Distribution of Microorganisms in Deep-Sea Hydrothermal Vent Chimneys Investigated by Whole-Cell Hybridization and Enrichment Culture of Thermophilic Subpopulations

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The microbial community structure of hydrothermal vent chimneys was evaluated by the combined use of enrichment cultures and whole-cell hybridizations with fluorescently labeled 16S rRNA-based oligonucleotide probes. Chimneys were collected during the Microsmoke cruise on the Mid-Atlantic Ridge and were subsampled on board and stored under reduced conditions or fixed. For estimation of culturable thermophiles, selective media were inoculated by dilution series of the samples and incubated at 65, 80, and 95°C. To analyze the microbial diversity of the samples, cells were extracted from the fixed chimney structure samples and hybridized with domain- and kingdom-specific probes. Quantification of the extracted cells was assessed by whole-cell hybridization on membrane filters. By both methods, the largest amounts of microorganisms were found in the upper and outer parts of the chimneys, although even the inner parts contained culturable and detectable amounts of cells. Different morphotypes of thermophilic and hyperthermophilic microorganisms were enriched and detected in samples. Our data clearly indicate that the morphological diversity observed by using whole-cell hybridization is much larger than that assessed by use of culture-based enrichments. This new approach, including culture-independent and -dependent methods to study hydrothermal vent chimneys, showed an uneven distribution of a diverse microbial community. Application of lower-level specific probes for known families and genera within each domain by our approach will be useful to reveal the real extent and nature of the chimney microbial diversity and to support cultivation attempts.

At submarine hydrothermal vent sites, one of the most extensively studied thermal environments are the spectacular black smokers and related sulfide structures. These structures form when metal sulfides precipitate from hot anaerobic fluids on contact with cold oxic seawater. Inside, outside, and across these chimneys, microniches for the occurrence of microbial processes are based on gradients of nutrients and extreme physicochemical conditions (temperatures from 350 to 2°C within a few centimeters, oxygen levels from highly reduced to oxygenated, and fluid velocities from high to low flow rates) (for a review, see reference 19). In the chimney conduits, the conditions are so extreme that the existence of life is unlikely.

Enrichment culture isolation experiments performed with samples collected from deep-sea hydrothermal fluids and chimneys revealed the presence of a physiologically diverse microbial community and led to the characterization of numerous bacterial and archaeal thermophiles (and hyperthermophiles), including both chemolithoautotrophic and chemoorganoheterotrophic strains. Most of the microorganisms known to thrive in the hottest parts of the ecosystem are anaerobes which fall into the domain *Archaea*. Representative species of both *Archaea* kingdoms, the *Euryarchaeota* and the *Crenarchaeota*, have been isolated from deep-sea vents, but the predominant group is composed of the heterotrophic sulfur metabolizers from the order *Thermococcales* (9, 13, 14, 18, 21, 26, 31,

33, 34). The other *Archaea* isolated at great depths so far were a sulfate reducer (5), chemolithotrophic methanogens (22, 23, 27, 42) within the *Euryarchaeota*, and a variety of fermenters within the *Crenarchaeota* (10, 20, 35). The occurrence in deep-sea hydrothermal fluids and chimneys of thermophilic aerobic heterotrophic bacteria from the genera *Thermus* and *Bacillus* was only recently reported (29, 30). However, the major limitation of culture-based techniques is that only a relatively small fraction of the microorganisms making up a natural community can generally be cultured. Consequently, results derived from elective culture studies may be of limited use in microbial ecology.

In addition to the direct isolation of strains from submarine vent environments, attempts have been made to detect their presence by either a biomass index or specific activity measurement. A molecular approach based on fatty acid analysis revealed a heterogeneous distribution of biomass in a chimney flange and resulted in the strongest evidence so far for the existence of a real thermophilic microbial community in sulfide structures (15). Particulate DNA was measured in hot fluids collected from a number of smokers and flanges on the Endeavour vent field (40). By measurements of the microbial reduction of $^{35}\text{SO}_4^{2-}$, Jørgensen et al. (24) found active sulfate reduction in Guaymas Basin sediments, where in situ temperatures of 70 to 130°C were recorded.

