostatic and hyperbaric effects on a deep-sea hyperthermophile (strain ES4; Table 1) was undertaken. The results dramatize the importance of mode of pressurization: hydrostatic pressure tripled the growth rate of ES4 at 95°C compared to hyperbaric pressure (NELSON et al., 1992).

In Table 2, we summarize the known growth responses of hyperthermophilic, heterotrophic deep-sea microorganisms to a range of hydrostatic pressures. Except for strains ES4 and SY, the responses at optimal growth temperature (determined at <10 atm pressure) indicate strong barotolerance or barophily, where barotolerance is defined as no effect of elevated pressure relative to low pressure and barophily, as preferential rate enhancement at elevated pressure. Without exception, the responses at supra-optimal growth temperatures are barophilic or obligately barophilic, the latter meaning

Table 2. Growth responses[a] of deep-sea hyperthermophilic heterotrophic[b] microorganisms to deep-sea hydrostatic pressures at optimal[c] and supra-optimal growth temperatures.

Isolate[e]	Temp (°C)	Incubation pressure (atm)[d]					References
		110[f]	220[f]	330	440	660	
AL1	90	+	=	=	-		Reysenbach & Deming, 1991
AL2	100	=	=	=	+		Reysenbach & Deming, 1991
ES1	85		-		-	-	Pledger, 1992; Pledger, Crump & Baross, 1993
	91		+		+	-	
	93		+				
ES4	89		-		-		Pledger, 1992; Pledger, Crump & Baross, 1993
	99		+		-		
	101		+		+		
	103		+		+		
GE5	95		+				Prieur et al., 1992; Erauso et al., 1993
	100		+		+		
	105		+		+		
	108		+		+		
GBD	95	=	+				Jannasch et al., 1992
	100	+	+				
	102	+	+				
	104	+	+				
GB4	88	=	+				Jannasch et al., 1992
	90	+	+				
	92	+					
SY	90	-	-				Jannasch et al., 1992
	94	-	-				
	96	+	+				

[a] Barosensitive (-; test pressure reduced growth rate relative to lower pressures); barotolerant (=; growth rate unaffected by test pressure); barophilic (+; growth rate stimulated by test pressure); obligately barophilic (+; growth enabled by test pressure; i.e., no growth at lower pressures, all other conditions being equal); no test made (blank space).
[b] Growth responses of hyperthermophilic methanogens have been shown to be barophilic (Miller et al., 1988; Jannasch et al., 1992).
[c] First incubation temperature listed for each isolate; determined at < 10 atm.
[d] Seafloor pressure for strains AL1, AL2, ES1 and ES4 was 220 atm; for GE5 and GBD, 200 atm; for SY, 250 atm.
[e] See Table 1 for additional isolate information.
[f] Jannasch et al. (1992) tested 100 and 200 atm, not 110 and 220 atm.

that the application of hydrostatic pressure is required for growth at the higher temperatures (see bold symbols in Table 2). Similar studies of two deep-sea hyperthermophilic methanogens have shown them to be barophilic as well (MILLER et al., 1988; JANNASCH et al., 1992).

YAYANOS (1986) has argued on the basis of pure culture work with psychrophilic (cold-loving) barophiles that the optimal growth pressure for a deep-sea microorganism significantly defines its preferred habitat depth. The same argument has been put forward for thermophilic barophiles (DEMING and BAROSS, 1986; REYSENBACH and DEMING, 1991). The information compiled in Table 2 helps to clarify two points in this regard: (1) limiting studies of pressure effects to seafloor pressures (JANNASCH et al., 1992) does not give a complete picture of habitat preference for hyperthermophilic vent bacteria; and (2) in studies where a wider range of pressures has been examined (PLEDGER, 1992; PLEDGER et al., 1993; PRIEUR et al., 1992; ERAUSO et al., 1993), the higher the test temperature, the greater the pressure required to achieve optimal growth. Strains ES1, ES4, and GE5, originally derived from smoker samples on the seafloor at 2000–2200 m depth (200–220 atm pressure), all demonstrate a capability if not preference for significantly greater hydrostatic pressure (440 atm) than that encountered at the seafloor. It is not difficult to model isotherms beneath an actively discharging sulfide structure and identify potential subsurface habitats for these microorganisms (BAROSS and DEMING, 1993).

The prediction that elevated hydrostatic pressures would enable the growth of deep-sea hyperthermophiles, at temperatures well above their limit at atmospheric pressure, (BAROSS et al., 1982) enjoys only modest support at this time. There seems to be little question that elevated pressure increases the upper temperature limit for growth by several degrees (true for all deep-sea strains examined, all other growth conditions being equal; see data in Table 2), but dramatic increases have not been observed among the isolates currently maintained in pure culture. From 1984 dive operations (Table 3), we obtained mixed cultures of hyperthermophiles whose upper temperature limit for growth was increased to 120°C by virtue of applied hydrostatic pressure (220 atm; DEMING and BAROSS, 1986). However, no higher temperatures or pressures were tested at the time; instead, we focused our efforts on purifying the cultures in response to reviewer concerns that 120°C would not be widely accepted on the basis of mixed culture work. The cultures resisted purification through many (unpublished) trials and approaches and eventually failed to subculture at all. This experience (along with our earlier work; BAROSS and DEMING, 1983; BAROSS et al., 1984) reinforces our belief that "superthermophilic" microorganisms do inhabit pressurized smoker environments but that novel approaches will be required to obtain them in sustainable culture.

