Relative abundance of *Archaea* and *Bacteria* along a thermal gradient of a shallow-water hydrothermal vent quantified by rRNA slot-blot hybridization

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Slot-blot hybridization of rRNA with domain-specific oligonucleotide probes targeting the 16S rRNA of *Archaea* and *Bacteria* was utilized to assess the relative abundance of these domains along a thermal gradient at a shallow submarine hydrothermal vent near Milos Island (Greece). The highest prokaryotic rRNA concentrations (defined as the sum of bacterial and archaeal rRNA) were found in the uppermost sediment surface (0–20 mm), decreasing strongly with depth. This indicates that the microbial activity was mainly occurring in the surface layer of this hydrothermal vent. Furthermore, rRNA concentrations were higher in regions closer to the vent, suggesting that the hydrothermal activity stimulated microbial activity. *Archaea* seemed to be a minor component of the microbial community at this vent site, even in the zones with higher temperatures. *Bacteria* made up at least 78% (mean 95%) of the prokaryotic rRNA. However, along the steepest temperature gradient, the proportion of archaeal rRNA increased. Nevertheless, even in the hottest sediment layer where a quantification was possible (*in situ* temperature 82 °C) archaeal rRNA made up only 11.9% of the prokaryotic rRNA. This suggests that *Archaea* were generally of minor importance at this vent site and were probably restricted to a narrow niche. The factors that allow *Bacteria* to dominate in a high temperature environment that was once believed to be the realm of *Archaea* remain elusive.

Keywords: rRNA slot-blot hybridization, *Archaea* and *Bacteria*, microbial activity, hydrothermal vent, Milos Island (Aegean Sea, Greece)

INTRODUCTION

The composition of microbial communities of high temperature hydrothermal vents was, until recently, mainly studied by cultivation-based techniques. Because of the frequent isolation of *Archaea* from these habitats it was assumed that *Archaea* dominate these communities (Stetter *et al.*, 1990; Baross & Deming, 1995). The first cultivation-independent support for that hypothesis came from the analysis of lipids extracted from sulfide structures of a black smoker (Hedrick *et al.*, 1992). However, the application of molecular-biological methods revealed a quite different picture. Although it could be shown that the diversity of *Archaea* was

unexpectedly high in terrestrial and marine hydrothermal systems (Barns *et al.*, 1994, 1996; Dando *et al.*, 1998; Takai & Sako, 1999), it seemed to be the *Bacteria* that dominated the microbial communities in most of these environments (Reysenbach *et al.*, 1994; Harmsen *et al.*, 1997a; Hugenholtz *et al.*, 1998; Takai & Sako, 1999). However, in none of these studies was the ratio of the two domains related to the *in situ* temperature nor was it investigated how this ratio changes along a thermal gradient. This is surprising, since temperature is the most obvious parameter that could determine the relative abundance of *Bacteria* and *Archaea* in hydrothermal habitats. In addition, most of these studies have used molecular techniques that are not a priori quantitative.

In the present study we have studied the relative abundance of two domains of life, *Archaea* and *Bacteria*

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(Woese *et al.*, 1990), along a thermal gradient at a shallow-water hydrothermal vent. We used slot-blot hybridization of rRNA directly extracted from the sediments to quantify the relative abundance of *Archaea* and *Bacteria* with domain-specific probes. This technique has been successfully applied in a variety of environments to estimate the relative abundance of specific groups of micro-organisms (Stahl *et al.*, 1988; Raskin *et al.*, 1994; MacGregor *et al.*, 1997; Massana *et al.*, 1998; Sahm & Berninger, 1998; Sahm *et al.*, 1999; Vetriani *et al.*, 1999). Concurrently with the sampling, the *in situ* temperature of the respective samples was determined.

METHODS

Study site. The study site was a solitary gaseous hydrothermal vent located in 8 m water in Palaeochori Bay (24° 31'220' E; 36° 40'391' N), a sandy bay in the south-eastern part of the island Milos in the Aegean Sea (Greece). For a more detailed site description see Sievert *et al.* (1999).