Fluorescence-labeled rRNA-targeted oligonucleotide probes have been introduced recently as powerful tools in microbial ecology (1, 2). This technique offers the advantage of circumventing the requirement for cultivation to identify and distinguish microorganisms and allowing their detection in samples either directly (in situ) or by preliminary cell extraction (2). Furthermore, whole-cell hybridization allows quanti-

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fication and identification of microbes from the highest to the lowest phylogenetic levels, which makes it now possible to define in detail the occurrence, the abundance, and the distribution of microorganisms in natural communities. These probes have successfully been applied to the hydrothermal environment, with the localization of endosymbiotic bacteria in host germ tissues (7) and the identification of yet-unculturable filamentous bacteria (37). In preliminary and semiquantitative experiments, the use of domain-specific rRNA probes revealed a ca. 1:10 relationship between *Archaea* and *Bacteria* in samples collected from various locations at Guaymas Basin vent sites, smoker wall, and *Beggiatoa* mats (19).

The Microsmoke cruise on the Mid-Atlantic Ridge (23°N) was organized by the Centre National de la Recherche Scientifique to study the microbial ecology of hydrothermal vent chimneys by using independent approaches. Samples of chimneys were collected and are now being investigated by using culture-based enrichments, lipid analysis, rDNA sequence analyses, and whole-cell hybridizations. We describe here the available results on the distribution of the thermophilic microbial community thriving in these chimneys. The diversity of the thermophilic microbial community was assessed by the combined use of traditional culture-based enrichments of strictly anaerobic thermophiles and whole-cell hybridization of cells extracted from the chimneys with domain- and kingdom-specific fluorescent-oligonucleotide probes based on 16S rRNA.

MATERIALS AND METHODS

Collection of chimney samples. Three beehive structures designated MS06CH, MS15CH, and MS17CH were collected in November and December 1995 from the Snake Pit vent field (23°22'118N, 44°56'984W) on the Mid-Atlantic Ridge at a depth of 3,500 m during the Microsmoke cruise. In contrast with the vertical pipes (black smokers), where a tight axial conduit focuses the flow of ascending fluid at high velocity, producing the black smoke at the top of the chimney, beehive structures are characterized by an axial zone of high porosity that favors diffusion of hot fluids at a low rate from all over their surfaces. For this reason, the beehive structures were termed diffusers (12). By using the port manipulator of the man-operated submersible *Nautille*, these chimneys were placed in the submersible insulated basket for the trip to the surface. Once they were transferred on board, subsampling across the sulfide structures was conducted as aseptically as possible according to three visible concentric mineralogical zones. From the inner to the outer parts of chimneys could be distinguished a pyrrhotite-rich, dark, and highly porous zone (zone 1) surrounded by a less-porous pyrite-marcasite layer (zone 2) and an external wall (zone 3) probably composed of melnikovite (around 1 to 5 mm in diameter) (11).

For further enrichments, chimney subsamples were transferred in 50-ml glass vials and flooded with a sterile solution of 3% (wt/vol) Sea Salts (Sigma Chemical Co., St. Louis, Mo.). The vials were then closed tightly with butyl rubber stoppers (Bellco, Vineland, N.J.), pressurized with N₂ (100 kPa), reduced with sodium sulfide when required, and stored at 4°C until processed.

Samples for whole-cell hybridization studies were fixed overnight in 4% paraformaldehyde in 3× phosphate-buffered saline (PBS) (38). The fixation buffer was then replaced by a mixture of equal volumes of 3× PBS and 100% ethanol. Fixed samples were then stored at -20°C.

Enrichment media. Enrichment cultures were performed anaerobically in Hungate tubes or in 50-ml vials as described by Balch and Wolfe (4).

The medium for the enrichment of methanogens consisted of (per liter of distilled water) Sea Salts, 30 g; NH₄Cl, 1 g; KH₂PO₄, 0.35 g; PIPES [piperazine-*N,N'*-bis(ethanesulfonic acid)] buffer, 3.46 g; NaHCO₃, 1 g; yeast extract, 2 g; peptone, 2 g; sodium acetate, 1 g; trace element mixture (41), 1 ml; selenite-tungstate solution (41), 1 ml; vitamin mixture (41), 1 ml; thiamine solution (41), 1 ml; vitamin B₁₂ solution (41), 1 ml; growth-stimulating factors (32), 1 ml; and resazurin, 1 mg. The pH was adjusted to 6.5. H₂-CO₂ (80:20; 200 kPa) was used as the gas phase.

