APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 4061–4068 Vol. 63, No. 10
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Group-Specific 16S rRNA-Targeted Oligonucleotide Probes To Identify Thermophilic Bacteria in Marine Hydrothermal Vents

HERMIE J. M. HARMSEN,[1]† DANIEL PRIEUR,[1,2] AND CHRISTIAN JEANTHON[1]*

Station Biologique, UPR9042, Centre National de la Recherche Scientifique and Université Pierre et Marie Curie, 29680 Roscoff,[1] and Université de Bretagne Occidentale, UFR des Sciences et Techniques, 29285 Brest Cedex,[2] France

Received 12 May 1997/Accepted 28 July 1997

Four 16S rRNA-targeted oligonucleotide probes were designed for the detection of thermophilic members of the domain *Bacteria* known to thrive in marine hydrothermal systems. We developed and characterized probes encompassing most of the thermophilic members of the genus *Bacillus*, most species of the genus *Thermus*, the genera *Thermotoga* and *Thermosipho*, and the *Aquificales* order. The temperature of dissociation of each probe was determined. Probe specificities to the target groups were demonstrated by whole-cell and dot blot hybridization against a collection of target and nontarget rRNAs. Whole-cell hybridizations with the specific probes were performed on cells extracted from hydrothermal vent chimneys. One of the samples contained cells that hybridized to the probe specific to genera *Thermotoga* and *Thermosipho*. No positive signals could be detected in the samples tested with the probes whose specificities encompassed either the genus *Thermus* or the thermophilic members of the genus *Bacillus*. However, when simultaneous hybridizations with the probe specific to the order *Aquificales* and a probe specific to the domain *Bacteria* (R. I. Amann, B. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl, Appl. Environ. Microbiol. 56:1919–1925, 1990) were performed on cells extracted from the top and exterior subsamples of chimneys, positive signals were obtained from morphologically diverse bacteria representing about 40% of the bacterial population. Since specificity studies also revealed that the bacterial probe did not hybridize with the members of the order *Aquificales*, the detected cells may therefore correspond to a new type of bacteria. One of the observed morphotypes was similar to that of a strictly anaerobic autotrophic sulfur-reducing strain that we isolated from the chimney samples. This work demonstrates that application of whole-cell hybridization with probes specific for different phylogenetic levels is a useful tool for detailed studies of hydrothermal vent microbial ecology.

Despite the extreme range of conditions displayed by submarine vent systems, there is evidence for the occurrence of metabolically and genetically diverse groups of thermophilic microorganisms interacting with hydrothermal fluids (24, 28). However, it is today generally accepted that the vast majority of microorganisms detectable by indirect methods in marine and other natural samples, including those collected from much less extreme environments, cannot be assessed by traditional methods that rely on laboratory cultivation (5, 51). In addition, substantial technical difficulties met in recovering black smoker samples, developing appropriate media, and recreating the extreme conditions of this environment in the laboratory have prevented microbiologists from gaining access to the real extent and nature of the existing microbial diversity.

These technical hurdles have been partly overcome by the development of culture-independent molecular methods that often lie in the universal distribution and sequence conservation of the rRNA molecules (52). In particular, the 16S rRNA molecule is perhaps the most widely used nucleic acid target for detection and identification of previously undetected organisms (5, 41, 52). Recently, 16S rRNA-targeted fluorescence in situ hybridization techniques have been successfully applied to the direct identification of microbial cells from the highest to the lowest phylogenetic levels and their accurate enumeration in genetically diverse natural assemblages (3–5, 17, 18, 44).

Recently, the whole-cell hybridization procedure was used in combination with culture-based enrichments to determine the abundance and distribution of thermophilic microorganisms in deep-sea hydrothermal chimneys (18). The use of domain- and kingdom-specific oligonucleotide probes (3, 11, 47) clearly demonstrated that the morphological diversity observed in the domains *Bacteria* and *Archaea* as well as in the archaeal kingdoms *Euryarchaeota* and *Crenarchaeota* was larger than that assessed by culture-based enrichments.

In this study, we report on the development and characterization of group-specific 16S rRNA-targeted oligonucleotide probes for four groups of thermophilic bacteria already isolated from marine coastal and/or deep-sea hydrothermal environments (20–23, 25, 34–37) and our attempts to identify and quantify cells extracted from some of the previously analyzed deep-sea vent chimney samples (18).

MATERIALS AND METHODS

Organisms and culture conditions. All the organisms used as reference strains in this study are listed below (see Table 1). The organisms were cultivated as described in the references cited in the table. The sheathed rod 429 (*Thermotogales*-like) was isolated from samples from a deep-sea hydrothermal system in the North Fiji Basin (37). The aerobic sporeformers SG1 and SG9 were isolated from deep-sea hydrothermal vents at the Lau Basin (35). From deep-sea hydrothermal samples collected from the Guaymas Basin were isolated the aerobic non-spore-forming rods 321 and B1 (34a) and the motile sulfate-reducing rod SL6 (25a). The sheathed rod ME4 (*Thermotogales*-like), the small motile rod BSA, and the aerobic sporeformers VT, VM22, VM27, VM34, and VM77 were isolated from deep-sea hydrothermal vents at the Mid-Atlantic Ridge (32a, 34a, 35).

* Corresponding author. Mailing address: Station Biologique, CNRS UPR 9042, Place Georges-Teissier, B. P. 74, F-29682 Roscoff Cedex, France. Phone: 02 98 29 23 09. Fax: 02 98 29 23 24. E-mail: jeanthon@sb-roscoff.fr.

† Present address: Department of Medical Microbiology, University of Groningen, 9713 GZ Groningen, The Netherlands.

