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Antje Rusch · Jan P. Amend

Order-specific 16S rRNA-targeted oligonucleotide probes for (hyper)thermophilic archaea and bacteria

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Abstract New oligonucleotide probes were designed and evaluated for application in fluorescence in situ hybridization (FISH) studies on (hyper)thermophilic microbial communities—Arglo32, Tcoc164, and Aqui1197 target the 16S rRNA of Archaeoglobales, Thermococcales, and Aquificales, respectively. Both sequence information and experimental evaluation showed high coverage and specificity of all three probes. The signal intensity of Aqui1197 was improved by addition of a newly designed, unlabeled "helper" oligonucleotide, hAqui1045. It was shown that in addition to its function as a probe for Aquificales, Aqui1197 is suitable as a supplementary probe to extend the coverage of the domain-specific bacterial probe EUB338. In sediments from two hydrothermal seeps on Vulcano Island, Italy, the microbial community structure was analyzed by FISH with both established and the new oligonucleotide probes, showing the applicability of Arglo32, Tcoc164, and Aqui1197/hAqui1045 to natural samples. At both sites, all major groups of (hyper)thermophiles, except for methanogens, were detected: Crenarchaeota (19%, 16%), Thermococcales (14%, 22%), Archaeoglobales (14%, 12%), Aquificales (5%, 8%), *Thermotoga*/*Thermosipho* spp. (12%, 9%), *Thermus* sp. (12%, none), and thermophilic *Bacillus* sp. (12%, 8%).

Keywords FISH · Oligonucleotide probes · 16S rRNA · Sediment · Thermophiles

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A. Rusch (✉) · J. P. Amend
Department of Earth and Planetary Sciences,
Washington University, One Brookings Drive,
St. Louis, MO 63130, USA
E-mail: rusch@levee.wustl.edu
Tel.: +1-314-9359088
Fax: +1-314-9357361

Introduction

Over the past decade, the application of cultivation-independent approaches in microbial ecology, including environmental clone libraries and fluorescence in situ hybridization (FISH), has increased tremendously. In a wide variety of natural and engineered habitats, microbial community structures have been investigated by probing with labeled, rRNA-targeted oligo- or polynucleotides, with phylogenetic resolution ranging from domain to species level (Böckelmann et al. 2000; Bond and Banfield 2001; Glöckner et al. 1999; Harmsen et al. 1997; Kalmbach et al. 1997; Llobet-Brossa et al. 1998; Pernthaler et al. 2002a; Ravenschlag et al. 2001; Snaidr et al. 1997; Zarda et al. 1997). Prokaryotic communities that inhabit geothermally heated fluids and sediments have received considerable attention, much of it focused on phylogenetic diversity (Brinkhoff et al. 1999; Ferris and Ward 1997; Huber et al. 2003; Jackson et al. 2001; Nold et al. 1996; Reysenbach et al. 2000; Sievert et al. 2000; Simmons and Norris 2002; Teske et al. 2000). Quantitative fluorescence in situ hybridization (FISH) studies in thermal environments, however, are rare (Harmsen et al. 1997), largely due to the lack of a comprehensive set of adequate probes. Thermophilic target groups of existing probes include the archaeal order Thermoplasmales (Bond and Banfield 2001), the bacterial order Aquificales, the genera *Thermotoga*/*Thermosipho* and *Thermus*, as well as the thermophilic *Bacillus* species (Harmsen et al. 1997). When compared to the most recent sequence databases, the probe Hydr540—which targets the Aquificales—is no longer satisfactory with respect to both specificity and coverage. Aquificales, as well as members of the archaeal orders Archaeoglobales and Thermococcales, although considered important inhabitants of hydrothermal environments, are precluded from quantification by the deficiency of appropriate FISH probes.

The cultivated members of Archaeoglobales are strictly anaerobic, obligate or facultative chemoauto-

trophs that grow optimally at temperatures of 80–88°C and circumneutral pH (Burggraf et al. 1990; Hafenbradl et al. 1996; Huber et al. 1997; Kashefi et al. 2002; Stetter 1988; Stetter et al. 1993). *Archaeoglobus* sp. use various oxidized sulfur compounds as electron acceptors, whereas *Geoglobus ahangari* and *Ferroglobus placidus* reduce Fe(III), and the latter is also capable of nitrate reduction (Hafenbradl et al. 1996; Kashefi et al. 2002). Culturing studies and environmental DNA sequence analyses have shown that *Archaeoglobus* inhabits continental hot springs, shallow and deep-sea hydrothermal vents, and oil reservoirs (Barns et al. 1994, 1996; Reysenbach et al. 2000; Takai et al. 2000).

The order Thermococcales is a large cosmopolitan group, with representatives found in deep-sea and shallow marine hydrothermal systems, geothermal wells, solfataras, oil reservoirs, and beach sediments. Isolated species of Thermococcales grow optimally at temperatures of 75–88°C (*Thermococcus* sp., *Palaeococcus* sp.) or 95–100°C (*Pyrococcus* sp.); most are strict anaerobes, but some are microaerophiles (Amend et al. 2003a); their optimal pH is circumneutral or, in a few cases, moderately alkaline (e.g., Amend et al. 2003a; Jurgens 2002; Takai et al. 2000). Thermococcales are exclusively heterotrophic, growing on a variety of organic compounds by fermentation or sulfur respiration.