SAMPLING CONSIDERATIONS

Sampling deep-sea smoker fluids, especially from a microbiological perspective, is not a trivial exercise. Some of the difficulties and limitations are discussed by KARL et al. (1988) and STRAUBE et al. (1990); recent developments in sampling equipment and protocols are reviewed by BAROSS and DEMING (1993). The basic unit of sampling equipment for fluids is a titanium (Ti) syringe with an intake pipe that is inserted directly into the orifice of the smoker (VON DAMM et al., 1985). The primary difficulty in sampling is obtaining "pure" smoker fluids undiluted by surrounding seawater; i.e., uncontaminated by microorganisms present in seawater. This can happen in two ways. Some smoker structures experience "natural" seawater intrusion as a result of porous wall materials or shallow subsurface entrainment. Such smokers are easily identified by the reduced temperature (significantly below 345°C) of the fluid emerging from the smoker orifice. Other smoker structures with nonporous walls, internally cemented by chalcopyrite minerals, emit fluids of consistently high temperatures (>345°C). These smokers are believed to be direct conduits to a much deeper hydrothermal system underlying the seafloor by tens of meters to several kilometers. However, collections of these superheated fluid emissions can still become contaminated with seawater as a result of the technical problems of sampling by submersible; e.g., inadvertent movement of the submersible or its manipulator arm during sample collection causing the intake pipe to shift from the stream of hydrothermal emissions into seawater.

The technical problems causing seawater entrainment have been reduced significantly by the use of a manifold sampling system that accomodates multiple Ti syringes and temporally couples sample intake with fluid temperature (MASSOTH et al., 1989b). The sampling process is monitored and filmed from the submersible while temperatures at the intake and flushing ends of the system are recorded and displayed continuously by computer. The trigger to capture samples is not released until the intake pipe and sampling chambers have flushed thoroughly with the smoker fluids and the system temperature has reached equilibrium with emission temperature at the smoker orifice. Ultimately, and regardless of the source of seawater contamination (entrained naturally through porous structures above or below seafloor or technically at sample intake), it is possible to assess the amount of seawater entrainment as a linear function of Mg content (pure hydrothermal fluids are Mg free; ambient seawater contains approximately 53 mmol kg^{-1}; EDMOND et al., 1979) or a number of other geomarkers (e.g., Si).

Table 3. Studies of deep-sea smoker fluids (>140°C) that address the question: are smoker fluids carriers of heat-tolerant microorganisms from a deep subsurface zone?[a]

Submersible cruise	References	Type of smoker sampler[b]	No. of smoker samples	Authors' conclusions
1979, 21°N (*RISE*)	Baross et al., 1982; 1984; Baross & Deming, 1983	Ti syringe	8	yes
1982, 21°N (*OASIS*)	Karl et al., 1984	smoker poker	1	no
not stated	Jannasch & Mottl, 1985	not stated	not stated	no
1984, 13°N (*BYOCYARISE*)	Deming, 1987	Ti syringe	2	yes
1984, Endeavour Segment (JFR)[c]	Deming & Baross, 1986	Ti syringe	3	yes
1987, 13°N (*HYDRONAUT*)	Floury et al., 1993; Deming, unpubl.	Ti syringe P-retaining Ti sampler	1 3	maybe[d]
1988, Guaymas Basin	Karl et al., 1988	Ti syringe smoker poker vent cap	3 2 2	no
1988, Endeavour Segment, JFR	Deming et al., 1989; Straube et al., 1990	Ti syringe[e] & Ti incubator	29[f]	yes
	Pace & Reysenbach, unpubl.	vent cap	2	N.A.[g]
1991, Endeavour Segment, JFR	Baross, unpubl.	Ti syringe[e]	9	N.A.
1991, 9°N	Deming, unpubl.[h]	Ti syringe[e]	28	N.A.
1992, Japan Trench	Pace & Reysenbach, unpubl.	vent cap	1	N.A.

[a] See Table 1 for studies leading to isolations of novel hyperthermophilic microorganisms.
[b] Descriptions of titanium (Ti) samplers, smoker poker and vent cap are reviewed by Baross and Deming (1993).
[c] Juan de Fuca Ridge in the NE Pacific.
[d] Results considered inconclusive.
[e] As part of an improved manifold intake system (see text).
[f] Includes 5 flange samples (Baross et al., 1989; Straube et al., 1990).
[g] Not available; conclusions not published or samples not analyzed yet.
[h] Samples collected by M.D. Lilley.

A second difficulty in collecting "uncontaminated" smoker fluids concerns the intake of material inadvertently knocked off the smoker walls during placement of the intake pipe. If smoker walls were sterile, this would not pose a problem to the question of determining microbial content of smoker fluids. However, external portions of some smoker walls have been shown to harbor significant numbers of microorganisms (Baross and Deming, 1985; Jannasch and Mottl, 1985; Juniper and Fouquet, 1988), some of which are hyperthermophilic (Table 1). A means to assess the amount of external wall material entrained in a fluid sample has not been developed; an analysis of total solid material in the sample is not helpful, since sulfides precipitate from "pure" smoker fluids upon cooling. Fortunately, use of the manifold sampling system greatly reduces the chance of inadvertent intake of solid materials as well as seawater. If the best sampling devices are used successfully (smoker fluids captured in a Ti syringe at emission temperature and subsequent chemical analyses prove insignificant seawater entrainment), then the potential sources of microorganisms in a smoker sample are limited to internal smoker wall surfaces or the largely uncharacterized (except theoretically; e.g., Norton, 1984) fracturing zone beneath the vent field through which the smoker fluids rise.