Heterotrophic sulfur reducers were enriched in a medium containing (per liter of distilled water) Sea Salts, 30 g; NH₄Cl, 1 g; KH₂PO₄, 0.35 g; PIPES buffer, 3.46 g; yeast extract, 1 g; peptone, 2 g; sulfur, 10 g; mineral solution (3), 10 ml; vitamin solution (3), 10 ml; and resazurin, 1 mg. The pH was adjusted to 7, and N₂ (100 kPa) was used as the gas phase. This medium was slightly modified to enrich for thiosulfate reducers; sulfur was replaced by 10 mM thiosulfate.

The medium for the enrichment of autotrophic sulfur reducers consisted of (per liter of distilled water) Sea Salts, 30 g; NH₄Cl, 1 g; KH₂PO₄, 0.35 g; MES [2-(*N*-morpholino)ethanesulfonic acid] buffer, 1.95 g; NaHCO₃, 1 g; sulfur, 10 g; trace element mixture (41), 1 ml; selenite-tungstate solution (41), 1 ml; vitamin mixture (41), 1 ml; thiamine solution (41), 1 ml; vitamin B₁₂ solution (41), 1 ml;

growth-stimulating factors (32); and resazurin, 1 mg. The pH was adjusted to 6. H₂-CO₂ (80:20; 200 kPa) was used as the gas phase.

Estimation of the culturable cell concentrations in chimney samples. To estimate the concentrations of culturable thermophilic microorganisms in the chimney samples, enrichment media were inoculated with multiple dilutions of chimney-seawater mixtures in an anoxic chamber (La Calhène, Vélizy, France) and incubated without shaking at 65, 80, and 95°C. Growth was recorded daily for at least 2 weeks. Each chimney-seawater mixture (1 ml) was incubated at 100°C for a few days until a constant dry weight could be measured.

Scanning electron microscopy (SEM). A few portions of sample from the external wall (A3) of chimney MS15CH were fixed with 2.5% (wt/vol) glutaraldehyde. After salt was eliminated and the preparation was dehydrated with increasing ethanol concentrations, samples were critical point dried after filtration with a 10-μm-pore-size Millipore membrane, coated with gold (Balzers, Balzers, Liechtenstein), and examined with a JEOL 5002 microscope.

Direct in situ hybridization of chimney cells. Roughly 25 mm³ of chimney samples were pretreated in a 1.5-ml Eppendorf tube for 30 min at 46°C with 1 ml of hybridization buffer containing 20% formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate, 0.4 mg of bovine serum albumin (BSA) per ml, and 0.2 mg of poly(U) per ml. After 5 min of centrifugation at 4,000 × g, the buffer was replaced by 100 μl of normal hybridization buffer containing 0.2 mg of BSA per ml, 0.1 mg of poly(U) per ml, and 50 ng of fluorescently labeled probe per ml. This mixture was incubated for 3 h at 46°C and centrifuged at 4,000 × g for 5 min. The pellet was washed with 1 ml of 0.9 M NaCl-20 mM Tris-HCl (pH 7.2)-0.1% sodium dodecyl sulfate at 48°C for 20 min and centrifuged as before. The pellet was washed with cold filtered water to remove the excess salt, centrifuged as before, and resuspended in 50 μl of 1× PBS (pH 9) containing 0.5% agarose prewarmed at 50°C. Aliquots were quickly smeared on a clean slide, covered with a cover slide, cooled, and viewed directly by epifluorescence.

Extraction, whole-cell hybridization, and quantification of chimney cells. (i) Extraction. For extraction of chimney cells, about 5 g (wet weight) of fixed chimney samples was centrifuged in a sterile 50-ml tube at 4,000 × g for 5 min. The cells were extracted from the pellets basically as described before (16) with some modifications. All solutions were sterile and filtered through a 0.2-μm-pore-size DynaGard syringe tip filter (Microgon Inc., Laguna Hills, Calif.). One gram of Chelex 100 (Bio-Rad, Ivry-Sur-Seine, France) and 4 ml of extraction buffer (1× PBS, 2% [vol/vol] polyethylene glycol 8000) were added to the pellet in a sterile 25-ml tube. The mixture was shaken on a wrist-action shaker for 1 h. The tube was centrifuged at 700 × g for 2 min. The supernatant was kept on ice while the pellet was reextracted with 3 ml of extraction buffer. After another cycle of centrifugation, the pellet was washed with 3 ml of extraction buffer and centrifuged again. The supernatants were pooled in a 10-ml polycarbonate tube and centrifuged at 700 × g. The cells were collected from the supernatant by centrifugation at 4,000 × g and resuspended in 1 ml of 1× PBS-0.1% (wt/vol) IGEAL (Sigma, Saint Quentin Fallavier, France). The cells were loaded on a layer of 2 ml of 1× PBS-0.1% IGEAL containing 50% (wt/vol) Ficoll (Sigma) in a 10-ml tube to separate the remaining sulfide minerals from the cells. After centrifugation at 4,000 × g for 10 min, the cells were recovered from the top of the Ficoll layer and resuspended in 9 ml of 1× PBS-0.1% IGEAL. The cells were collected by centrifugation at 4,000 × g for 10 min, washed with 1 ml of 1× PBS-0.1% IGEAL, and resuspended in 60 μl of 1× PBS-0.1% IGEAL and 60 μl of 100% ethanol. The cell extracts were stored at -20°C for several months.