Sampling and temperature measurements. Sampling was carried out along a transect from the centre of the almost circular vent to the surrounding area. Sediment cores were taken by scuba-divers at specified distances from the vent centre in June 1997. Vertical profiles of temperature were determined *in situ* up to 12 cm sediment depth in 1 cm increments along the same transect, as described by Sievert *et al.* (1999).

Slicing of sediment cores. Sediment cores were immediately subsampled by extruding the sediment from the polycarbonate tubes and slicing each core. The following depth intervals were sampled: 0–2, 2–7, 7–12, 12–17, 17–27, 27–37, 37–57, 57–77, 77–97 and 97–117 mm.

RNA extraction. Subsamples (5 cm³) from sliced sediment cores were immediately deep-frozen in liquid nitrogen. For long-term storage, the samples were kept at –80 °C. RNA was isolated directly from the sediments by bead-beating, phenol extraction and ethanol precipitation, based on methods described elsewhere (MacGregor *et al.*, 1997; Sahm & Berninger, 1998; Sahm *et al.*, 1999). The extracted RNA was stored at –80 °C until further analysis. The quality of the RNA was checked by polyacrylamide gel electrophoresis.

Oligonucleotides. These were purchased from Biometra. The probes used were S*-Univ-1392-a-A-15 (Stahl *et al.*, 1988), which targets most known forms of life, S-D-Bact-0338-a-A-18 (Amann *et al.*, 1990), which is specific for *Bacteria* and S-D-Arch-0915-a-A-20 (Stahl & Amann, 1991), targeting *Archaea*.

Quantification of domains in environmental samples. For quantitative rRNA hybridization experiments, RNA extracts from natural samples and from suitable reference organisms (*Escherichia coli* and *Methanobacterium bryantii*) were loaded onto nylon membranes (Magna Charge; Micron Separations) using a slot-blotting apparatus (Minifold II; Schleicher and Schüll) and probed with radioactively labelled oligonucleotides as described by Stahl *et al.* (1988). Because the amount of extractable RNA from environmental samples was in some cases very low, i.e. below the detection limit of the gel electrophoresis, we could not blot equal amounts of RNA. When possible, approximately 50 ng RNA was blotted in triplicate. However, in some cases triplicate blotting was not possible due to the low amount of RNA.

After blotting, the membranes were baked at 80 °C for 1 h. Membranes were further prehybridized at 40 °C for 1 h with 10 ml hybridization buffer (0.9 M NaCl, 50 mM NaPO₄, pH 7.2, 5 mM EDTA, pH 7.2, 10× Denhardt's solution, 0.5 mg polyadenosine ml⁻¹, 0.5% SDS), followed by the addition of the 16S rRNA oligonucleotide probes (see below) end-labelled with ³²P. After overnight hybridization at 40 °C, membranes were washed twice at 40 °C for 1 h with washing buffer (1% SDS, 1× SSC). Membranes were then transferred to the same buffer pre-equilibrated to the high-stringency washing temperature (40 °C for S*-Univ-1392-a-A-15, 54 °C for S-D-Bact-0338-a-A-18 and 56 °C for S-D-Arch-0915-a-A-20), washed twice for 15 min, removed and air-dried.

The dried membranes were exposed to Storage Phosphor-Imager screens (Molecular Dynamics). Subsequently, the hybridization signal intensity was measured with a Phosphor-Imager (Molecular Dynamics) and quantified with the software package ImageQuant (Molecular Dynamics) relative to suitable reference RNA. For each probe, an individual set of membranes was prepared to which a reference dilution series of reference RNA and the environmental samples were applied. In addition, one membrane was prepared with the dilution series of all reference RNAs for the universal hybridization. This membrane was used to quantify the reference RNA of *M. bryantii* according to an *E. coli* standard (Boehringer Mannheim).