TABLE 1. Organisms used in the specificity studies

Species of isolate[a]	Position on blot[b]	Reference
Thermus sp. (DSM579, *Thermus thermophilus*)	A1	40
Thermus thermophilus (DSM9247)	A2	34
Thermus sp. (DSM674, *Thermus flavus*)	A3	45
Thermus aquaticus (DSM625[T])	A4	9
Thermus scotoductus (ATCC 27978)	A5	30
Rhodothermus marinus (DSM4252[T])	A6	1
Strain B1 (Guaymas Basin)	A7	34a
Strain 321 (Guaymas Basin)	A8	34a
Bacillus sp. (DSM411, *Bacillus caldovelox*)	B1	19
Bacillus sp. (DSM406, *Bacillus caldotenax*)	B2	19
Bacillus sp. (DMS405, *Bacillus caldolyticus*)	B3	19
Bacillus thermoleovorans (DSM5366[T])	B4	54
Bacillus stearothermophilus (DSM22[T])	B5	13
Bacillus thermocatenulatus (DMS730[T])	B6	16
Bacillus sp. (DSM466, *Bacillus thermodenitrificans*)	B7	6
Bacillus thermoglucosidasius DSM2542[T])	B8	50
Strain VM77 (Mid-Atlantic Ridge)	C1	35
Strain VM22 (Mid-Atlantic Ridge)	C2	35
Strain VM34 (Mid-Atlantic Ridge)	C3	35
Strain VM27 (Mid-Atlantic Ridge)	C4	35
Strain VT (Mid-Atlantic Ridge)	C5	34a
Strain SG9 (Lau Basin)	C6	35
Strain SG1 (Lau Basin)	C7	35
Strain BSA (Mid-Atlantic Ridge)	C8	32a
Thermotoga subterranea (DSM9912[T])	D1	26
Thermotoga maritima (DSM3109[T])	D2	20
Thermotoga neapolitana (DMS4359[T])	D3	25
Fervidobacterium islandicum (DSM 5733[T])	D4	23
Fervidobacterium nodosum (DSM 5306[T])	D5	42
Thermosipho africanus (DSM 5309[T])	D6	22
Strain 429 (North Fiji Basin)	D7	37
Strain ME4 (Mid-Atlantic Ridge)	D8	34a
Methanococcus jannaschii (DSM2661[T])	E1	27
Thermococcus celer (DSM2476[T])	E2	55
Pyrococcus furiosus (DMS3638[T])	E3	15
Archaeoglobus furiosus (DSM4304[T])	E4	48
Sulfolobus acidocaldarius (DSM639[T])	E5	8
Methanothermus fervidus (DMS2088[T])	E6	49
Saccharomyces cerevisiae (DSM3797)	E7	32
Escherichia coli (DSM3950)	E8	53
Aquifex pyrophilus (DSM6858[T])	F1	21
Calderobacterium hydrogenophilum (DSM2913[T])	F2	31
Hydrogenobacter thermophilus (DSM6534[T])	F3	29
Strain SL6 (Guaymas Basin)	F4	25a
Methanococcus igneus (DSM5666[T])	F5	10
Desulfurococcus mucosus (DSM2162[T])	F6	56
Pyrococcus abyssi (CNCM I-1302[T])	F7	14
Thermococcus litoralis (DSM5473[T])	F8	38

[a] Collection numbers of species or origins of isolates are shown in parentheses. Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany); ATCC; American Type Culture Collection (Rockville, Md.); CNCM, Collection Nationale de Culture de Microorganismes (Institut Pasteur, Paris, France).

[b] For example, A1 represents lane A, blot 1.

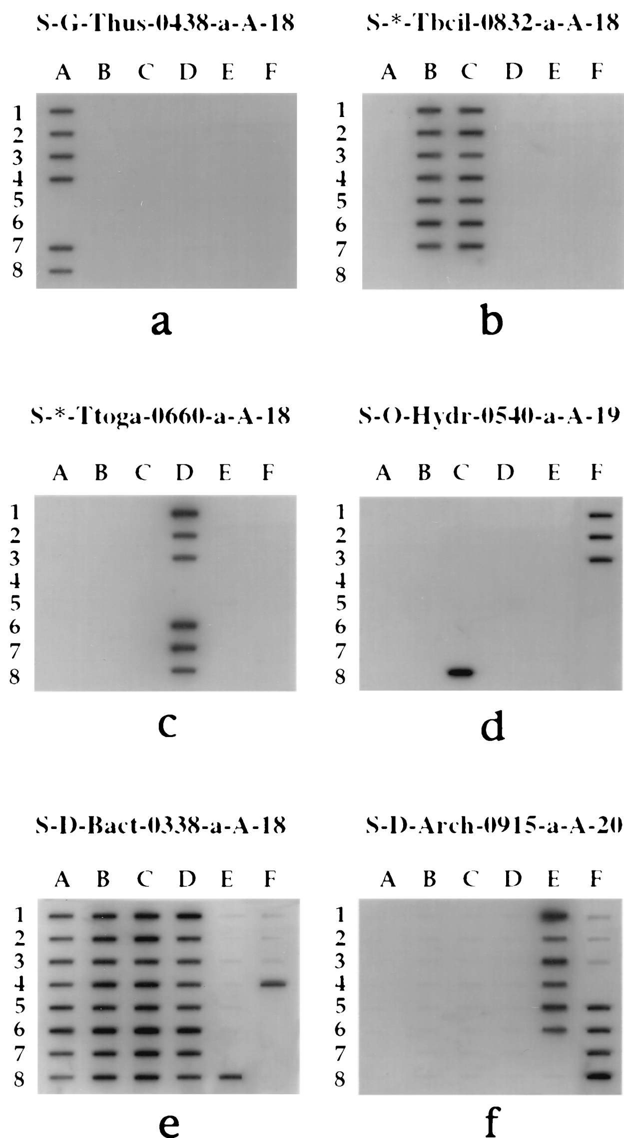


FIG. 1. Slot blot analysis of the probe specificities. Hybridization responses of radioactively labeled probes to nucleic acids from 48 target and nontarget strains were scanned and printed with Adobe (Seattle, Wash.) Photoshop 3.0. The layout of nucleic acid samples on the blots (names of individual organisms) is shown in Table 1. The blots were hybridized with the following probes: S-G-Thus-0438-a-A-18 (a), S-*-Tbcil-0832-a-A-18 (b), S-*-Ttoga-0660-a-A-18 (c), S-O-Hydr-0540-a-A-19 (d), S-D-Bact-0338-a-A-18 (e), and S-D-Arch-0915-a-A-20 (f).