The order Aquificales not only includes the most thermophilic bacterial genus *Aquifex*, but is also metabolically unique among inhabitants of hydrothermal systems. The species isolated to date are obligate or facultative aerobes that oxidize H_2, reduced sulfur compounds, formate, or formamide (Deckert et al. 1998; Huber et al. 1992, 1998; Kawasumi et al. 1984; Kryukov et al. 1983; L'Haridon et al. 1998; Shima and Suzuki 1993; Stöhr et al. 2001). The majority of representatives cannot be detected by the domain-specific probe EUB338 (Daims et al. 1999), emphasizing the need for a probe that covers the Aquificales. To remedy this situation, we designed a new probe for Aquificales as well as probes for Archaeoglobales and Thermococcales. The present study paves the way towards FISH analyses of microbial communities in hydrothermal systems.

Materials and methods

Probe design

All probes and the accessory oligonucleotide were designed with the PC software PRIMROSE, which allows for nucleotides with degenerate positions (Ashelford et al. 2002), and the current version of the rRNA database RDP-II (http://rdp.cme.msu.edu/), amended with additional sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/). The database probeBase (http://www.microbial-ecology.de/probebase/) was consulted for information on oligonucleotide probes known to date (Loy et al. 2003).

Custom-synthesized oligonucleotides were obtained from ThermoHybaid, Ulm, Germany. The order-specific probes were 5′-labeled with Oregon Green 488, the domain-specific ones with 5,5′-disulfo-1,1′-(γ-carbopentynyl)-3,3,3′,3′-tetramethylindolocarbocyanin-N-hydroxysuccinimide ester (Cy3), and the helper oligonucleotide remained unlabeled.

Probe evaluation

For each probe, whole-cell hybridization conditions (stringency) were optimized using cell cultures of a target organism and the nontarget organism with the lowest number of mismatches to the probe. Further reference strains belonging to the target order were then hybridized at optimal stringency with the newly designed oligonucleotide, the domain-specific probe ARCH917 (Loy et al. 2002) or EUB338 (Daims et al. 1999) as a positive control, and NON338 (Ravenschlag et al. 2001) as the negative (or background) control probe.

Organisms

All reference strains used in this study are listed in Table 1. It should be noted that many of these organisms are difficult to grow optimally in culture, resulting in heterogeneous populations with respect to cell wall structure and ribosome content, and thus, detectability by FISH.

Cell fixation

Three commonly applied fixation protocols were compared with respect to their efficiency in permeabilizing archaeal thermophiles: treatment with formalin (as described in the following paragraph), ethanol (Roller et al. 1994), and lipase/proteinase (Davenport et al. 2000). Each protocol was applied to cells from growing cultures of *Thermococcus celer* DSM 2476. The fixed cells were then examined by FISH as described below to evaluate the permeabilization of their cell walls by the different treatments. Around 80% of the *T. celer* cells were detected by whole-cell hybridization with ARCH917 (negative control subtracted), without significant differences between the fixation protocols. The lipase/proteinase treatment was accompanied by considerable cell loss.

Based on these results, the following protocol was applied to all cell cultures: Aliquots of pure cultures were fixed with phosphate buffered saline solution (PBS)-buffered formalin (final concentration: 2%) for 3 h at 4°C. After centrifugation (14,000 *g*, 5 min), the supernatant was removed, and the pellet was washed twice with 1.5 ml PBS buffer (pH = 7.3). The pelleted cells were preserved by addition of 0.5 ml PBS buffer and 0.5 ml ethanol and stored at −20°C until analysis.

Table 1 Reference strains used in the specificity studies

	Source[a]
Archaeoglobus fulgidus DSM 4304^T	DSMZ[b]
A. profundus DSM 5631^T	CH
A. veneficus DSM 11195^T	DSMZ[b]
Ferroglobus placidus DSM 10642^T	DSMZ[b]
Geoglobus ahangari	KK (Kashefi et al. 2002)
Pyrodictium occultum DSM 2709^T	DSMZ
Pyrolobus fumarii DSM 11204^T	DSMZ[b]
Thermococcus celer DSM 2476^T	DSMZ
T. litoralis DSM 5473^T	DSMZ
T. alcaliphilus DSM 10322^T	DSMZ[b]
T. gorgonarius DSM 10395^T	DSMZ
T. acidaminovorans DSM 11906^T	DSMZ[b]
T. aegaeus DSM 12767^T	DSMZ
Palaeococcus furiosus DSM 3638^T	CH
Pyrococcus woesei DSM 3773^T	DSMZ
Palaeococcus ferrophilus DSM 13482^T	DSMZ
P. helgesonii DSM 15127^T	Vulcano Island, Italy
Hot vent isolate LS1	Hawaii (unpublished data)
Hot vent isolate CS1	Panarea, Italy (unpublished data)
Stetteria hydrogenophila DSM 11227^T	DSMZ
Aquifex pyrophilus DSM 6858^T	DSMZ
Hydrogenobacter hydrogenophilus DSM 2913^T	DSMZ
H. thermophilus DSM 6534^T	DSMZ
H. acidophilus DSM 11251^T	DSMZ
Desulfurobacterium thermolithotrophum DSM 11699^T	DSMZ
Hydrogenothermus marinus DSM 12046^T	DSMZ
Thermocrinis ruber DSM 12173^T	DSMZ[b]
Dictyoglomus thermophilum DSM 3960^T	DSMZ