(ii) Whole-cell hybridization. For whole-cell hybridization, 1 to 4 μl of the extracted cells was smeared on the gelatin-coated slide, dried, rinsed in series of alcohol concentrations, and hybridized with fluorescent probes as described before (39).

(iii) Quantification. To quantify extracted cells, an appropriate amount of cell extract (4 to 30 μl) was diluted in 5 ml of 1× PBS-0.1% IGEAL and filtered through a 0.2-μm-pore-size Nucleopore filter. The filter was rinsed with 5 ml of deionized water, and dried at 48°C for 5 min. The filters were hybridized on a normal slide with 20 μl of hybridization buffer containing 5 ng of probe per μl under a cover slide. After 3 h, the filters were washed in hybridization buffer without BSA and poly(U) at 48°C for 15 min, rinsed with cold water, dried at 48°C, and mounted in Citifluor (Citifluor Ltd., London, United Kingdom). The cells were viewed and counted on a Olympus epifluorescence microscope. Minimums of 25 cells per field, 10 fields per filter, three filters per cell extract, and two cell extractions per sample were counted, using a fluorescein-labeled EUB338 bacterial probe (1) and a rhodamine-labeled ARC915 archaeal probe (39).

To estimate the relative amount of crenarchaeal cells, cell smears on slides were hybridized simultaneously with a rhodamine-labeled CREN499 probe (6) and a fluorescein-labeled ARC915 probe. For each extraction of cells, five double-exposed photomicrographs with approximately 100 archaeal cells were taken with both filter sets.

RESULTS

Chimney samples. Three beehive structures were sampled during the Microsmoke cruise, subsampled on board, and processed on the same day for further microbiological investiga-

FIG. 1. (A) Photograph of the site of chimney MS15CH (arrowhead) taken by the fixed camera of the submarine *Nautil* just before sampling. The shrimps dwelling on the chimney are indicated by an arrow. (B) Schematic drawings of the chimney MS15CH and its subsampling. The top drawing shows a frontal view, and the bottom drawing shows a horizontal cross section. The letters and numbers indicate the subsamples. The part marked X was lost during deposition of the chimney in the submarine basket.

tions. The diffuser MS06CH, of approximately 20 cm in length and up to 15 cm in width, allowed the definition of three vertical layers of around 7 cm in length each (A, B, and C, from the top to the bottom). As diffuser MS17CH was a small sulfide structure, it was subsampled only in its width. MS15CH (Fig. 1A) was composed of a vertical pipe (Top and X in Fig. 1B) overlapping a beehive structure. At the top of the beehive structure and the bottom of the pipe, the presence of shrimps (Fig. 1A) indicated a moderate temperature range. A black hydrothermal fluid circulated at a medium flow rate at the top of the vertical pipe. Whereas the venting hydrothermal fluids had temperatures of around 300°C, the outer chimney walls were exposed to the 2°C ambient temperature. The temperature gradient within the chimneys is not known; it probably fluctuated and was very difficult to measure.

Enrichment culture of thermophilic subpopulations. The presence and the abundance of thermophilic microorganisms in the chimney samples designated MS06CH, MS15CH, and MS17CH were evaluated by inoculating enrichment media with serial dilutions of chimney subsamples and incubating at 65, 80, and 95°C (Table 1). The sole positive enrichment at 95°C was obtained from the MS15CHTOP subsample in medium designed for heterotrophic sulfur reducers. On the three chimneys, the highest numbers of anaerobic thermophilic microorganisms (up to 10^6 per g [dry weight]) were measured mainly in the upper external wall (MS06CHA3, MS15CHA3, and MS17CHA3) and in the pipe of MS15CH (MS15CHTOP subsample). As an exception, the upper inner part of MS06CH (MS06CHA1 subsample) also yielded large numbers of thermophiles of all metabolic types. In most of these samples, the community growing at 65 and 80°C was composed mainly of heterotrophic sulfur-reducing cocci. The same distribution was observed for high numbers of thiosulfate-reducing microorganisms (up to 10^5 per g [dry weight]) in MS17CH and MS15CH. However, thiosulfate reducers (mainly cocci with a few small rods and sheathed cells) were enriched mainly at