Design and validation of oligonucleotide probes. (i) Design. Alignments of 16S rRNA sequences from the Ribosomal Database Project (RDP) (33) and from the GenBank database (recently published sequences) were screened for group-specific signature sequences distinguishing the genus *Thermus*, thermophilic members of the genus *Bacillus*, the order *Thermotogales*, and the *Hydrogenobacter-Aquifex* group. The selected oligonucleotide sequences were tested for specificity against the ribosomal sequences available in the RDP by use of the CHECK_PROBE analysis function of the RDP. Fluorescein- and rhodamine-labeled and unlabeled oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium).

(ii) Probe optimization and specificity studies. The oligonucleotides were labeled at their 5′ ends with T4 polynucleotide kinase (phosphatase-free; Boehringer Mannheim Biochemicals, Meylan, France) and [γ-^{32}P]ATP (Amersham, Les Ulis, France).

Pure cultures (10 to 25 ml) of the reference strains were centrifuged (5,000 × *g* for 10 min at 4°C), and the pellets were stored at −20°C until they were used for nucleic acid extraction. Nucleic acids were extracted from frozen cell pellets for slot blot hybridization as described before (17) with the following modifications. Gram-positive cells were lysed in STE buffer (46) by a 15-min incubation at room temperature with 20 mg of lysozyme per ml. Sodium dodecyl sulfate (SDS; final concentration, 0.1%) and proteinase K (20 mg/ml) were added, and the mixture was incubated for 15 min at 45°C. All other bacterial and archaeal cells were lysed by vortexing in 10 mM Tris-HCl (pH 8)–1 mM EDTA–0.1% SDS for 10 min. Total nucleic acids were recovered from the lysates by phenol-chloroform extraction and then by ethanol precipitation.

Reference nucleic acids (approximately 30 ng of rRNA in 20 μl of demineralized water) were mixed with 30 μl of denaturing buffer (4% glutaraldehyde in demineralized water with a little drop of 1% bromophenol blue) and incubated for 5 min at room temperature (43). Reference nucleic acids (representing both target and nontarget strains) were applied in replicate to nylon membranes (Zeta-Probe GT; Bio-Rad, Hercules, Calif.) by use of a slot blot format unit

TABLE 2. SSU rRNA sequences of target and nontarget species, probe sequences, and optimized experimental conditions[a]

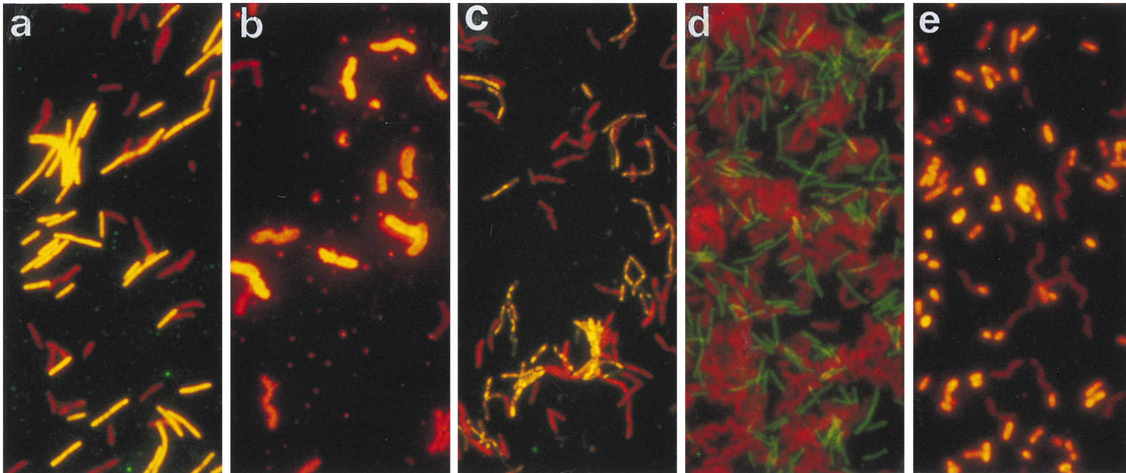
Target and nontarget organisms	Probe names and target sequences[b]	Expt T_d (°C)	% Formamide in FISH[c]
Thermus spp.	S-G-Thus-0438-a-A-18 3′-CTTGGGCCCTGCTTTGGG-5′	52	60
Thermus thermophilus DSM579	5′-GAACCCGGGACGAAACCC-3′		
Thermus flavus DSM675	5′-..................-3′		
Thermus aquaticus ATCC27978	5′-..................-3′		
Thermus filiformis ATCC43280	5′-......A........U..-3′		
Rhodothermus marinus DSM4252	5′-...A.....G.U..UA..-3′		
Meiothermus ruber DSM1279	5′-....U........U.AUG-3′		
Meiothermus silvanus DSM9946	5′-....AG.......UGAUG-3′		
Meiothermus chliarophilus DSM9957	5′-....U........UGGUG-3′		
Thermophilic *Bacillus* sp.	S-*-Tbcil-0832-a-A-18 3′-CAATCTCCCCAGTGTGGG-5′	58	40 (+lysozyme)
Bacillus thermoleovorans DSM5366	5′-GUUAGAGGGGUCACACCC-3′		
"Bacillus caldovelox" DSM411	5′-..................-3′		
"Bacillus caldotenax" DSM406	5′-..................-3′		
"Bacillus caldolyticus" DSM405	5′-..................-3′		
Bacillus stearothermophilus DSM22	5′-..................-3′		
Bacillus thermocatenulatus DSM730	5′-..................-3′		
Bacillus kaustophilus DSM7263	5′-..................-3′		
"Bacillus thermodenitrificans" DSM466	5′-..................-3′		
Bacillus thermoglucosidasius DSM 2542	5′-...........U.UU...-3′		
Bacillus smithii DSM4216	5′-.........C.UC.....-3′		
"Bacillus flavothermus" DSM2641	5′-.........UAUC.....-3′		
Thermotogales	S-*-Ttoga-0660-a-A-18 3′-CCATCTCCCTCTGCCTTG-5′	58	20
Thermotoga subterranea DSM9912	5′-GGUAGAGGGAGACGGAAC-3′		
Thermotoga elfii DSM9442	5′-..................-3′		
Thermotoga maritima DSM3109	5′-..................-3′		
Thermotoga thermarum DSM5069	5′-..................-3′		
Thermosipho africanus DSM5309	5′-..................-3′		
Fervidobacterium islandicum DSM 5733	5′-..C.....C.........-3′		
Fervidobacterium gondwanense ACM5017	5′-A.C.....C.........-3′		
Fervidobacterium nodosum DSM5306	5′-..C.....C...U.....-3′		
Geotoga petraea ATCC51226	5′-ACC.....U........U-3′		
Geotoga subterranea ATCC51225	5′-ACC.....U....A...U-3′		
Petrotoga miotherma ATCC51224	5′-A.C.....U........U-3′		
Aquificales	S-O-Hydr-0540-a-A-19 3′-CCAGGGCTCGCAACGCGCT-5′	54	60
Hydrogenobacter thermophilus DSM6534	5′-GGUCCCGAGCGUUGCGCGA-3′		
Hydrogenobacter acidophilus strain 3H-1	5′-...................-3′		
Calderobacterium hydrogenophilum DSM2913	5′-...................-3′		
Aquifex pyrophilus DSM6858	5′-...................-3′		
Thermus aquaticus ATCC 27978	5′-..G.G........A.C..G-3′		
Thermotoga maritima DSM3109	5′-..GGG.A......A.C..G-3′		