[a] *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; *CH* kindly provided by C.H. House, Pennsylvania State University, University Park, Penn., USA; *KK* kindly provided by K. Kashefi, University of Massachusetts, Amherst, Mass., USA

[b] Deposited in the DSMZ by Prof. K.O. Stetter, Universität Regensburg, Germany

Sediment sampling sites

Vulcano Island (38.4°N, 015.0°E) is part of the volcanic Aeolian Arc in a convergent tectonic environment of the southern Tyrrhenian Sea, where venting and diffuse emission of hydrothermal fluids and gases are ubiquitous. Our sampling sites were Stinky Surf Rock and Punto Sette, two hydrothermal seeps on Vulcano Island. Stinky Surf Rock is located in the surf zone and discharged acidic (pH = 3.7), hot (85°C), saline fluid. Punto Sette is a subaerial sediment seep that emitted acidic (pH = 2.7) fluids of low salinity at 81°C (Amend et al. 2003b).

Sample collection and processing

In June 2001, geothermally heated sediments were sampled at Stinky Surf Rock and Punto Sette. From each site, 50 cm^3 of sediment was collected and stored at −20°C until analysis. Aliquots (0.5 cm^3) were fixed with PBS-buffered formalin (final concentration: 2%) for 3 h at 4°C. After centrifugation (14,000 *g*, 5 min), the supernatant was removed, and the pellet was washed twice with 1.5 ml PBS buffer (pH = 7.1). The pelleted cells were preserved by addition of 0.5 ml PBS buffer and 0.5 ml ethanol and stored at −20°C until analysis.

Prior to hybridization, cells were dislodged from mineral surfaces by mild ultrasonic treatment (2 min, pulsed) and suspended in 12×1.5 ml PBS buffer; PBS and ethanol were added to a total volume of 50 ml (final concentration: 50% ethanol).

Hybridization

The fixed cells were concentrated on polycarbonate membrane filters (0.2-μm pore size), allowed to air dry, and stored in a petri dish at −20°C until hybridization. Filter sections were incubated with fluorescently labeled (Cy3, OregonGreen 488) oligonucleotide probes and, if applicable, with equimolar amounts of helper oligonucleotide in an equilibrated humidity chamber at 46°C for 2–3 h (Manz et al. 1992; Snaidr et al. 1997). Information on the probes, their targets, and optimal stringency to be applied is given in Table 2 (and references therein). To minimize unspecific binding of probes to particles, 2% blocking reagent was added to the hybridization buffer (Nogales et al. 2001). Excess reagent was removed with washing buffer at 48°C for 15 min. After rinsing with distilled water, the hybridized filter sectors were counterstained with 4′-6′-diamidino-2-phenylindole (DAPI), rinsed briefly with distilled water, air dried in the dark, and mounted on glass slides in Citifluor AF87.

Microscopy and evaluation

The slides were examined by epifluorescence microscopy (Zeiss Axioskop2) using a magnification of 1,300×. On 1–3 filters, cells showing probe-conferred fluorescence and DAPI-stained cells in the same field of view were enumerated in 12–24 randomly chosen counting grids each, corresponding to 1,300–8,900 cells. The unspecific background signal (NON 338) was subtracted from the signal obtained with the other probes. Signal intensity was quantified indirectly as the percentage of cells whose brightness exceeded the visual detection limit.

To compare the number of probe-detected cells to the number of false positives, we applied a *t*-test or a Behrens/Fisher test, depending on the result of an *F*-pretest for possible differences in variance (Sachs 1997). The same tests were also applied to compare the number of cells detected, with the newly designed probes and those detected with well-established domain-specific probes. Lastly, these tests were employed to compare cell detection between hybridizations with and without addition of the helper oligonucleotide.

Table 2 Oligonucleotide probes applied in this study and their phylogenetic target groups. Coverage, i.e., the number of complementary sequences relative to the total number of sequences in the target group, was inferred from the current version of the rRNA database RDP-II (http://rdp.cme.msu.edu/). *Stringency* refers to the concentration of formamide in the hybridization buffer. The probe Aqui1197 was applied with equimolar amounts of the unlabeled helper oligonucleotide hAqui1045