If microbial contamination has occurred, for whatever reason and regardless of specific peripheral source, then the contaminants must survive exposure to the extreme conditions of smoker fluids to be detected as "false positives" in the search for heat-tolerant microorganisms flushed from deep subsurface habitats. Little is known about the ability of microorganisms of any thermal class (psychrophiles to hyperthermophiles) to survive typical smoker temperatures and pressures. It is probably safe to assume that organisms normally inhabiting cool environments (ambient seawater and surfaces in the vent field) are not able to survive even brief exposure to the known range of smoker temperatures (140–420°C). Jannasch et al. (1992) tested several hyperthermophilic strains for survival at elevated temperatures under 200 atm hydrostatic pressure and found that none survived above 120°C. On the other hand, when a different vent hyperthermophile, strain AL1, was first allowed to acclimate to 110°C (above its normal range for growth), survival was observed at 150°C under 220 atm hydrostatic pressure (though not at <10 atm pressure) for periods of minutes to hours (Reysenbach and Deming, 1990). The latter is suggestive of heat-shock response in which the organism produces heat-protective proteins upon exposure to temperatures above its growth range, as has been documented for thermophiles derived from shallow or terrestrial environments (Trent et al., 1990; Phipps et al., 1991) and for the deep-sea hyperthermophile ES4 from Endeavour Segment (Holden and Baross, 1993). It may be significant that the heat-intolerant strains tested by Jannasch et al. (1992) were isolated from collections of peripheral smoker wall material or sediment originally at moderate temperatures (<120°C), while strain AL1 was isolated from a sample of smoker fluid originally at 350°C. (The original temperature of the flange solids from which ES4 was isolated, though potentially very high, is not known; Baross et al., 1989; Pledger and Baross, 1991).

Prior to the Fall of 1988, all attempts to sample smoker fluids for the purpose of evaluating microbial content were dependent on very limited sample sizes (typically 1–3 samples) from sporadic cruise opportunities (Table 3), sometimes in the absence of any illuminating geochemical or temperature measurements. These limited studies produced contradictory conclusions regarding the question of smoker fluids as carriers of heat-tolerant microorganisms from a deep subsurface zone (Table 3). Those investigators offering "no" to the question always invoked sample contamination to explain the presence of any microorganisms in their smoker samples and, by inference, in others. To better address the issue of contamination and the larger question at hand (the hypothesized subsurface biosphere), we proposed and obtained a submersible cruise to the Endeavour Segment of the Juan de Fuca Ridge dedicated in large part to a systematic microbial-geochemical sampling of as many smoker fluids as possible. We hoped to obtain enough information to assess the subsurface biosphere question from statistically, geochemically, and geographically sound perspectives. We were fortunate in the timely development of the manifold sampling system described earlier so that the best possible (least contaminated) collections of smoker fluids could be obtained on the cruise. We also tested a new Ti incubator, designed to capture smoker fluids and hold them at in situ temperature and pressure for a period of minutes to hours, which yielded four excellent smoker samples (Deming et al., 1989; Baross and Deming, 1993). Some of the microbial analyses of the samples collected on this cruise have already been examined statistically and published: DNA extracted from the particulate fraction (pDNA) in Deming et al. (1989), Straube et al. (1990), and Baross and Deming (1993); and microscopic counts in Baross and Deming (1993). Others await funding support for completion (e.g., lipid analyses and DNA sequencing).

With the availability of geochemical and thermal analyses in parallel with microbial data (pDNA), obtained from more smoker samples than the sum of all those collected in previous studies (Table 3), it became clear that the same microbial methods that failed to detect microorganisms in some smoker fluids (in keeping with the "no" conclusions in Table 3) succeeded in detecting levels in other smoker fluids as much as 56 times higher than those found in surrounding seawater (Straube et al., 1990; in keeping with the "yes" conclusions in Table 3). We concluded from earlier analyses of these data (Straube et al., 1990) that the microbial content of emissions from a given smoker must be more dependent on the geochemical nature and history of the smoker itself than on specific sampling problems (entrainment of contaminants). This idea is explored further in the next section as we re-evaluate the pDNA data in light of new information, and will be addressed in the future via analyses of additional large sample sets from more recent cruises (Table 3).

THE ENDEAVOUR MODEL

The Endeavour Segment of the Juan de Fuca Ridge as it appeared during the *Alvin* dives of 1988 is shown in Fig. 1, a simplified version of the more detailed geologic map published by Delaney et al. (1992). The vent field is characterized by a series of large sulfide mounds that have formed along a northeast to southwest geographic gradient parallel to fault lines and depth contours. The mounds, colorfully named by their mappers, can be ordered from northeast to southwest along this gradient as follows: Hulk (HU), Crypto (CR), Dante (DA), Lobo (LO), Grotto (GR), and Peanut (PN). Each mound is topped by one or more black smokers actively discharging superheated hydrothermal fluids.

The smokers sampled for pDNA in 1988 (Fig. 1) can be distinguished according to the temperature of their emission fluids. The hottest fluids (345–357°C) were discharging from four of the smokers (8A, 16, 9, and 8E), which therefore represent the most direct conduits to the deep subsurface fracture zone. To put bounds on potential contamination of these "pure" hydrothermal fluids during sampling, ambient seawater samples were collected in the vicinity of three of the smokers (especially 16; see Fig. 2) between LO and GR. Lower temperature fluids (175–341°C) were discharged by nine of the smokers (N8F, 36, 8F, 86, APL, 12, 14, N8A, and N16), indicating entrainment of some cooler waters either through porous wall structures or in subsurface mixing zones. The remaining smoker (98) was anomalous for its extreme variability in temperature (dropping 47°C between samplings, from 221 to 174°C) and flow rate (JAB, pers. observation); all other smokers[†] were stable, discharging fluids at constant temperature and flow rate. Smoker 98 was also anomalous by location, being the only smoker to have formed in a faulted zone (west of GR) instead of atop a sulfide mound (Fig. 1). *Alvin* dives to the Endeavour Segment

[†] No temperature measurements were made at smoker N16.