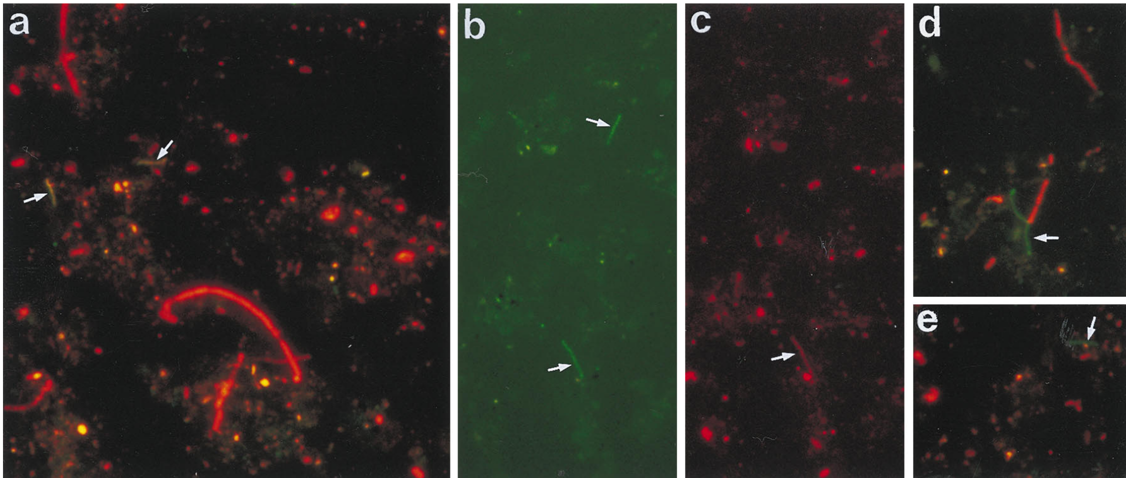
[a] The top SSU rRNA sequence for each group of organisms is the target sequence. Periods in the succeeding sequences for that group signify identical nucleotides, and capital letters indicate nucleotide differences from the target sequence at the respective positions.
[b] Probe nomenclature are based on the oligonucleotide probe database (2). Sequence data are from the RDP database (33) and the GenBank database.
[c] FISH, fluorescence in situ hybridization.

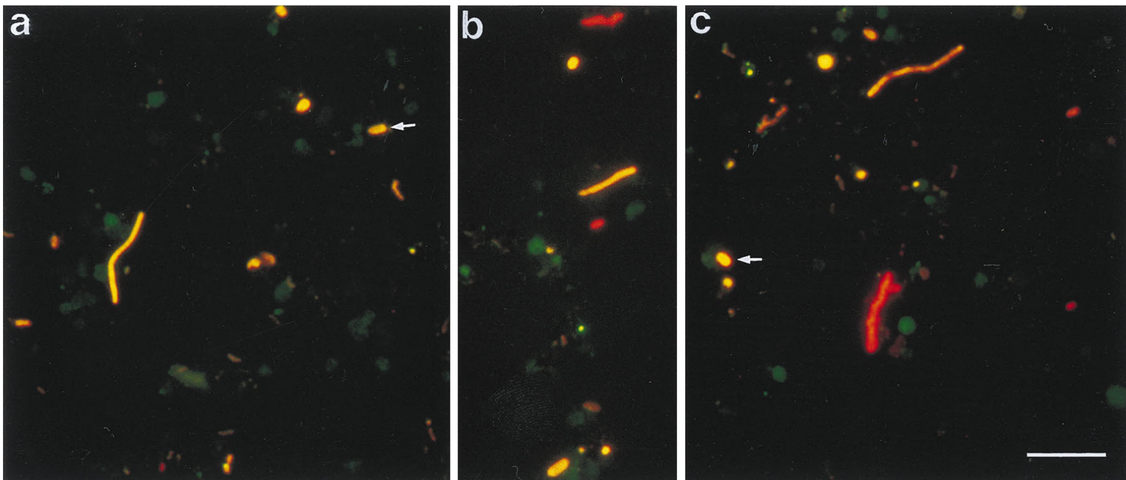
(Bio-Rad). The membranes were pretreated with hybridization buffer (0.5 M phosphate buffer, 7% SDS, 1% bovine serum albumin [BSA], 1 mM of EDTA [pH 7.2]) for 30 min in screw-cap hybridization tubes prior to hybridization with 100 ng of labeled probe. The membranes were hybridized overnight at 40°C (12) and washed with 1% SDS–1× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate [pH 7]) buffer initially at 40°C.