Probe	Target organisms	Coverage (% of target sequences)	Stringency (% of formamide)	References
NON 338	Negative control		35	Ravenschlag et al. (2001)
EUB 338	Domain Bacteria (excl. most Aquificales)	90	35	Amann et al. (1990); Daims et al. 1999
ARCH 917	Domain Archaea	72	0, 20	Loy et al. (2002)
ARC 344	Domain Archaea	61	0, 20	Raskin et al. (1994)
Cren 499	Thermophilic Crenarchaeota	31	0	Burggraf et al. (1994)
Aqui 1197	Bacterial order Aquificales	79	30	This study
Arglo 32	Euryarchaeal order Archaeoglobales	75	25	This study
Tcoc 164	Euryarchaeal order Thermococcales	86	20	This study
Ttoga 660	Bacterial genera *Thermotoga, Thermosipho*	35	20	Harmsen et al. (1997)
Thus 438	Bacterial genus *Thermus*	45	60	Harmsen et al. (1997)
Tbcil 832	Thermophilic species of the genus *Bacillus*	47	40	Harmsen et al. (1997)
EUB 338/Aqui 1197	Domain Bacteria (incl. Aquificales)	91	30	
ARCH 917/ARC 344	Domain Archaea	93	0	

Results

Archaeoglobales—sequence information

In this study, we designed the oligonucleotide probe 5′-TTA GTC CCA GCC GGA TAG CA-3′, hereafter referred to as Arglo32. Its target region on the 16S rRNA comprises *Escherichia coli*-equivalent positions 32–51. The accessibility of this target region, according to models of rRNA secondary structure and rated on a scale of I for best to VI for worst (Behrens et al. 2003; Fuchs et al. 1998), was predicted to be class II, compared to classes II–III accessibility for the domain-specific probe ARCH917.

Within the target group—the order Archaeoglobales—complementary rRNA sequences occur in all six cultured species as well as six clones from environmental samples; one clone has one mismatch, and three clones have at least three mismatches to the probe sequence. Thus, depending on the stringency applied, Arglo32 covers 75–81% of the known target sequences. Outside the target group, there is no sequence complementary to Arglo32, and only one cloned sequence with a single mismatch to the probe. The cultured species with the lowest number of mismatches to the probe sequence are *Pyrodictium occultum* and *Pyrolobus fumarii*, both with mismatches in three positions.

Archaeoglobales—experimental evaluation

The negative control organisms, *P. occultum* and *P. fumarii*, were hybridized with Arglo32 at stringencies between 15 and 40% formamide. The number of detected cells did not differ significantly ($\alpha = 0.10$) from that of the false positives obtained with the background control probe NON338. Significantly more cells ($\alpha < 0.01$) were detected with the probe ARCH917.

Archaeoglobus fulgidus, serving as the main positive control organism, was hybridized with Arglo32 at stringencies between 20 and 50% formamide. Cell detection was found to decline when the formamide concentration in the hybridization buffer exceeded 25–30%. A stringency of 25% formamide was applied in all following hybridizations with Arglo32 because it was considered sufficiently discriminating against nontarget organisms, while avoiding a loss of signal in *A. fulgidus*. The positive control organisms to the order Archaeoglobales (Table 1) were hybridized with Arglo32, ARCH917, and NON338 at their optimal stringencies. In all *Archaeoglobus* species and in *Geoglobus ahangari*, no significant differences were noted in cell detection between Arglo32 and ARCH917 ($\alpha = 0.10$). With *Ferroglobus placidus*, more cells were detected with Arglo32 than with ARCH917 ($\alpha < 0.01$).

Thermococcales—sequence information

We designed the degenerate oligonucleotide 5′-CAV RCC TAT GGG GGA TTA GC-3′, hereafter referred to as Tcoc164. Its target region on the 16S rRNA comprises *E. coli*-equivalent positions 164–183. The accessibility of this target region was rated class IV. Within the target group—the order Thermococcales—complementary rRNA sequences occur in 37 cultured species as well as in 27 clones from environmental samples; *Thermococcus sibiricus* and one clone have one mismatch, *Palaeococcus helgesonii* has a terminal and a central mismatch, and seven clones have at least three mismatches to the probe sequence. Thus, Tcoc164 covers 86–90% of the known target sequences at stringencies allowing for zero to two mismatches,

respectively. Outside the target group, no sequence complementary to Tcoc164 was identified, and one mismatch to the probe occurs in *Stetteria hydrogenophila*.

Thermococcales—experimental evaluation

S. hydrogenophila and *T. celer* were used as the negative and main positive control organisms, respectively. They were hybridized with Tcoc164 at stringencies between 0 and 30% formamide; the results are shown in Fig. 1. At stringencies between 15 and 20% formamide, Tcoc164 discriminates well against a single-mismatch organism, but still fully detects cells of *T. celer*. In all following hybridizations with Tcoc164, a stringency of 20% formamide was applied.

The positive control organisms to the order Thermococcales (Table 1) were hybridized with Tcoc164, ARCH917, and NON338 at their optimal stringencies, and, with the exception of *P. helgesonii*, no significant differences in cell detection were observed between Tcoc164 and ARCH917 ($\alpha = 0.10$). In *S. hydrogenophila*, significantly more cells were detected with ARCH917 than with Tcoc164 ($\alpha < 0.01$). The number of cells of *P. helgesonii* detected with Tcoc164 did not differ significantly ($\alpha = 0.10$) from that of the false positives; significantly more cells ($\alpha < 0.01$) were detected with the probe ARCH917.