RESULTS

The profiles of the *in situ* temperature illustrate that the studied vent system was well suited to study the relative distribution of *Archaea* and *Bacteria* along an environmental thermal gradient (Fig. 1). It has been shown before that temperature is an important parameter in determining the bacterial community structure (Sievert *et al.*, 1999) and it was expected that it would also influence the relative distribution of *Archaea* and

Fig. 1. Profile of *in situ* temperature along a transect from the centre of the vent to the surrounding area at the following sediment depth: 0 cm (○), 1 cm (□), 2 cm (◇), 3 cm (×), 4 cm (△), 5 cm (+), 6 cm (●), 7 cm (■), 8 cm (◆), 9 cm (▲), 10 cm (◐), 11 cm (◑) and 12 cm (◒). The arrows mark the positions where the sediment cores for the molecular analysis were taken.

Fig. 2. Profiles of temperature (○) and the concentration of prokaryotic rRNA (sum of bacterial and archaeal rRNA) (□) with sediment depth in the four different cores along the transect: (a) centre, (b) 1.2 m from vent, (c) 2.2 m from vent, (d) 4 m from vent.

Bacteria. Four cores along the transect, which varied in their temperature gradient with sediment depth, were selected to address this question. Apart from temperature, other physico-chemical parameters have previously been determined at this vent.

As a general feature, it was observed that the amount of prokaryotic rRNA (defined as the sum of bacterial and archaeal rRNA) rapidly decreased with sediment depth in all cores, although the decrease seemed to be more pronounced in the zones with the steepest temperature gradient (Fig. 2). In the deeper sediment layers of the cores generally, only very low amounts of rRNA were detected [<1 ng (g sediment wet weight)⁻¹] and a quantification of the two domains was not attempted in these cases. Interestingly, the upper sediment layers of the inner two cores contained a higher amount of prokaryotic rRNA than the outer two cores. The lowest concentrations of prokaryotic rRNA were found 2.2 m from the vent. *Bacteria* constituted the most important component of the prokaryotic community in all regions and depths, making up at least 78% (mean 95%) of the prokaryotic rRNA (data not shown). *Archaea* constituted only a small fraction of the prokaryotic community (Table 1). However, there was a trend of an increasing archaeal contribution to prokaryotic rRNA with increasing temperature at the centre of the vent and in the core from 1.2 m from the centre (Table 1a and b). The increase was from 0.7% at the surface to 11.9% at 27 mm sediment depth at the centre (concurrently, the temperature increased from 42 to 82 °C), and from 1.1% at the surface to 22.2% at 27 mm sediment depth 1.2 m from the centre (here the temperature increased from 23.5 to 40.5 °C). Such a trend was not apparent in the cores 2.2 m and 4 m from the vent (Table 1c and d).

DISCUSSION

Methodological considerations

We have quantified the relative abundance of *Archaea* and *Bacteria* at a shallow-water hydrothermal vent by using the hybridization of environmental rRNA with domain-specific oligonucleotides targeting the 16S rRNA. Although this method, like any other molecular method, has the advantage that it circumvents selective and potentially ineffective cultivation, it also has limitations. 16S rRNA-targeted probes are widely used in molecular ecology. However, their sequences are based on known sequences in the database and thus new populations that may not contain the target signature sites are not detected (see, for example, Daims *et al.*, 1999). This limitation is more severe in environments where many unknown or new populations are suspected, such as hydrothermal vents (Barns *et al.*, 1994, 1996; Harmsen *et al.*, 1997b; Dando *et al.*, 1998; Hugenholtz *et al.*, 1998; L'Haridon *et al.*, 1998; Takai & Sako, 1999). The first step in all studies of microbial community analysis by molecular methods is extraction of nucleic acids from the environment. Especially in sediments and soils this poses some challenges, and much remains to be learned about the extraction efficiencies of nucleic acids from this kind of samples. In addition, it is unknown whether there is a difference in the lysing efficiency of archaeal and bacterial cells, for example due to the difference in cell-wall composition and structure (MacGregor *et al.*,

Table 1. Profile of temperature, prokaryotic rRNA, relative abundance of *Archaea* and the ratio of *Bacteria* to *Archaea* in the different cores along the transect

NA, Not analysed; —, not calculated.