For the determination of the dissociation temperature (T_d) values of the oligonucleotides, eight replicate filters with reference nucleic acids for each probe were then washed in the same buffer at 3°C temperature increments (43 to 66°C), 30 min at each increment (12). Probe remaining in association with the membrane at each wash temperature was quantified by scintillation counting. The T_d (temperature at which one-half of the hybridized probe remained on the filter under the conditions described above) was used as the wash temperature in subsequent hybridization experiments.

The specificity of the oligonucleotide probes for intended target groups was examined by use of membranes blotted with nucleic acids from the strains listed in Table 1 and visualized by exposure of the membranes to Hyperfilm MP (Amersham) for 24 h.


a
b
c
d
e


a
b
c
d
e


a
b
c

(iii) Whole-cell hybridization. Cells of the reference strains were washed once in 3× phosphate-buffered saline (PBS; pH 7.2) (46), fixed in 3% paraformaldehyde in 3× PBS for 3 to 16 h, and stored in 50% (vol/vol) ethanol–1× PBS at −20°C until further use (4). Cells (1 μl) were applied to gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$] with Teflon-preprinted 8-mm-diameter wells. The slides were dried and hybridized at 45°C in a humid chamber as described before (47). The hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS, 0.2 mg of BSA per ml, 0.1 mg of poly(U) per ml] contained 5 ng of fluorescently labeled probe per μl. The optimal formamide concentrations were determined by varying the formamide concentration from 0 to 60% and comparing the fluorescent signals of the target organisms to those of nonspecific hybridizations with negative controls. For the hybridizations of the cells from *Bacillus* spp., a lysozyme pretreatment was needed. The cell smears were incubated with 10 μl of a 2-mg/ml concentration of lysozyme in 100 mM Tris-HCl (pH 7.2) for 5 min on ice. After hybridization for 3 to 5 h, the slides were washed with washing buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.1% SDS) at 48°C when hybridization buffers contained no formamide or 10% formamide. They were washed in low-salt wash buffer (0.18 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.1% SDS) at 48°C when hybridization buffers contained 20% formamide or more.

Extraction, whole-cell hybridization, and quantification of chimney cells. Three beehive structures, designated MS06CH, MS15CH, and MS17CH, were collected in November and December 1995 from the Snake Pit vent field (23°22′118″N, 44°56′984″W) on the Mid-Atlantic Ridge at a depth of 3,500 m during the Microsmoke cruise (18). The chimneys were subsampled on board, stored under reduced conditions at 4°C for cultivation, and fixed with 4% paraformaldehyde in 3× PBS before storage in 50% ethanol in 3× PBS at −20°C for whole-cell hybridization as described before (18). The procedure of cell extraction from chimneys was described in detail previously (18). For whole-cell hybridization, 1 to 4 μl of the extracted cells was smeared on a gelatin-coated slide, dried, rinsed in a series of alcohol, and hybridized with fluorescent probes as described before (47). To quantify the amount of cells hybridizing with probe S-O-Hydr-0540-a-A-19, an appropriate amount of cell extract (4 to 30 μl) was hybridized on a 0.2-μm-pore-size filter with 20 μl of hybridization buffer containing 5 ng of probe per ml under a cover slide. The cells were viewed and counted with an Olympus (Hamburg, Germany) BX60 epifluorescence microscope equipped with a 100-W halogen light source, a 100X U-PlanFI oil lens (Olympus) with a numerical aperture of 1.3, and U-MWB (for fluorescein) and U-MSWG (for rhodamine) filter cubes (Olympus). For the enumeration of microorganisms, the size and shape of the fluorescent signals were the criteria used to discriminate the cells from the sulfide particles. A minimum of 25 cells per field, 10 fields per filter, three filters per cell extract, and two cell extractions per sample were counted by use of the fluorescein-labeled S-O-Hydr-0540-a-A-19 probe simultaneously with the bacterial probe S-D-Bact-0338-a-A-18 (3).

RESULTS

Probe design. The design of the oligonucleotide probes was based on comparative analysis of all small-subunit (SSU) rRNA sequences from the RDP and, in addition, of newly deposited sequences from GenBank. Alignments of 16S rRNA sequences were screened to find signatures that distinguish four different groups of thermophilic microorganisms of the domain *Bacteria*, i.e., (i) most species of the genus *Thermus*, (ii) the thermophilic members of the genus *Bacillus*, (iii) two genera of the order *Thermotogales*, and (iv) the order *Aquificales*. Signature sequences that had three mismatches or more with sequences of nongroup members were checked for secondary structure and reasonable theoretical T_ds. Table 2 shows the selected probe sequences and their corresponding target groups. The sequence of probe S-G-Thus-0438-a-A-18 perfectly matches the sequences of all *Thermus* spp. except those of *Thermus filiformis* (one C-A and one G-U mismatch at positions 444 and 453, respectively [*Escherichia coli* numbering]) and the species recently transferred to the genus *Meiothermus* (39). The sequence of probe S-*-Tbcil-0832-a-A-18 perfectly matches the sequences of the thermophilic *Bacillus* spp. of the rRNA group 5 defined by Ash et al. (7) except that of *Bacillus thermoglucosidasius* (2 G-U mismatches and one T-U mismatch at positions 843, 845, and 846, respectively [*E. coli* numbering]). The sequence of probe S-*-Ttoga-0660-a-A-18 matches perfectly the sequences of species of the genus *Thermotoga* and *Thermosipho africanus* and has two or three mismatches with sequences of the species of the genus *Fervidobacterium*. The sequence of probe S-O-Hydr-0540-a-A-19, which was designed for all the species of the order *Aquificales* rooting deeply on the bacterial phylogenetic tree, shows no mismatches with the 16S rRNA sequences from this group available to date in the RDP.