Aquificales—sequence information

We designed the degenerate oligonucleotide probe 5′-GCA TAA AGG GCA TAM TGA YC-3′, hereafter referred to as Aqui1197. Its target region on the 16S rRNA comprises *E. coli*-equivalent positions 1197–1216. The accessibility of this target region was rated class IV, compared to classes II–III accessibility for the domain-specific probe EUB338. Within the target group—the bacterial order Aquificales—complementary rRNA sequences occur in 13 cultured species as well as 71 clones from environmental samples. Further, a terminal mismatch occurs in *Hydrogenothermus marinus* and a central mismatch is noted in one cloned sequence. Four species (*Balnearium lithotrophicum, Desulfurobacterium crinifex, D. thermolithotrophicum, Thermovibrio ammonificans*) and 13 clones have two mismatches to the probe sequence, and three clones differ in more than three positions from the exact complement of the probe. Thus, Aqui1197 covers 79–97% of the known target sequences at stringencies allowing for zero to two mismatches, respectively. Outside the target group, no sequence complementary to Aqui1197 and one cloned sequence with one mismatch to the probe are observed. Among cultured nontarget organisms, the lowest number of mismatches to the probe sequence is found in *Dictyoglomus thermophilum* (Table 1) and 14 other bacterial species.

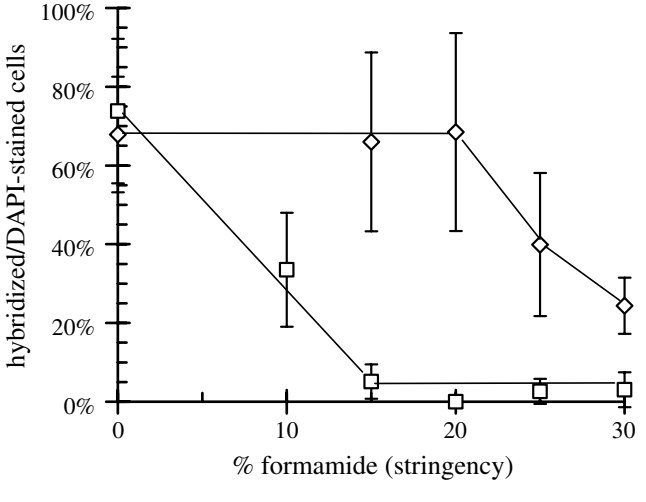

Fig. 1 Number of reference cells detected by hybridization with Tcoc164, given as percentage of total [4′-6′-diamidino-2-phenylindole (*DAPI*)-stained] cell number, at different formamide concentrations in the hybridization buffer. *Diamonds T. celer* (positive control), *squares Stetteria hydrogenophila* (negative control). *Error bars* represent the standard deviation between 12 and 48 evaluated fields of view

To improve the accessibility of the probe's target region, a helper nucleotide was designed. The oligonucleotide 5′-ACG GCC ATG CAC CAC CTG TG-3′, hereafter referred to as hAqui1045, targets *E. coli*-equivalent positions 1045–1064, which base pair with the target region of Aqui1197, forming helix 39 in bacterial 16S rRNA. The helper effect is achieved by hAqui1045 competing with intramolecular base pairings, thus liberating the target region of Aqui1197. Among Aquificales, all cultured species—except *Tv. ammonificans*—and most cloned sequences can form the desired hybrid with hAqui1045 if stringency allows for zero or one mismatch. Under these conditions, only 54 hybrids are possible between hAqui1045 and nontarget sequences from thermophilic bacteria. Hence, we expect moderate dissipation of the helper effect by the presence of nontarget cells in natural samples.

Aquificales—experimental evaluation

The negative control organisms, *D. thermophilum* and *D. thermolithotrophicum*, were hybridized with Aqui1197 at stringencies between 25 and 45% formamide, both with and without addition of hAqui1045. The number of detected cells did not differ significantly ($\alpha = 0.10$) from the number of false positives, as determined with the background control probe NON338. Significantly more cells ($\alpha < 0.01$) were detected with the probe EUB338.

Aquifex pyrophilus, serving as the main positive control organism, was hybridized with Aqui1197 at stringencies between 0 and 45% formamide. Cell detection decreased both with and without hAqui1045 when the formamide concentration in the hybridization