65°C. The autotrophic thermophilic community was dominated by numerous small, highly motile sulfur-reducing rods with rare cocci enriched mostly at 65°C. In some samples, these autotrophic sulfur reducers represented a significant fraction of the culturable anaerobic thermophiles. Dispersed and less abundant methanogenic cocci were enriched at both temperatures. In most of the inner subsamples, numbers of culturable anaerobic thermophiles were more than 2 orders of magnitude lower than those recorded in the outer subsamples.

SEM and in situ hybridization of the chimney samples. SEM analysis of sample MS15CHA3 revealed that most of the mineral sulfides consisted of diamond-shaped crystals with no or very few microbial cells on them. Nevertheless, some grains had a rough organic-like surface with cracks. These grains were colonized with filamentous, rod-shaped, and coccoid microbes. Figure 2 shows a photomicrograph of such a grain colonized by mainly filamentous and some rod-shaped cells.

Direct in situ hybridization of the mineral sulfides with fluorescently labeled probes resulted in photomicrographs of poor quality, caused by an overwhelming autofluorescence due to autofluorescence of the sulfides and binding of the oligonucleotides to the probably charged minerals. However, bacterial and archaeal cells and microcolonies were detected by using pretreatment of the minerals in a hybridization buffer with twice the amounts of BSA and poly(U), thus blocking the charged sites. After the pretreatment, in situ hybridization was possible by using fluorescein-labeled probes, because most of the background was reduced and appeared to be yellowish when a fluorescein-specific filter was used. Per 25 mm³ of chimney sample, we detected only a few microbial cells and some microcolonies, either of rods, of filaments, or mixed (Fig. 3A and B). Because of the autofluorescence and the large amount of sulfide minerals (Fig. 3C), cell extraction seemed to be a logical step to eliminate the sulfide minerals and to concentrate the cells from the samples.

TABLE 1. Culturable thermophilic anaerobic microorganisms enumerated by enrichment culture of serial dilutions of chimney subsamples at 65 and 80°C

Subsample ^a	No./g (dry wt)							
	Heterotrophic S ⁰ reducers		Autotrophic S ⁰ reducers		Methanogens		Thiosulfate reducers	
	65°C	80°C	65°C	80°C	65°C	80°C	65°C	80°C
MS06CHA1	5.1×10^4	5.1×10^5	5.1×10^3	25	5.1×10^2	25	5.1×10^3	0
MS06CHA2	10	0	10	0	0	0	0	0
MS06CHA3	5.6×10^3	5.6×10^4	5.6×10^2	0	0	0	0	0
MS06CHB1	0	10	10	0	0	0	0	0
MS06CHB2	0	2	3.0×10^2	0	0	2	0	0
MS06CHB3	20	20	20	0	0	0	0	0
MS06CHC1	20	4.0×10^3	20	0	0	0	0	0
MS06CHC2	0	10^3	0	0	0	0	0	0
MS06CHC3	5.0×10^2	5.0×10^2	2	0	0	0	5.0×10^2	0
MS15CHA1	0	40	40	0	0	2	4.0×10^2	0
MS15CHA2	30	0	10	0	0	0	30	0
MS15CHA3	4.0×10^4	4×10^6	0	0	2	2	4.0×10^3	0
MS15CHTOP	3.0×10^6	3.0×10^5	3.0×10^4	3.0×10^4	1	1	3.0×10^5	3×10^4
MS15CHB2	8.0×10^2	8.0×10^2	0	0	4	0	8.0×10^2	0
MS17CHA1	7.0×10^2	7.0×10^2	0	0	0	0	3	70
MS17CHA2	3.0×10^3	3.0×10^2	3.0×10^2	0	2	0	30	0
MS17CHA3	10^5	10^5	0	0	0	10^2	10^5	10^5

^a In the last two positions of the designation, the letters A, B, and C correspond to successive vertical sections of the chimneys from top to bottom. The numbers 1, 2, and 3 correspond to successive horizontal layers of the chimneys from the inner part to the outer part.