Specificity studies. The specificity of selected oligonucleotide sequences revealed by comparison with existing rRNA sequence databases was also ensured by optimization of experimental hybridization conditions. The T_d values were experimentally determined from target and nontarget 16S rRNAs for the four probes characterized in this study (Table 2). The T_d values were then used as the posthybridization washing temperature to test the specificity of each of these probes by slot blot hybridization of the nucleic acids from 48 target and nontarget species. Autoradiographs of the six identical slot blots containing nucleic acids from the reference strains mentioned in Table 1 are shown in Fig. 1. Slot blot membranes were hybridized with probes S-G-Thus-0438-a-A-18 (Fig. 1a), S-*-Tbcil-0832-a-A-18 (Fig. 1b), S-*-Ttoga-0660-a-A-18 (Fig. 1c), and S-O-Hydr-0540-a-A-19 (Fig. 1d). Two of them were then washed for reprobing with the bacterial probe S-D-Bact-0338-a-A-18 (Fig. 1e) and the archaeal probe S-D-Arch-0915-a-A-20 (47) (Fig. 1f).

Figure 1a shows that probe S-G-Thus-0438-a-A-18 is specific to the genus except for *Thermus scotoductus* (lane A, blot 4) and hybridizes with deep-sea isolates (lane A, blots 7 and 8). Hybridization with probe S-*-Tbcil-0832-a-A-18 (Fig. 1b) re-

FIG. 2 (top five panels). Whole-cell hybridizations of different mixtures of fixed cells with fluorescein-labeled (green fluorescence) and rhodamine-labeled (red fluorescence) probes. Epifluorescence photomicrographs (double exposures) are shown at a magnification of ×1,140 (scale bar, 10 μm). (a) Cells of *Thermus thermophilus* and *Rhodothermus marinus* simultaneously hybridized with probes S-G-Thus-0438-a-A-18 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence); the double labeling of *Thermus thermophilus* results in yellow fluorescence. (b) Cells of "*Bacillus caldotenax*" and *Bacillus thermoglucosidasius* simultaneously hybridized with probes S-*-Tbcil-0832-a-A-18 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence). The double labeling of "*B. caldotenax*" results in yellow fluorescence. (c) Cells of *Thermosipho africanus* and *Fervidobacterium islandicum* simultaneously hybridized with probes S-*-Ttoga-0660-a-A-18 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence). The double labeling of *Thermosipho africanus* results in yellow fluorescence. (d) Cells of *Hydrogenobacter thermophilus* and *Thermotoga maritima* simultaneously hybridized with probes S-O-Hydr-0540-a-A-19 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence). (e) Cells of isolate BSA and *Thermotoga maritima* simultaneously hybridized with probes S-O-Hydr-0540-a-A-19 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence). The double labeling of isolate BSA results in yellow fluorescence.

FIG. 3 (middle five panels). Whole-cell hybridizations of cells extracted from chimney sample MS17CH3 with fluorescein-labeled (green fluorescence) and rhodamine-labeled (red fluorescence) probes. Epifluorescence photomicrographs are shown at a magnification of ×1,140 (scale bar, 10 μm). (a) Probe S-*-Ttoga-0660-a-A-18 (green fluorescence) and probe S-D-Bact-0338-a-A-18 (red fluorescence). Two long thin rods (indicated by arrows) that hybridized with both probes appeared yellowish on the double-exposed film. (b) Probe S-*-Ttoga-0660-a-A-18 (green fluorescence) hybridizing with two long thin rods indicated by arrows. (c) The same field as that of panel b, showing cells that hybridized with probe S-D-Bact-0338-a-A-18 (red fluorescence). It is important to note that only one of the rods indicated by an arrow in panel b hybridized with this probe. (d and e) Simultaneous hybridizations with probes S-*-Ttoga-0660-a-A-18 (green fluorescence) and S-D-Bact-0338-a-A-18 (red fluorescence). Arrows indicate cells that hybridized only with probe S-*-Ttoga-0660-a-A-18.

FIG. 4 (bottom three panels). Whole-cell hybridizations of cells extracted from chimney sample MS15CHA3 with fluorescein-labeled probes S-O-Hydr-0540-a-A-19 and S-D-Arch-0915-a-A-20 (green fluorescence) and rhodamine-labeled probe S-D-Bact-0338-a-A-18 (red fluorescence). Epifluorescence photomicrographs (double exposures) are shown at a magnification of ×1,140 (scale bar, 10 μm). (a to c) Cells that appear yellowish, which hybridized with probes S-O-Hydr-0540-a-A-19 and S-D-Bact-0338-a-A-18, are predominant. The cell with a morphotype similar to that of isolate BSA (Fig. 2e) is indicated by arrows in panels a and c of this figure.

sulted in a positive signal for all thermophilic members of the genus *Bacillus*, except *B. thermoglucosidasius* (lane B, blot 8) and for all of the isolates previously assigned to this genus (lane C, blots 1 to 7). The genus-specific probe S-*-Ttoga-0660-a-A-18 is specific for all *Thermotoga* spp. and *Thermosipho africanus* and discriminates the species of the genus *Fervidobacterium* (Fig. 1c, lane D, blots 4 and 5); this probe allowed us to identify two deep-sea isolates (Fig. 1c). Specific hybridization with probe S-O-Hydr-0540-a-A-19 (Fig. 1d) was obtained with all tested members of the order *Aquificales* (lane F, blots 1 to 3) and an isolate from the Mid-Atlantic Ridge (C8). Figure 1e shows that all of the nucleic acids isolated from strains belonging to the domain *Bacteria* exhibited a positive signal when hybridized with probe S-D-Bact-0338-a-A-18, except *Aquifex pyrophilus* (F1), *Calderobacterium hydrogenophilum* (F2), and *Hydrogenobacter thermophilus* (F3). As expected, the probe S-D-Arch-0915-a-A-20 yields a hybridization signal with all members of the domain *Archaea*. However, as shown in Fig. 1f, there was also a weak but distinct positive response with *Aquifex pyrophilus*, *Calderobacterium hydrogenophilum* and *Hydrogenobacter thermophilus.*