Depth (mm)	Temp. (°C)	Prokaryotic rRNA [ng (g sediment) ⁻¹]	Relative abundance of <i>Archaea</i> (% prokaryotic rRNA)	Ratio <i>Bacteria:Archaea</i>
(a) Centre of vent				
2	42	1749.3	0.7	139
7	54	1051.3	1.9	51
12	64	48.0	7.3	13
17	74	14.7	7.8	12
27	82	2.2	11.9	7
37	86	< 1	—	—
57	92	< 1	—	—
77	95	< 1	—	—
97	98	NA	—	—
117	100	NA	—	—
(b) 1.2 m from vent				
2	24	2714.8	1.1	88
7	27	354.8	3.5	28
12	30	99.4	4.8	20
17	34	45.0	5.2	18
27	41	17.5	22.2	3
37	47	< 2	—	—
57	58	< 2	—	—
77	65	< 2	—	—
97	70	< 2	—	—
117	75	< 1	—	—
(c) 2.2 m from vent				
2	22	111.6	3.2	30
7	23	122.8	2.7	36
12	25	371.1	2.3	43
17	27	123.9	2.7	36
27	30	97.8	3.4	29
37	32	53.1	4.9	19
57	36	29.6	8.2	11
77	38	< 2	—	—
97	42	< 2	—	—
117	45	< 2	—	—
(d) 4 m from vent				
2	21	703.2	2.4	41
7	21	435.4	3.0	32
12	21	182.2	3.8	25
17	21	153.0	2.5	38
27	21	176.7	2.4	40
37	21	26.9	4.6	21
57	21	NA	—	—
77	21	NA	—	—
97	21	NA	—	—
117	21	NA	—	—

1997). It is possible that the much lower yield of archaeal rRNA from sediments as compared to pelagic environments is associated with these two biases (Sahm & Berninger, 1998). Another potential caveat that affects quantification is the unknown relationship between the

abundance of rRNA and actual cell numbers, since the content of rRNA per cell varies between species and with growth rate (Devereux *et al.*, 1996; Kemp *et al.*, 1993). Thus, it is possible that certain organisms contribute proportionally more to the total rRNA than

their cell number would suggest and vice versa. Whether there is a systematic difference in rRNA content per cell between *Archaea* and *Bacteria* is not known. The universal probe used in the present study has been shown to overestimate the contribution of *Archaea* consistently about twofold (Massana *et al.*, 1998). Thus, the data presented here are likely to be conservative with respect to the contribution of bacterial rRNA, and the actual contribution of *Archaea* may be even lower.

Spatial distribution of prokaryotic rRNA

If it is assumed that rRNA is an integrated measure of cell number and growth, most of the microbial biomass and growth at the studied hydrothermal vent seems to be present in the first 10–20 mm of the sediments in all zones. This has also been observed by others, although in these cases the decrease was not as steep (MacGregor *et al.*, 1997; Sahm & Berninger, 1998; Sahm *et al.*, 1999). However, a similar drastic decrease of rRNA has been observed in sediment samples from a deep-sea vent site in the Guaymas Basin (B. J. MacGregor, personal communication). The analysis of lipids extracted from sediments at a deep-sea hydrothermal vent in the Guaymas Basin also revealed that the active microbial biomass was concentrated in the upper first centimetre and decreased drastically with depth (Guezennec *et al.*, 1996). If a mean rRNA content of 3 fg cell⁻¹ is considered (Sahm & Berninger, 1998), the calculated cell numbers in the upper layers are in the range of total cell counts that have been determined previously at the same vent (Sievert *et al.*, 1999). In the lower layers there is a discrepancy, which might be related to the fact that a higher percentage of cells was not active and thus contained less rRNA. Interestingly, we found higher concentrations of rRNA in the zones closer to the vent centre. This indicates that the cellular concentration of rRNA was higher at these sites than in the outer zones. This is surprising considering that the highest total cell numbers were found in the outer zone (Sievert *et al.*, 1999). This suggests that the percentage of active cells was higher in the inner zones compared to the outer zones, and it might be related to stimulated microbial activities due to the higher temperatures and certain constituents of the hydrothermal fluid.