Detection of cells of different microbial groups by whole-cell hybridization. Cells were extracted from the subsamples by shaking in extraction buffer containing Chelex 100 and polyethylene glycol 8000 and further differential centrifugations. Two cycles of extraction were found to be optimal, because additional cycles yielded only a few more cells, while the amount of small minerals increased dramatically. As the cell extracts obtained still contained too many black minerals for whole-cell hybridization, centrifugation on a Ficoll layer was needed to separate the cells from the minerals. During this step, the minerals sedimented at the bottom of the tube, while

the cells just penetrated the Ficoll layer. A few minerals still remained between the cells recovered from the Ficoll layer, but the extracts were sufficiently cleaned for whole-cell hybridization.

The extracted cells were used for whole-cell hybridization of cell smears on slides with probes specific for *Bacteria*, *Archaea*, and *Crenarchaeota* to detect these different microbial groups. With the fluorescein-labeled probe EUB338 and the rhodamine-labeled probe ARC915, whole-cell hybridizations with extracts from subsamples MS15CHA3 and MS15CHTOP demonstrated the presence of morphologically different bacterial

FIG. 2. Scanning electron micrograph of subsample MS15CHA3, showing a mixed microcolony of microorganisms. Bar, 5 μ m.

FIG. 3. In situ hybridization of microbial cells directly on the minerals from subsample MS15CHA3. (A and B) The minerals were hybridized with the fluorescein-labeled bacterial probe EUB338 and photographed at two following plains of focus to show a microcolony (cells are indicated by arrows). (C) The phase-contrast micrograph of the same field as in panel B shows the minerals present. Bar, 10 μ m.

rods and filaments associated with archaeal cocci and thin long rods (Fig. 4A, B, C, and D). In most of the subsamples from the external part of the three chimneys, a similar diversity was observed. As shown by a simultaneous hybridization with rhodamine-labeled probe EUB338 and fluorescein-labeled probe ARC915 with sample MS17CHA3, whole-cell hybridizations were specific and no artifacts were caused by the differently labeled probes (Fig. 4D). Furthermore, the diversity of the bacterial morphologies seems to be higher in this sample than in the MS15CH samples. Large bacterial cells forming chains and long filaments were detected next to small and very small cocci and rods. The persistence of bacteria in chains in the extracts indicated that the extraction method is gentle enough to avoid their breaking. The archaeal population from MS17CHA3 appeared to be similar to that from other samples. A similar diverse population was observed in subsample MS06CHB3, another external wall, when hybridized with fluorescein-labeled probe EUB338 and rhodamine-labeled probe ARC915 (Fig. 4E).

Archaeal cocci from MS15CHA3 were distinguished as *Euryarchaeota* and *Crenarchaeota* when hybridized with the domain-specific probes CREN499 and EURY498 (Fig. 4F). However, the weakness of the fluorescent signals obtained with probe EURY498 made it doubtful that all euryarchaeal cells hybridized with the probe. Simultaneous hybridizations with probe ARC915 and probe CREN499 were performed to estimate the *Crenarchaeota/Archaea* ratio and revealed that the thin and long archaeal rods hybridized only with probe CREN499 (Fig. 4G).

Quantification of the cells from chimney MS15CH. The extracted cells of the subsamples of chimney MS15CH were quantified by whole-cell hybridization on filters (Fig. 4H). Despite the low background on the filter, the fluorescent signals were strong enough to allow discrimination and counting of the rhodamine-labeled *Archaea* and the fluorescein-labeled *Bacteria*. The efficient binding of DAPI (4,6-diamidino-2-phenylindole), acridine orange, or YOYO dye to the remaining minerals made our attempts to measure the total cell counts unsuccessful. Therefore, the sum of the bacterial cells and archaeal cells enumerated in a sample by using both specific probes was considered the total cell count. The subsample MS15CHTOP was unfortunately not fixed on board but was stored under reduced conditions at 5°C for 6 months. How-

ever, after fixation and cell extraction, large amounts of cells could be obtained from this subsample.

The enumerations of the cells detected by whole-cell hybridization are reported in Table 2. The counts obtained from two independent cell extractions revealed some variability but showed similar trends. The results clearly indicated that the highest microbial densities were measured at the top (mean, 3.9×10^7 per g [dry weight]) and the external wall (A3) (mean, 3.8×10^6 per g [dry weight]) of the chimney. The abundance of microorganisms decreased significantly in the inner part (A1) of the chimney (mean, 1.4×10^5 per g [dry weight]), in the middle part (A2) (mean, 0.8×10^5 per g [dry weight]), and in the lower layer (B2) (mean, 4.1×10^5 per g [dry weight]).