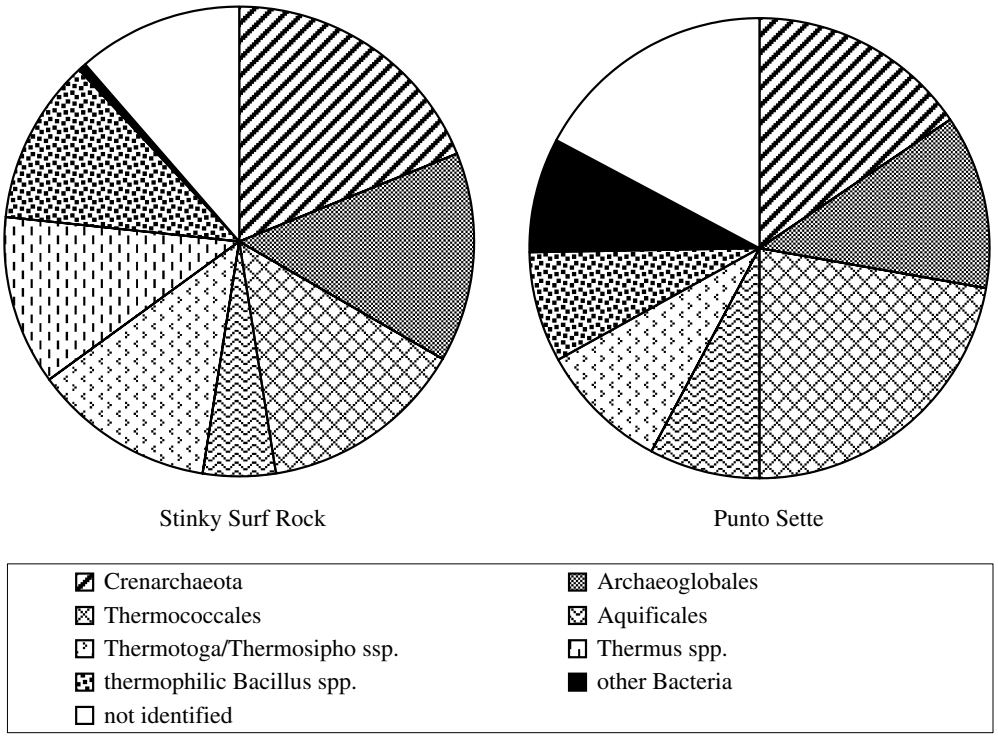

Fig. 2 Community structure of sedimentary prokaryotes as analyzed by fluorescence in situ hybridization

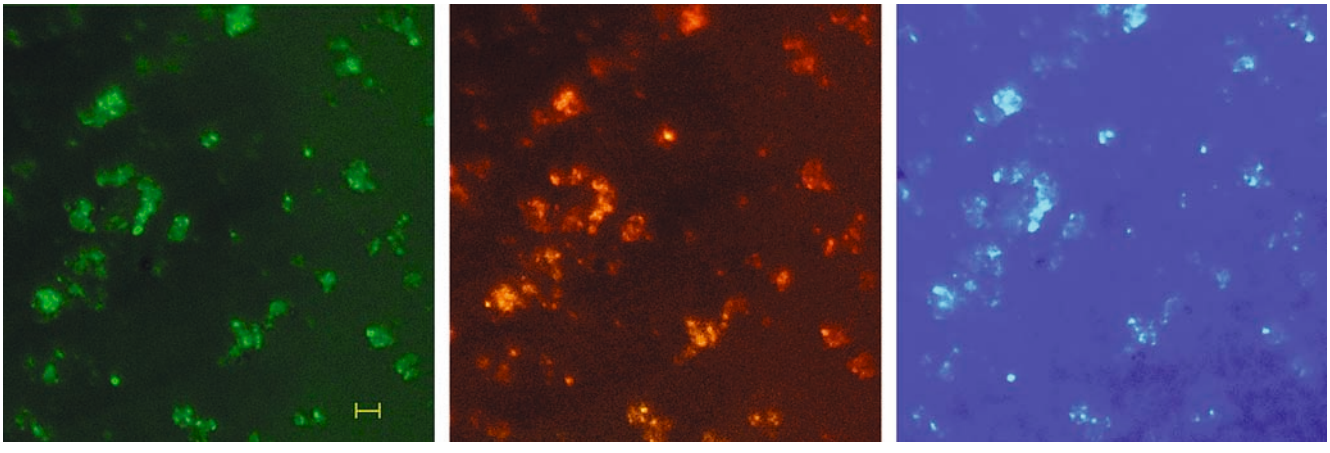

Fig. 3 Epifluorescence microphotograph of benthic microbes from Punto Sette. *Scale bar* 2 μm, *Green* Thermococcales detected by OregonGreen-labeled Tcoc164, *red* archaea detected by Cy3-labeled Arch917 and Arc344, *blue* DAPI-stained cells. Brightness, shape, and size of the luminescent objects were used to distinguish cells from other particles

buffer exceeded 30–35%. A stringency of 30% formamide was applied in all following hybridizations with Aqui1197, because it was considered sufficiently discriminating against nontarget organisms while avoiding a loss of signal in *A. pyrophilus*. The positive control organisms to the order Aquificales (Table 1) were hybridized with Aqui1197, Aqui1197/hAqui1045, and NON338 at their optimal stringencies. These species (with the exception of *H. marinus*) are among the few bacteria not covered by EUB338 (Daims et al. 1999), and hence, they were not double checked with this domain-specific probe. The addition of hAqui1045 improved the detection of all positive control organisms, with a significant increase observed in *A. pyrophilus* ($\alpha < 0.01$) and *Hydrogenobacter acidophilus* ($\alpha < 0.05$).

Field samples

Cell abundances in sediment samples from Stinky Surf Rock and Punto Sette were 2.6×10^8 cm^{-3} and 1.3×10^9 cm^{-3}, respectively. The results of our FISH analyses on these samples are summarized in Fig. 2. As an illustrative example, Fig. 3 shows microphotographs of cells from Punto Sette, as visualized by FISH with Tcoc164 and Arch917/Arc344.