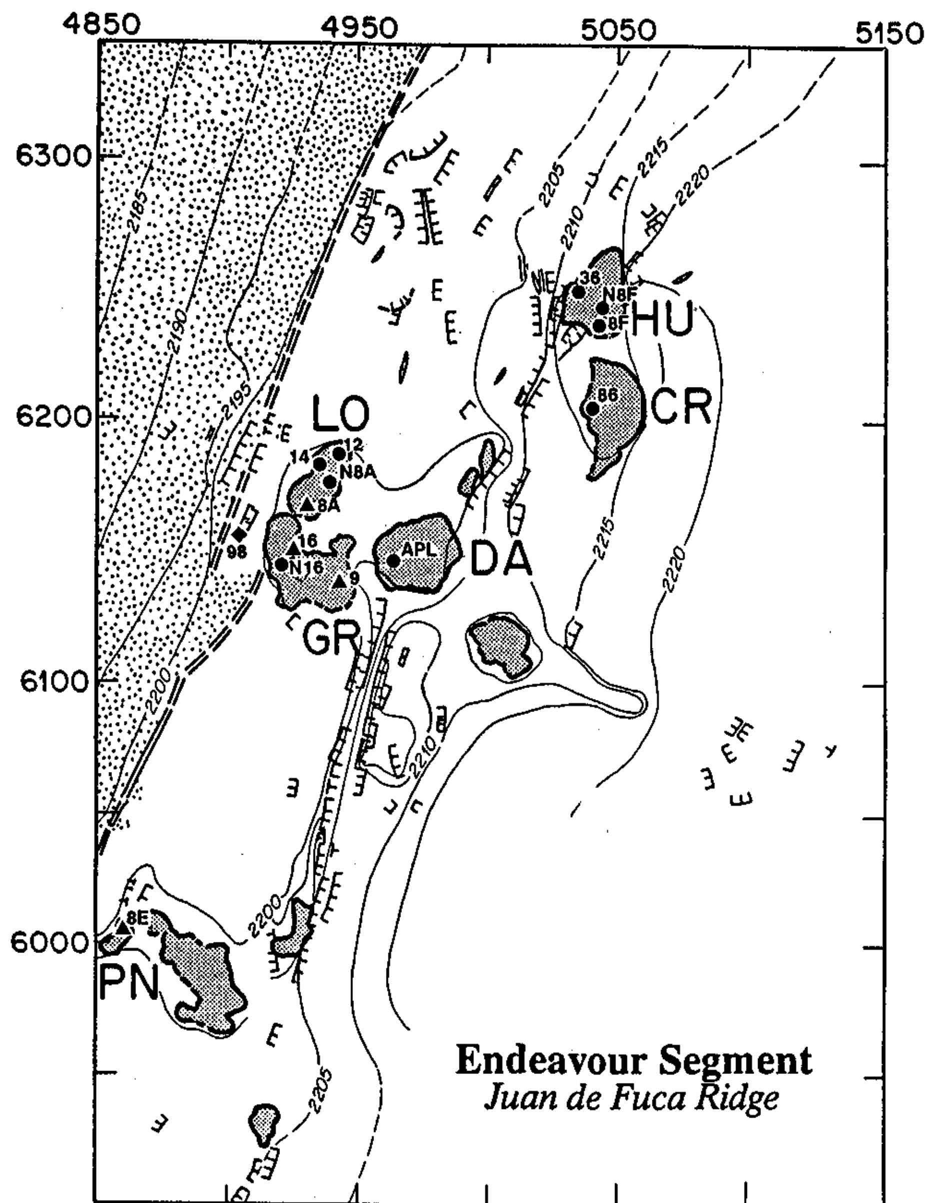

FIG. 1. Geologic map of the Endeavour vent field on the Juan de Fuca Ridge at 47°57′N, 129°06′W, modified from DELANEY et al. (1992) and LILLEY et al. (1993). Sulfide mounds with active smokers sampled in 1988 for microbial analyses are designated with abbreviations of names given in DELANEY et al. (1992): HU for Hulk, CR for Crypto, DA for Dante, LO for Lobo, GR for Grotto, and PN for Peanut. Solid circles and triangles mark locations of the smokers sampled; circles indicate emission temperatures at <345°C; triangles, 345°C or higher (see Fig. 4). Note smoker 98 (solid diamond), anomalous for its location in a faulted zone (west of GR) rather than atop a mound, for instability of temperature (221–174°C) and flow rate, and for its demise prior to return dives at the site in 1991 (J. A. Baross, pers. observation). Axes and depth contours in meters; scale, 11,250.

in 1991 confirmed that all but one of the fourteen smokers sampled in 1988 were still actively discharging fluids at temperatures similar to those recorded previously (J. A. Baross and M. D. Lilley, pers. observations). Smoker 98 no longer existed; the site of its original location west of GR had transformed into a diffuse hydrothermal flow field.