The estimation of the abundance of crenarchaeal cells was technically possible only when the subsample yielded a large amount of extracted cells. In subsample MS15CHA3, cells hybridizing with the crenarchaeal probe represented 19% of those detected with the archaeal probe (about 1,000 archaeal cells counted). The *Crenarchaeota/Archaea* ratio in subsample MS15CHTOP was very low (<1%).

DISCUSSION

The microbial distribution in deep-sea hydrothermal chimneys was analyzed here by the combined use of whole-cell hybridizations and enrichment culture of thermophilic populations. Whole-cell hybridization is based on phylogenetic staining and does not give information on the growth temperature range and the metabolic traits of the detected cells. On the other hand, culture-based enrichment is dependent on the culturability of the cells, but appropriate media and incubation temperatures can provide valuable information on the culturable fraction of the whole community. Other limitations of both techniques when applied to studies of environmental communities (2, 6), and specifically to those of hydrothermal vents (25), have been noted. Cell counts by whole-cell hybridization could take into account thermophilic and mesophilic prokaryotes, since this technique can detect all physiological cell types, and contamination during sampling, which can not be excluded in our case, might cause biases. Moreover, cells which are impermeable to the probes by our fixation and hybridization methods, those which do not contain enough rRNA, or those which have no target sites for the archaeal or

FIG. 4. Whole-cell hybridizations of cells extracted from different chimney samples hybridized simultaneously with fluorescein-labeled (green fluorescence) and rhodamine-labeled (red fluorescence) probes. Epifluorescence photomicrographs (double exposures) were done. Magnification, $\times 1,060$. Bar, 10 μm . (A) Probe EUB338 (green fluorescence) and probe ARCH915 (red fluorescence) were used with subsample MS15CHA3 (second extraction). (B) Probe EUB338 (green fluorescence) and probe ARCH915 (red fluorescence) were used with subsample MS15CHTOP. (C) Probe EUB338 (green fluorescence) and probe ARCH915 (red fluorescence) were used with subsample MS15CHA3 (first extraction). (D) Probe EUB338 (red fluorescence) and probe ARCH915 (green fluorescence) were used with subsample MS17CH3. (E) Probe EUB338 (green fluorescence) and probe ARCH915 (red fluorescence) were used with subsample MS06CHB3. (F) Probe EURY498 (green fluorescence) and probe CREN499 (red fluorescence) were used with subsample MS15CHA3. Cells are indicated by arrows. (G) Probe ARCH915 (green fluorescence) and probe CREN499 (red fluorescence) were used with subsample MS15CHA3. Thin long rods and cocci that hybridized with both probes appeared yellowish on the double-exposed film. (H) Hybridization on a filter with probe EUB338 (green fluorescence) and probe ARCH915 (red fluorescence) was used with subsample MS15CHA3.

TABLE 2. Enumeration of cells detected by whole-cell hybridization on filters after extraction from mineral sulfides from chimney MS15CH

Subsamples of MS15CH and extraction	No./g (dry wt) ^a		
	<i>Bacteria</i>	<i>Archaea</i>	Total
Top			
First	$(1.5 \pm 0.3) \times 10^7$	$(1.6 \pm 0.2) \times 10^7$	$(3.1 \pm 0.4) \times 10^7$
Second	$(2.4 \pm 0.3) \times 10^7$	$(2.5 \pm 0.3) \times 10^7$	$(4.8 \pm 0.5) \times 10^7$
A1			
First	$(1.5 \pm 0.4) \times 10^5$	$(1.9 \pm 0.4) \times 10^3$	$(1.5 \pm 0.4) \times 10^5$
Second	$(1.3 \pm 0.4) \times 10^5$	$(1.8 \pm 0.4) \times 10^3$	$(1.3 \pm 0.4) \times 10^5$
A2			
First	$(1.1 \pm 0.3) \times 10^5$	$(1.7 \pm 1.3) \times 10^4$	$1.2 \pm 0.3 \times 10^5$
Second	$(2.6 \pm 0.6) \times 10^4$	$(1.2 \pm 0.6) \times 10^4$	$(3.7 \pm 0.8) \times 10^4$
A3			
First	$(2.0 \pm 0.4) \times 10^6$	$(2.7 \pm 0.5) \times 10^6$	$(4.7 \pm 0.7) \times 10^6$
Second	$(7.1 \pm 1.4) \times 10^5$	$(2.3 \pm 0.4) \times 10^6$	$(3.0 \pm 0.5) \times 10^6$
B2			
First	$(4.3 \pm 1.4) \times 10^5$	$(1.9 \pm 0.9) \times 10^5$	$(6.2 \pm 1.8) \times 10^5$
Second	$(8.3 \pm 2.7) \times 10^4$	$(1.1 \pm 0.3) \times 10^5$	$(2.0 \pm 0.4) \times 10^5$