Archaea

At both sites, almost half the cells (SSR 46%, PtoS 47%) could be identified as archaeal by cohybridization with Arch917 and Arc344. While all archaea at Punto Sette were also detected by Arch917 alone, this was not the case at Stinky Surf Rock (data not shown), where cohybridization significantly improved the detection of archaea compared to Arch917 alone and Arc344 alone. As differences in target coverage between these two probes are dispersed over the domain, this result does not allow for conclusions on the presence or absence of certain phylogenetic groups within Archaea.

All archaea detected on the domain level were also detected by one of the probes targeting Crenarchaeota, Archaeoglobales, or Thermococcales. Thermococcales were significantly ($\alpha < 0.01$) more abundant at Punto Sette (22%) than at Stinky Surf Rock (14%), whereas the relative abundances of Crenarchaeota (SSR 19%, PtoS 16%) and Archaeoglobales (SSR 14%, PtoS 12%) did not differ significantly ($\alpha = 0.10$) between the two sites.

Bacteria

The relative abundance of bacterial cells was significantly ($\alpha < 0.05$) higher at Stinky Surf Rock (41%) than at Punto Sette (33%). The sites also differed significantly ($\alpha < 0.05$) in the detection of each targeted subgroup within the domain. At Stinky Surf Rock, 5% of all cells could be assigned to the order Aquificales, and the other three groups comprised 12% each. At Punto Sette, no *Thermus* sp. were detected, 9% of all cells belonged to the genera *Thermotoga* or *Thermosipho*, and 8% each were identified as Aquificales or thermophilic *Bacillus* species. At both sites, the overall detection of bacterial cells was increased by cohybridization with EUB338 and Aqui1197 compared to hybridization with EUB338 alone (data not shown).

Discussion

Among the array of molecular methods applied in environmental studies, FISH is appreciated as a versatile, inexpensive, and quantitative tool to analyze microbial community structures. The dearth of adequate probes, however, has largely precluded the application of FISH to hydrothermal ecosystems. To remedy this situation, we developed new oligonucleotide probes that target three major orders of (hyper)thermophilic organisms.

Coverage and specificity

To aid in their meaningful application, probes should be designed to maximize both coverage and specificity, i.e., to detect most sequences within their target taxon, while hybridizing with only a minimum number of nontarget sequences (Ashelford et al. 2002). Arglo32, Tcoc164, and Aqui1197 cover more than 75, 86, and 79% of their target orders, respectively, while clearly discriminating against even single-mismatch nontargets (Fig. 1), rendering them comprehensive and specific tools for the detection of Archaeoglobales, Thermococcales, and Aquificales.

As a consequence of continuously growing sequence databases, coverage and specificity of any probe may change over time. As an example, we briefly discuss Hydr540, the previously applied probe for Aquificales (Harmsen et al. 1997). Though originally well designed, it now covers only a third of the target sequences known, and complementary sequences have been found in at least six nontarget organisms from environments where cooccurrence with members of the Aquificales is likely. Based on the currently known 16S rRNA sequences, Aqui1197 should prove superior to Hydr540 in detecting Aquificales with respect to both coverage and specificity.

Even well-designed oligonucleotides are not necessarily successful probes in whole-cell hybridization analyses, and hence, we evaluated Arglo32, Tcoc164, and Aqui1197 experimentally with reference strains (Table 1). The hybridizations in this study confirmed the expected discrimination against nontarget organisms. They also confirmed the detection of all positive control organisms to match or exceed the detection by well-established domain-specific probes. Thus, our newly designed oligonucleotides are suitable for practical application as probes in whole-cell FISH analyses.

Signal intensity

Detection limits in whole-cell FISH are determined by cell wall permeability, cellular ribosome content, accessibility of the target site (secondary/tertiary structure), fluorescence intensity, and the sensitivity of the detecting eye or camera. Among the various approaches to improve the overall sensitivity of FISH (deLong et al. 1999; Hodson et al. 1995; Nogales et al. 2002; Ouverney and Fuhrman 1997; Pernthaler et al. 2002a, b), unlabeled helper nucleotides were applied to aid the hybridization of probes to target sites that are base paired within the secondary structure of the rRNA molecule (Fuchs et al. 2000). In the present study, the accessory oligonucleotide hAqui1045 slightly improved cell detection by Aqui1197; signal intensity increased significantly in two out of six positive control organisms. In the other species, base pairing may have less impact on the ribosomal tertiary structure than, for example, proteins, rendering the helper nucleotide comparatively ineffective. The indigenous variation of fluorescence intensity within a batch culture, which determines the resolution of our indirect brightness scale, is also likely to differ between cultures; in some cases, small enhancements may simply not be resolved. As no negative side effects were observed, we recommend the use of hAqui1045 in all hybridizations with Aqui1197.