Optimization of whole-cell hybridization conditions. The specificity of the fluorescently labeled probes was tested by whole-cell hybridization of paraformaldehyde-fixed cells of the reference strains. The formamide concentration and permeability treatments were optimized by using mixtures of cells for which positive and negative signals had been detected on the slot blots. The determined optimal formamide concentrations used in the whole-cell hybridizations are listed in Table 2. All strains that were positive on the slot blots and a selection of the negative strains were tested for hybridization with the corresponding probes. Representative epifluorescence micrographs of whole-cell hybridizations with each fluorescein-labeled specific probe against a target organism and a negative control hybridized at optimal formamide conditions are shown in Fig. 2. The hybridizations were performed simultaneously with a rhodamine-labeled probe, S-D-Bact-0338-a-A-18. A remarkable observation was that the *Thermus* spp. gave only weak fluorescent signals when hybridized at low formamide concentrations with both the S-D-Bact-0338-a-A-18 and S-G-Thus-0438-a-A-18 probes but strong and specific signals at high formamide concentrations (60%), indicating that formamide increased either the permeability of the cells or the availability of the target sites (Fig. 2a). All positive strains on slot blots did react to the fluorescent probes, and no significant cross-hybridizations were found. As an example, *Fervidobacterium islandicum*, which had only two mismatches with the S-*-Ttoga-0660-a-A-18 probe, did not cross-react under the conditions used (20% formamide). Cells of strains that were positive on slot blots with probe S-O-Hydr-0540-a-A-19 (namely, *Aquifex pyrophilus*, *Calderobacterium hydrogenophilum*, *Hydrogenobacter thermophilus*, and isolate BSA) and cells of *Thermotoga maritima* (used as a control) were hybridized simultaneously with this probe and probe S-D-Bact-0338-a-A-18. Strong positive signals were observed with the four first strains when they were hybridized with the fluorescein-labeled probe S-O-Hydr-0540-a-A-19, whereas only cells of isolate BSA and *Thermotoga maritima* hybridized strongly with the rhodamine-labeled probe S-D-Bact-0338-a-A-18 (Fig. 2d and e). These observations confirmed results obtained from slot blot hybridization studies.

Detection and quantification of thermophilic bacteria in chimney samples. The designed and validated probes were used to detect and identify thermophilic bacteria among cells extracted from the deep-sea vent chimney samples. No unambiguous signals were detected when cells extracted from all of the tested samples were hybridized with probes S-G-Thus-0438-a-A-18 and S-*-Tbcil-0832-a-A-18. The S-*-Ttoga-0660-a-A-18 probe detected thin rods in the subsample MS17CH3 only (Fig. 3), a chimney fragment from the exterior wall of chimney MS17CH. This probe hybridized simultaneously with probe S-D-Bact-0338-a-A-18 on some of the rod-shaped cells (Fig. 3a to c). However, similar morphotypes hybridizing with only the group-specific probe were also observed (Fig. 3b to e).

Probe S-O-Hydr-0540-a-A-19 hybridized with small and long rods extracted from all exterior parts of the chimney samples (Fig. 4). Cells hybridizing simultaneously with probes S-O-Hydr-0540-a-A-19 (fluorescein labeled) and S-D-Bact-0338-a-A-18 (rhodamine labeled) were enumerated in separate extractions from subsample MS15CHA3, the external wall of chimney MS15CH. Of the cells recovered from the first extraction, 41% yielded a positive signal with both probes ($1.5 \times 10^6 \pm 0.4 \times 10^6$ cells/g [dry weight] of chimney for a total count of *Bacteria* [cells hybridizing with only probe S-D-Bact-0338-a-A-18] of $3.9 \times 10^6 \pm 0.6 \times 10^6$ cells/g [dry weight]). Of the bacterial cells from the second extraction, 34% hybridized with both probes ($3.2 \times 10^5 \pm 0.9 \times 10^5$ cells/g [dry weight] of chimney for a total count of *Bacteria* of $9.8 \times 10^5 \pm 1.7 \times 10^5$ cells/g [dry weight]).

DISCUSSION

In the course of this study, four oligonucleotide probes targeting 16S rRNAs of defined groups of thermophilic bacteria known to thrive in marine hydrothermal vents were developed. A target site for probes to detect the complete genera of thermophilic *Bacillus* and *Thermus* and the order *Thermotogales* was not found within the 16S rRNA sequences existing in databases without the use of more than one degenerated (e.g., wobbled) nucleotide position. Therefore, target sites that matched 16S rRNA sequences of the most numerous members of each target group were chosen. As a consequence, species that were moderately thermophilic or isolated from terrestrial sites were excluded. Thermophilic or mesophilic members of these genera, which might be present in marine hydrothermal vents, could therefore escape detection by these probes.

The probes designed to detect thermophilic *Bacillus* spp. and *Thermus* spp. in cell extracts from deep-sea hydrothermal chimneys failed to do so. Nevertheless, strains of both of these genera have already been isolated from chimney structures collected from the same and other study sites (34a, 35, 36). The failure to detect cells of these bacterial groups could be attributed to their absence or scarcity in the samples we analyzed or, in the case of cells of the *Bacillus* spp., to their occurrence as spores in the habitat.