^a The *Bacteria* and *Archaea* were counted by hybridization with probes EUB338 and ARCH915, respectively. The total is the sum of the detected *Bacteria* and *Archaea*. Results are means for three different filters per sample, with the geometric mean of the standard deviation per filter.

the bacterial probe might escape detection. Despite the limitations of the techniques used, the results clearly show that an active and diverse community of microorganisms is associated with the hydrothermal vent chimneys.

Although the above-mentioned methods used independent approaches, they enumerated microorganisms in the same order of magnitude in the external and upper parts of the chimney MS15CH. Since chimneys were transferred to the surface in the insulated basket of the submersible, contamination by mesophilic microorganisms could also be responsible for the high numbers of cells detected by in situ hybridization in these external parts. However, these numbers are consistent with those measured here by culture enrichments and those estimated earlier in discrete mineral layers within sulfide flanges (15). Our complementary approaches also revealed that the microbial concentrations increased gradiently (i) from the inner to the outer parts of the upper sulfide layers and (ii) from the lower to the upper chimney sections. This distribution was confirmed for chimneys MS06CH and MS17CH, which were analyzed only by using culture-based enrichments. In the sampled chimneys, the fluid diffuses through an axial zone of high porosity up to the surface and the sides of the beehive structures. Therefore, the growth of the structure occurs both vertically and horizontally, giving a typical regular conical shape (12). As a consequence of these features, the inner parts are likely older and hotter than the outer walls, and the mineralogical successions within the beehive structure are related to this thermal gradient. This could also likely explain the distribution of the thermophilic community.

By using the different domain- and kingdom-specific probes and analyzing the observed morphologies, at least three different morphotypes of *Archaea* and four morphotypes of *Bacteria* could be distinguished. The abundantly present large coccoid *Archaea*, hybridizing with both archaeal and euryarchaeal probes, may correspond to the predominantly enriched heterotrophic sulfur reducers and may be partly assigned to the order *Thermococcales*. Whole-cell hybridizations with the crenarchaeal probe detected cocci and long thin rods. Representative species isolated up to now from deep-sea hydrothermal vents

that belong to the domain *Crenarchaeota* are the heterotrophic sulfur-metabolizing cocci *Staphylothermus marinus* (10), *Pyrodictum abyssi* (35), and members of the genus *Desulfurococcus* (20). The relatively abundant long and thin archaeal rods do not correspond by their size and shape to well-known microorganisms, and unfortunately, they have not been observed in the designed enrichment media. Among the detected bacteria, rod-shaped cells surrounded by a sheath-like envelope have been observed and preferably enriched in medium supplemented with thiosulfate. This characteristic shape and thiosulfate reduction are common morphological and physiological features of the members of the order *Thermotogales* (17, 36). The abundant small bacterial rods observed in the chimney samples correspond mostly to the autotrophic sulfur reducers enriched preferably at 65°C (data not shown). The enrichment media and conditions did not allow growth of large rods like those observed in in situ hybridization experiments. However, a few aerobic heterotrophic bacteria were cultured from these samples at 65°C (28). The large filamentous bacteria observed in high numbers in chimney subsamples failed to grow in the enrichment media. Considering the predicted temperatures near the chimney outlet, they could be better affiliated with the *Aquificales*, such as the pink filaments from hot springs in Yellowstone National Park (37), than to the mesophilic mat-forming *Beggiatoa* (8) even if mesophilic space-limited niches are present as revealed by the shrimps dwelling on chimney MS15CH.

Our data clearly indicate that the morphological diversity observed by using whole-cell hybridization is much larger than that assessed by culture-based enrichments. The real extent and nature of the chimney microbial diversity might be better estimated by using a spectrum of lower-level specific probes to identify the known families, genera, and species within each domain. Additional probes can be constructed for our as-yet-unculturable organisms by using PCR for retrieval of rRNA sequences directly from the biotope, and this will give assistance in cultivation efforts.

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