The sampling strategy of the 1988 cruise included a survey of the emission fluids from the fourteen smokers shown in Fig. 1 (one sample each, except for smokers 36, 98, and 9, which were sampled two, three, and four times, respectively) and repeated sampling in and around the best candidate for a direct conduit to a deep subsurface zone, smoker 16 (Fig. 2). Methods and other particulars concerning the pDNA measurements made on the various samples are provided in DEMING et al. (1989) and STRAUBE et al. (1990), published prior to the availability of the geological information shown in Fig. 1 and many of the geochemical analyses addressed in the following text. STRAUBE et al. (1990) applied two DNA

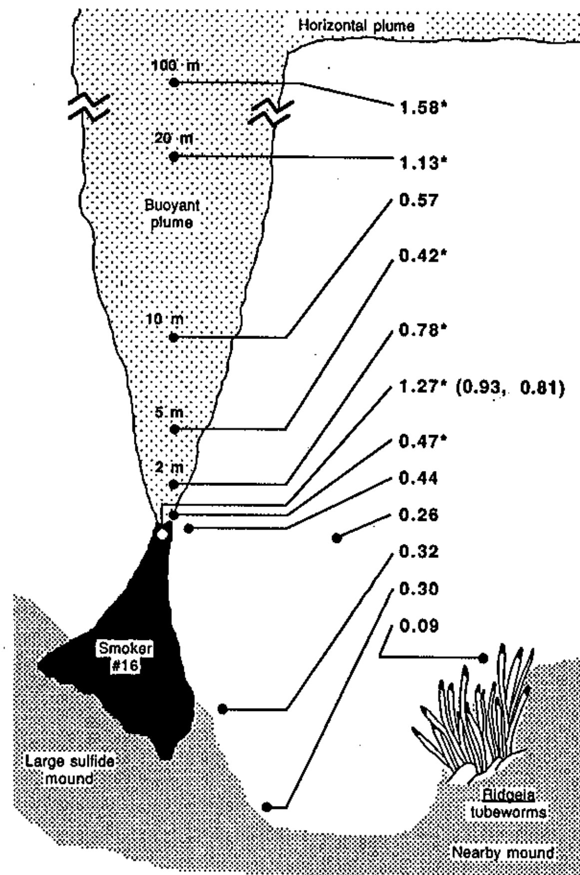

FIG. 2. Comprehensive sampling plan at smoker 16 (adapted from Fig. 1 in STRAUBE et al., 1990), showing smoker pDNA concentrations (corrected for seawater input as described in the text using data from Table 3 in STRAUBE et al., 1990) and amount of pDNA in ambient seawater (lower 5 values; from Table 1 in STRAUBE et al., 1990). Note highest values from interior of smoker orifice and in distant plume where secondary microbial populations were encountered (DE ANGELIS et al., 1993). Values in parentheses (from Table VI in BAROSS and DEMING, 1993) were obtained using a Ti incubator (see text). Asterisks indicate pDNA samples amplified by polymerase chain reaction using universal 16S rRNA primers for subsequent DNA sequencing (P. S. Kessler and J. W. Deming, unpubl. data).

extraction techniques to each sample to maximize DNA recovery from potentially recalcitrant microorganisms. In this paper, we use the higher amount of DNA recovered for each sample (nevertheless a conservative value, since neither extraction procedure was completely efficient) and refer to that as "total pDNA." "Smoker pDNA" refers to total pDNA reduced by the probable maximal input of seawater microbial DNA, calculated from percent seawater in the sample (determined by geomarker content) and the highest amount of pDNA extracted from the ambient seawater controls. By making the (unlikely) assumption that all entrained "contaminant" organisms survived exposure to superheated temperatures, we arrive at conservative estimates of smoker pDNA; i.e., DNA attributable to the hypothesized heat-tolerant microorganisms from a deep subsurface zone.

In STRAUBE et al. (1990), smoker samples were analyzed on an individual basis to assess the problem of seawater contamination as reflected by Mg content. At that time, it was not possible to analyze two of the smoker samples (from 9 and 98) in this way due to lack of Mg measurements. The latter have since been obtained (BUTTERFIELD, 1990), allowing us to plot all of the total pDNA measurements obtained from the 1988 smoker samples against percent entrained seawater (Fig. 3). This analysis reveals two key points relevant to an assessment of smoker pDNA as evidence for a subsurface biosphere.

First, unlike some vent fields (e.g., Guaymas Basin, Galapagos, 9°N), ambient seawater near the Endeavour smokers was not loaded with free-living or free-floating mats of bacteria (see seawater values in Fig. 3). Total pDNA values for the five ambient seawater samples, including samples taken a few centimeters from animal colonies and sulfide wall structures (Fig. 2), were uniformly low (Fig. 3). Thus, the maximal amount of pDNA in smoker samples that could be attributed to ambient seawater (100% entrainment of seawater into the Ti syringe) was 0.32 ng mL^{-1}. In contrast, Guaymas vent fields are notorious for pervasively high densities of free-floating bacteria and thick microbial mats (JANNASCH et al., 1989); it is here that KARL et al. (1988) sampled three smokers and (logically) projected a positive relationship between seawater entrainment and measures of the microbial content of smoker samples.

Second, there is no predictable relationship between total pDNA extracted from Endeavour smoker samples and seawater content (Fig. 3), indicating the inadequacy of seawater entrainment as an explanation for smoker pDNA content. In fact, the total pDNA values for five samples of smoker 16 fluids showed an inverse relationship with seawater content (Fig. 3), with the poorest sampling effort (99% seawater entrained at the sampling intake point) yielding the lowest amount of pDNA. Thus, at this smoker, seawater entrainment at the orifice appeared to dilute the amount of pDNA, not (falsely) increase it through the introduction of intact microorganisms from surrounding seawater. This is all the more striking since, of the smokers sampled, smoker 16 was the least porous structure emitting the hottest of fluids (357°C). Neither of the other stable smokers that were sampled more than once (9 at 350°C and 36 at 289°C) provided any clear evidence that seawater entrainment increased pDNA values (Fig. 3).