Applications

The probes Arglo32, Tcoc164, and Aqui1197 serve as powerful tools in investigations of (hyper)thermophilic microbial communities. Together with the probes Cren499, Ttoga660, Thus438, and Tbcil832 (Table 2), many major groups of bacteria and archaea known to inhabit hydrothermal systems could be quantified (Fig. 2). Although reliable FISH analyses of sediment samples are not trivial, the actual presence of the probe-detected groups is highly credible. The hydrothermal environments of Vulcano Island have repeatedly been the source of newly isolated members of these groups, and environmental rDNA clone libraries (Rogers and Amend, in preparation), enrichment experiments (Rusch and Amend, in preparation), and metabolic studies (Tor et al. 2003) give affirmative evidence.

Further phylogenetic resolutions, e.g., within the Crenarchaeota, may be desirable for certain investigations in microbial ecology, but they are beyond the scope of the present study. The order Aquificales, which is largely missed by the domain-specific probe EUB338 (Daims et al. 1999), is well covered by Aqui1197, with almost identical stringency requirements. Thus, Aqui1197 can serve as a supplement to EUB338 to extend its coverage. Supplementary probes targeting mesophilic bacteria were developed earlier (Daims et al. 1999), and Aqui1197 will further improve the coverage of the bacterial domain, particularly in hydrothermal environments.

Field samples

Besides serving as the first examples for the successful application of our newly designed probes, the sediment samples from Vulcano Island also provide insight into the microbial ecology of hydrothermal systems. The local vent fluids contain significant concentrations of a variety of short-chain fatty acids (Amend et al. 1998), aldoses (Skoog et al., in preparation) and amino acids (Svensson et al., in preparation), and some of these organic compounds were shown to be metabolized at 90°C in anoxically incubated sediment collected only a few meters from Stinky Surf Rock (Tor et al. 2003). However, in that study, the microbial community responsible for fermentation and sulfate reduction of organic compounds was not investigated. The present FISH study is the first to identify and quantify (hyper)thermophiles in the shallow marine hydrothermal system at Vulcano Island (see Fig. 2).

Earlier studies of hydrothermal sediments at shallow marine sites strongly suggest the presence of not only (hyper)thermophilic, but also mesophilic prokaryotes (Sievert et al. 1999; Takai and Sako 1999), and preliminary FISH data of sediments collected at Stinky Surf Rock in 2003 (Rusch et al., in preparation) also indicate a mixed population of thermophiles and mesophiles. In the porous sediments at Vulcano Island, convective mixing of hydrothermal fluids with cool seawater will not only mix the microbial populations they carry, but may also create microniches that allow for thermophiles and mesophiles to thrive in very close proximity of each other. This view finds some support in our data of bacterial community structure (Fig. 2), viz., the significant presence of *Bacillus* sp., which are only moderately thermophilic, and of Bacteria that remained undetected by probes targeting thermophilic groups. Evidence of archaeal mesophiles, however, was not found in our sediment samples, as the archaeal community entirely consisted of Archaeoglobales, Thermococcales, and thermophilic Crenarchaeota (Fig. 2). Mesophilic marine Crenarchaeota are missed by the probe Cren499, while 51% of the published sequences in this group are detectable with the probes Arch917/Arc344. Although we cannot rule out the presence of those mesophilic Crenarchaeota that escape detection by the domain specific probes, it is more likely that the cooling impact of seawater was of minor importance at the sampling sites.

With the exception of *Thermus* sp., all detected groups include cultured representatives that were originally isolated from Vulcano Island. The FISH analyses, together with the in vitro metabolic properties of these and other isolates, provide insight into which metabolic reactions are likely to occur in situ. Fermentation and sulfate reduction were found to be the dominant anaerobic processes (Tor et al. 2003), although thermodynamic calculations show significantly higher energy yields from iron or nitrate reduction (Amend et al. 2003b). The order Archaeoglobales alone, which was well represented in the microbial community at both study sites (Fig. 2), comprises sulfate, nitrate, and iron reducing species, and the diversity of detected groups alludes to an even greater variety of consortia and metabolic interactions.

Sediment from Punto Sette not only contained more cells than sediment from Stinky Surf Rock, but it also had a significantly higher share of Thermococcales. This group of obligate heterotrophs may be supported by very high organic carbon concentrations measured in the fluids at Punto Sette compared to various other hydrothermal sites on Vulcano Island (Svensson et al., in preparation).

Concluding remarks

The FISH analyses of sediment samples at Vulcano Island clearly illustrate that microbial community structure can be linked to the activity of archaea and bacteria in hydrothermal ecosystems. Quantitative studies applying highly specific oligonucleotide probes for major groups of (hyper)thermophiles, including those developed here, can complement investigations that inventory microbial populations or subpopulations, compute the in situ energetic yields of key metabolic processes, or measure turnover rates. At Vulcano Island, this multipronged approach is beginning to paint a clear picture of

the highly diverse (hyper)thermophilic communities involved in complex metabolic interactions. Given the relatively routine analyses of environmental rDNA sequences, the development of new FISH probes, recent advances in anaerobic culturing techniques, and reliable thermodynamic data at elevated temperatures and pressures for hundreds of organic and inorganic compounds, the multidisciplinary approach employed at Vulcano Island is also amenable to other hydrothermal ecosystems.

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