Relative abundance of *Archaea*

Based on the hybridization analysis *Archaea* seemed to be of minor importance for the microbial community at this vent site, even in the zones with higher temperatures. This finding was somewhat surprising, but is in line with recent findings by others. As there are *Bacteria* known that can grow at 80–90 °C, but none that can grow above 97 °C, one may argue that the temperature limit where *Archaea* will dominate over *Bacteria* may not have been reached in our investigation. However, in this case one would expect an increase in rRNA at the depth where the temperature seems to be optimal for the *Archaea* that were isolated from this environment, i.e. at around 90–95 °C (Jochimsen *et al.*, 1997). Even in this layer, however, the total prokaryotic rRNA concen-

tration was very low. Nevertheless, the trend of an increasing contribution of archaeal rRNA with sediment depth in the two inner cores might be seen as an indication for a greater importance of *Archaea* at higher temperatures. The values reported for the lower layers, i.e. 11.9% and 22%, are at the upper range of values reported for sediments so far (MacGregor *et al.*, 1997; Sahm & Berninger, 1998; Sahm *et al.*, 1999; Vetriani *et al.*, 1999), supporting this assumption. However, because of an insufficient RNA recovery, these samples could not be plotted in triplicate. Although this affects the reliability of these data, we are confident that the data are reflecting a real trend, because the variation between triplicate samples was generally low (data not shown) and such a trend was not found in the two outer cores. By using whole-cell hybridization with the same domain-specific probes as used in the present study, it was shown that *Archaea* constituted up to 77% of the microbial community in the sulfide structures of a black smoker (calculated from Harmsen *et al.*, 1997a). However, this number was found in the supposedly coldest, outer part of the structure, whereas the lowest percentage of *Archaea* (1.3 and 1.4%) was found in the presumably hottest, inner part (data in Harmsen *et al.*, 1997a). Because the absolute yield of archaeal rRNA was quite low in the present study, the actual numbers of archaeal cells are also expected to be small. This suggests that the hyperthermophilic *Archaea* were occupying only a narrow niche. In future studies, it should be worthwhile to investigate the ecological role of these *Archaea* in more detail, e.g. to reveal the causes of their high diversity (Dando *et al.*, 1998), despite their apparently low numbers. It would have been interesting to see whether the proportion of *Archaea* would have continued to increase at even higher temperatures. However, the amount of rRNA that we could extract from these layers was too low to make reasonable quantifications. This problem has been noted before by Hugenholtz *et al.* (1998), who used the amplification of 16S rDNA by PCR because the amount of rRNA was too low to evaluate the relative abundance of *Archaea* and *Bacteria* directly. However, the ratio of *Bacteria* to *Archaea* they found for three different hot springs (80, 75 and 1, respectively) are comparable to ours and also suggest that the dominance of *Archaea* in high-temperature environments is the exception rather than the rule. In line with this, Guezennec *et al.* (1996) also found that *Archaea* contributed insignificantly to the total microbial biomass at a deep-sea hydrothermal vent in the Guaymas Basin, even in sediments with high *in situ* temperatures, and by using domain specific rRNA probes H. W. Jannasch and C. O. Wirsen (unpublished, cited in Jannasch, 1995) found a *circa* 10:1 ratio of *Bacteria* to *Archaea* at various locations at the same site.

Overall, the data presented in this paper indicate that the main microbial processes at this hydrothermal vent are occurring in the upper sediment layers and that *Bacteria* rather than *Archaea* principally carry out these processes. Furthermore, the hydrothermal activity seemed to stimulate the microbial activity as indicated

by the higher prokaryotic rRNA concentrations in the regions more strongly affected by the hydrothermalism. However, more studies are required to investigate the ecological roles of *Archaea* and *Bacteria* in high-temperature environments in more detail and to unravel the forces that govern their relative abundances.

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