The results from smoker 98, with its uniquely unstable temperature and flow characteristics, present a different picture. The smoker was sampled on consecutive days: once on the first day, using the manifold system which verified a temperature of 221°C; and twice on the second day, using Ti syringes independently of the manifold system. On the second day, the *Alvin* temperature probe recorded 174°C within the smoker orifice, indicating a substantial natural infusion of cooler waters from a subsurface mixing zone. Low pDNA levels comparable to surrounding seawater were measured in the first two samples, which in fact contained 57 and 81% seawater by Mg content. The third sample, comprised of just 10% more seawater than the second, contained ten times the amount of pDNA measured in ambient seawater and thus the highest total pDNA concentration (3.1 ng mL^{-1}; Fig. 3) of the entire study (DEMING et al., 1989; STRAUBE et al., 1990). We cannot exclude the possibility that wall material

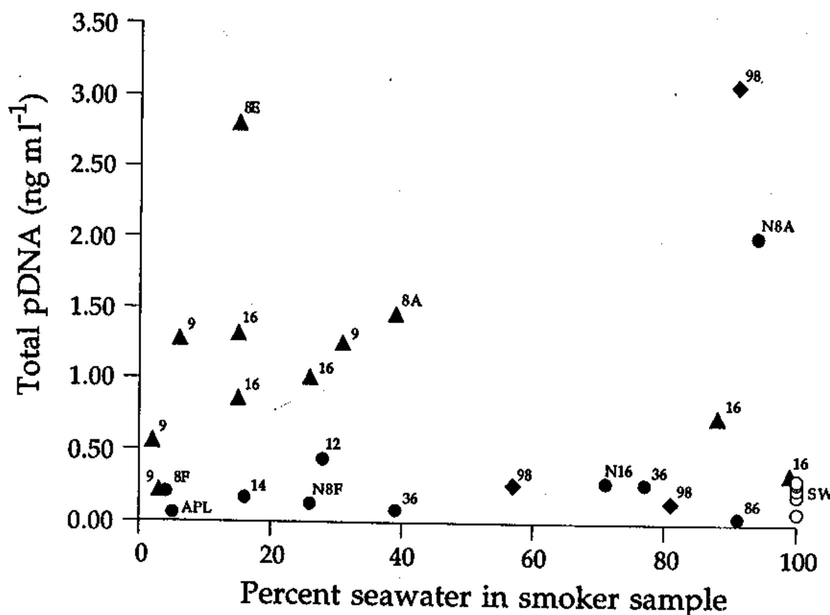

FIG. 3. Absence of relationship between total pDNA concentration and seawater content, determined by Mg content, in samples of emission fluids from 14 Endeavour smokers (data from Tables 1 and 3 in STRAUBE et al., 1990, and Table VI in BAROSS and DEMING, 1993). Solid symbols indicate smoker samples: circles, emission temperatures at <345°C; triangles, 345°C or higher; diamond, anomalous smoker 98 (221–174°C). Open circles indicate ambient seawater.

and associated microorganisms were inadvertently collected in the third sample (accounting for the high value), but no evidence of this was apparent from observations made during sampling. Furthermore, the results of a "vent cap" deployment on smoker 98 at the end of the cruise (N. R. Pace and A. L. Reysenbach, unpubl. data) suggest that the high pDNA value was not a freak measurement; solid surfaces positioned in the stream of the emission fluids (then measuring 140°C as they emerged from the top of the vent cap) were heavily coated with microorganisms upon recovery (J. W. Deming and J. A. Baross, pers. observations). Thus, we offer an alternate explanation for the shift from low to high smoker pDNA during the sampling period that considers the impending "death" of smoker 98; i.e., that a rapid shift in flow path occurred in the mixing zone subsurface to site WGR, exposing a previously stable and rich zone of heat-tolerant microbial inhabitants to hydrothermal flushing. That other Endeavour smokers with lower temperatures and chemical indicators of subsurface mixing (e.g., those on HU and CR) were not releasing high levels of pDNA may be related to the long-term stability (>3 y) of their flow paths.

In Fig. 4, all of the available smoker pDNA values (now corrected for seawater input, as described earlier) are plotted against emission temperature. With the single exception of the third sample from smoker 98, the pattern is unmistakable; the highest smoker pDNA values were obtained from smokers with hotter fluids more directly tapping deep subsurface zones, while the lowest values were measured in lower temperature smokers (significance of two-tailed Spearman's rank order correlation coefficient = 0.02, n = 22 [all data]; 0.001, n = 21 [high smoker 98 value omitted]; 0.005, n = 19 [all 98 values omitted]). Clearly, the prediction that lower temperature smokers (e.g., Endeavour smokers 86, 36, N8F, 8F) should contain greater numbers of microorganisms (KARL et al., 1988) is not verified by this data set (Fig. 4).

The results from close sampling in and around the hottest smoker 16 (Fig. 2) also contradict the notion that pure hydrothermal fluids emerging from nonporous smokers are initially sterile, entraining detectable numbers of microorganisms only at some distance (in centimeters) above the orifice (KARL et al., 1988). The amount of smoker pDNA in the purest samples taken centimeters within (down into) the smoker orifice were about three times higher than those observed not only in surrounding ambient seawater but also in the sample tripped at the lip of the smoker orifice (Fig. 2), an intake point most likely to entrain smoker solids. Concentrations actually decreased in the rising plume to background seawater levels before increasing at altitudes of 20 m and higher (Fig. 2), where secondary microbial populations were encountered (DE ANGELIS, 1989; DE ANGELIS et al., 1993).

As chemical oceanographers completed their analyses of Endeavour smoker fluids from the 1988 cruise, we began to search for a broader geophysical or geochemical understanding of the microbial parameters than had been possible earlier (STRAUBE et al., 1990). In particular, we examined microbial parameters from a geographic perspective and in relation to chloride measurements, converted to endmember (undiluted hydrothermal) values from data in Table B-1 of BUTTERFIELD (1990). Ordering the pDNA analyses and available microscopic counts (BAROSS and DEMING, 1993), according to sulfide mound occurrence on the NE–SW geographic gradient of the region, revealed a strong trend towards microbially rich smoker samples in the SW (Fig. 5). It also revealed a

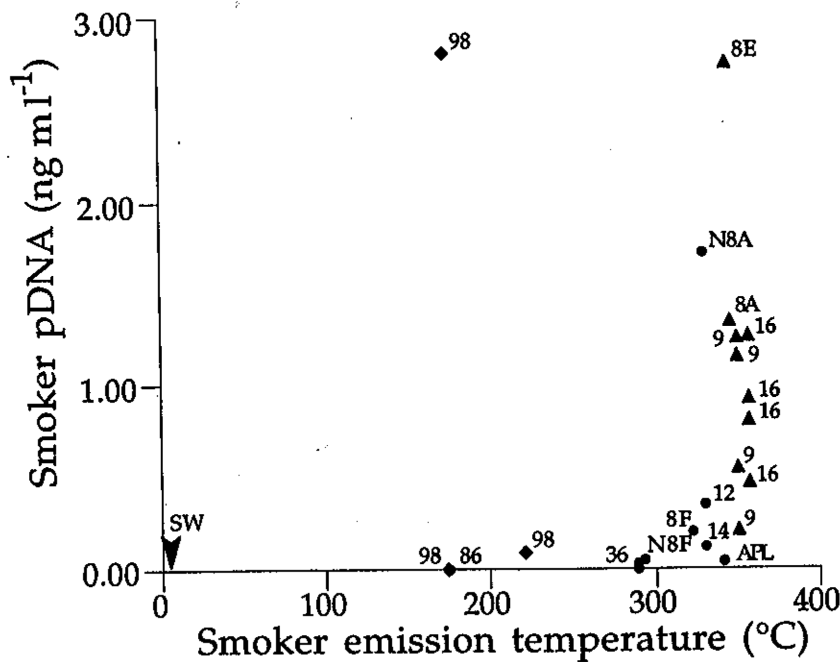

FIG. 4. Smoker pDNA concentrations (corrected for seawater input as described in text using data from Tables 1 and 3 in STRAUBE et al., 1990, and Table VI in BAROSS and DEMING, 1993) in samples of emission fluids from 13 Endeavour smokers as a function of emission temperature (see text for statistical analyses). N16 does not appear in the figure because no temperature measurement was made. Symbols are the same as in Fig. 3; arrow indicates seawater (SW) temperature.

positive relationship between pDNA and microbial counts (by linear regression analysis, $P = 0.074$, $r^2 = 0.708$, $n = 5$). Regressing smoker pDNA against endmember chloride concentrations revealed a significant inverse relationship ($P = 0.0005$, $r^2 = 0.723$, $n = 12$; chloride measurements were not available for all pDNA samples) and the segregation of

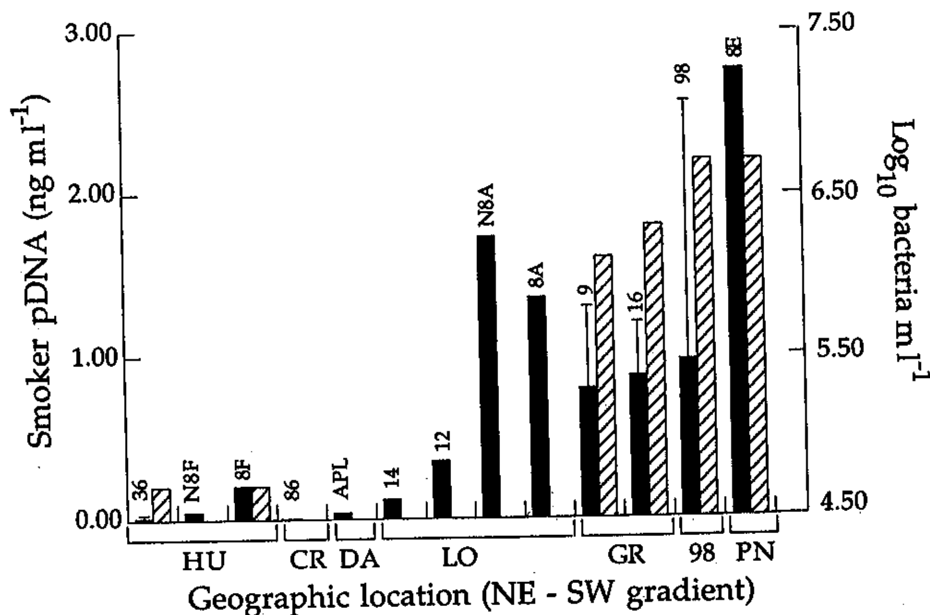

FIG. 5. Smoker pDNA concentrations (as in Fig. 4) and bacterial counts by epifluorescence microscopy (from BAROSS and DEMING, 1993) per smoker, ordered according to mound location along the NE–SW geographic gradient (see Fig. 1 for mound abbreviations and locations). Error bars indicate standard deviation of the mean for smokers sampled more than once (for smokers 36, 9, 16, and 98, n = 2, 4, 5, and 3, respectively).

mounds along the geographic gradient according to northeast (HU, DA, and CR), central-west (GR and LO), and southwest (PN) regions (Fig. 6).

BUTTERFIELD (1990) had already determined from his complete set of measurements that endmember chloride concentrations decreased fairly linearly from 506 mmol kg^{-1} in the northeast section of the Endeavour Segment to 255 mmol kg^{-1} in the southwest portion. Any degree of chloride depletion in smoker fluids from the seawater level of 547 mmol kg^{-1} is considered evidence for two-phase separation (segregation into brine and vapor phases; boiling) in a deep subsurface zone (DELANEY and GOLDFARB, 1987; MASSOTH et al., 1989a; BISCHOFF and ROSENBAUER, 1989; BUTTERFIELD, 1990). Finding variable degrees of two-phase separation along a gradient within one vent field was unprecedented, nor are there any geophysical models to explain the observed trend on this scale. BUTTERFIELD (1990) suggested that each Endeavour mound caps a unique hydrothermal source and that the southwest portion of the Endeavour Segment experiences deeper, more dynamic flushing of the subsurface by its phase-segregated source fluids. The inverse relationship between endmember chloride and smoker pDNA suggests to us that deeper subsurface boiling provides for more extensive and dynamic flushing of subsurface habitats, thereby releasing higher levels of microorganisms in chloride-depleted smoker fluids.

The latest chemical analyses from the 1988 cruise (LILLEY et al., 1993) documented ammonium and methane concentrations in the smoker emissions that are unusually high for a vent field not overlain by organic-rich sediments (like Guaymas Basin). These findings led LILLEY et al. (1993) to postulate that the Endeavour vent field may be underlain by a vast source of organic material. Of the alternative hypotheses considered, the most parsimonious explanation of the data is a vast layer of subsurface sediments. The possibility of subsurface methanogenic microorganisms contributing to the methane emissions observed could not be excluded, but more research on stable isotope partitioning by hyperthermophilic methanogens is required to settle the issue. In keeping with the microbial parameters discussed in this paper, we offer the explanation that at least some portion of the vast source of organic material required to explain the ammonium and methane data in LILLEY et al. (1993) is, in fact, a living biomass of heat-tolerant microorganisms.

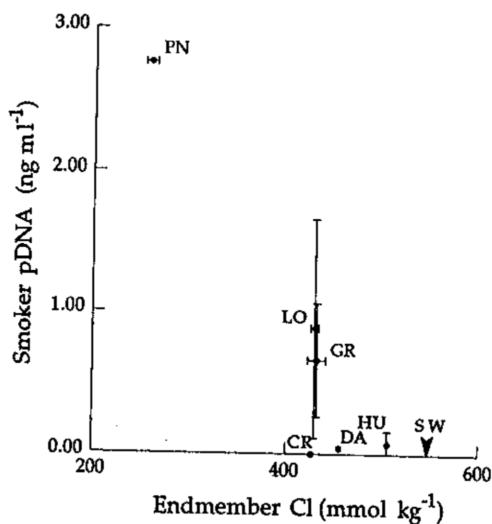

FIG. 6. Inverse relationship between smoker pDNA (as in Fig. 4) and endmember chloride concentrations (calculated from data in Table B-1 in BUTTERFIELD, 1990) in Endeavour smoker fluids, according to sulfide mound location on the NE–SW geographic gradient (see Fig. 1). Error bars indicate standard deviation of the mean value of measurements from a given mound. Linear regression analysis of the subset of smoker samples for which both measurements were available confirmed the significance of the inverse relationship ($P = 0.0005$, $r^2 = 0.723$, $n = 12$). Chloride analyses were not available for pDNA samples from smokers N8A or N16; smoker 98 is not included due to incomplete chloride data as well as its anomalous off-mound location. Arrow indicates chloride concentration in ambient seawater (SW).

The proposed existence of a biosphere subsurface to the Endeavour Segment and perhaps elsewhere along seafloor spreading zones could be tested in several ways. The most direct means would be to drill into the subsurface region and sample for heat-tolerant microorganisms, using the deep-sea drilling vessel (see Ocean Drilling Program Leg 139, 1992). We expect definitive tests of the hypothesis to emerge from such work. Related phenomena being explored at terrestrial sites (e.g., under the auspices of the US Department of Energy program on Subsurface Microbiology) may also provide supportive information. Short of direct drilling, however, the use of accessible smoker vents as potential "windows" to a subsurface biosphere could be expanded. Additional efforts could be made to obtain unusually heat-tolerant microorganisms from relatively pure smoker emissions or to screen directly for the presence of known hyperthermophiles using immunofluorescent or genetic probes (HUBER et al., 1990). The stability of hyperthermophiles or potential activity of superthermophiles in smoker fluids could be determined via in situ experimentation (an improved version of our Ti incubator is currently under construction for this purpose). Their presence in cooler diffuse vent fields (e.g., where smoker 98 clogged and died) might signal the location of a subsurface habitat in the absence of smokers. Finally, DNA extracted directly from the purest smoker emissions could be amplified and sequenced to document the presence (or absence) not only of known hyperthermophilic genes but also of novel, potentially superthermophilic sequences (Fig. 2; STRAUBE et al., 1990; P. S. Kessler and J. W. Deming, unpubl. data). Confirmation of the latter, which ultimately will require obtaining superthermophilic microorganisms in sustainable culture, would have significance to evolutionary questions and the origins of life, as well as to the concept of a subsurface biosphere.

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