A few cells hybridizing simultaneously with probes S-*-Ttoga-0660-a-A-18 and S-D-Bact-0338-a-A-18 were detected only in subsample MS17CH3 (Fig. 3a to c). Cells with morphologies characteristic of the members of the order *Thermotogales* (i.e., rod-shaped cells surrounded by a sheath-like envelope) were previously enriched in thiosulfate-supplemented medium inoculated with this subsample (18). Some other cells with this typical shape were also observed in MS17CH3; they hybridized with probe S-*-Ttoga-0660-a-A-18 but did not yield any signal with probe S-D-Bact-0338-a-A-18 (Fig. 3b to e). This could indicate that these cells might be members of an unknown phylum related to the *Thermotogales.*

Probe S-O-Hydr-0540-a-A-19 was specific for the complete *Hydrogenobacter-Aquifex* group. However, control hybridizations showed that all members of this group failed to hybridize with bacterial probe S-D-Bact-0338-a-A-18. A check of the corresponding sequences showed that the 16S rRNA se-

quences of these organisms had one A-C and one T-G internal mismatch at positions 340 and 349, respectively (*E. coli* numbering) with the sequence of bacterial probe S-D-Bact-0338-a-A-18. As previously reported (11), nucleic acids of these organisms were found to hybridize weakly with probe S-D-Arch-0915-a-A-20. The sequence of this probe has three mismatches with the target sequence of the *Aquificales*, but the mismatches are located near the end of the duplex. As suggested by Stahl and Amann (47), a mismatch at this position is generally less destabilizing than an internal mismatch. Our results support this relationship in the fact that only one additional internal mismatch (C-A) at position 923 (*E. coli* numbering) is sufficient to avoid a very weak signal when probe S-D-Arch-0915-a-A-20 is hybridized against *Thermotoga maritima*, *Fervidobacterium nodosum*, and *Thermus thermophilus*. The enriched isolate BSA from Mid-Atlantic Ridge chimney samples hybridizes strongly with both probe S-O-Hydr-0540-a-A-19 and probe S-D-Bact-0338-a-A-18. The analysis of its complete 16S rRNA sequence confirmed that the sequences of both probes have no mismatches with the sequence of this strain and revealed that this strictly anaerobic autotrophic sulfur-reducing organism represents a new genus and a new species rooting on the phylogenetic tree between the orders *Aquificales* and *Thermotogales* (32a).

Probe S-O-Hydr-0540-a-A-19 was further used in combination with probe S-D-Bact-0338-a-A-18 for whole-cell hybridization experiments with cells extracted from deep-sea vent chimneys. Our specificity studies indicated clearly that members of the order *Aquificales* did not hybridize simultaneously with both of these probes. Consequently, cells enumerated by this approach differ from the known members of the order *Aquificales*. The combination of probes S-O-Hydr-0540-a-A-19 and S-D-Bact-0338-a-A-18 allowed the quantification of morphologically diverse cells, ranging from small to long thick rods or chains of rods and representing a large proportion of the population, of up to 40% of the number determined by use of the general bacterial probe. Since one of the morphotypes observed has now been isolated and identified phylogenetically and metabolically (isolate BSA), we can conclude that there are unknown rod-shaped bacteria in the marine hydrothermal systems which have sequences identical to the target sequences of probes S-O-Hydr-0540-a-A-19 and S-D-Bact-0338-a-A-18. Given that the probes used to label cells in the natural samples were designed from sequences of cultured isolates, it is not surprising that previously unidentified or noncultured microorganisms were labeled with the probes. However, since the amount of phylogenetic information obtained is limited because of the probe sizes, the phylogenetic diversity and the physiological role of these new organisms remain unclear.

This study demonstrates, therefore, that the combination of rRNA-directed oligonucleotide probes specific for different phylogenetic levels and traditional cultivation-based methods (the whole-cell hybridization method presented here and the enrichments described in reference 18) allows the identification and quantification of specifically stained thermophilic bacteria from deep-sea hydrothermal chimneys. Further probe development and the use of other enrichment procedures are necessary to provide new insights into the microbial community structure of this thermophilic habitat.

ACKNOWLEDGMENTS

We thank Jacques Orillon and Gwenola Omnes for their professional photographic work.

The Microsmoke cruise was organized by CNRS (D. Prieur, chief scientist), with the NO *Le Nadir* and the DSV *Nautile* operated by Ifremer. We thank the captain and the crew of NO *Le Nadir* and the DSV *Nautile* pilots for skillful operations and support crew.

This work was supported by CNRS, GDR 1006 CNRS/Ifremer, CPER 94-95 (Contrat de Plan Etat-Région), and Fonds Structurel Européen (FEDER 5b). H.J.M.H. was supported by a postdoctoral grant from ECC (Human Capital and Mobility Network contract CHRX-CT93-0194).

REFERENCES

1. **Alfredsson, G. A., J. K. Kristjansson, S. Hjörleifsdöttir, and K. O. Stetter.** 1988. *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine host springs in Iceland. J. Gen. Microbiol. **134:** 299–306.
2. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557–3559.
3. **Amann, R. I., B. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
4. **Amann, R. I., M. Krumholtz, and D. A. Stahl.** 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
5. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
6. **Ambroz, A.** 1913. *Denitrobacterium thermophilum* spec. nova, ein Beitrag zur Biologie der thermophilen Bakterien. Zentralbl. Bakteriol. II **37:**3–16.
7. **Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins.** 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. **13:**202–206.
8. **Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss.** 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Microbiol. **84:**54–68.
9. **Brock, T. D., and H. Freeze.** 1962. *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J. Bacteriol. **98:**289–297.
10. **Burggraf, S., H. Fricke, A. Neuner, J. Kristjansson, P. Rouviere, L. Mandelco, C. R. Woese, and K. O. Stetter.** 1990. *Methanococcus igneus* sp. nov., a novel hyperthermophilic methanogen from a shallow submarine hydrothermal system. Syst. Appl. Microbiol. **13:**33–38.
11. **Burggraf, S., T. Mayer, R. Amann, S. Schadhauser, C. R. Woese, and K. O. Stetter.** 1994. Identifying members of the domain *Archaea* with rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **60:**3112–3119.
12. **Devereaux, R., M. D. Kane, J. Winfrey, and D. A. Stahl.** 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol. **58:**614–623.
13. **Donk, P. J.** 1920. A highly resistant thermophilic organism. J. Bacteriol. **5:**272–276.
14. **Erauso, G., A.-L. Reysenbach, A. Godfroy, J.-R. Meunier, B. Crump, F. Partensky, J. A. Baross, V. T. Marteinsson, G. Barbier, N. R. Pace, and D. Prieur.** 1993. *Pyrococcus abyssi sp*. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Arch. Microbiol. **160:**338–349.
15. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. **145:**56–61.
16. **Golovacheva, R. S., L. G. Loginova, T. A. Salikhov, A. A. Kolesnikov, and G. N. Zaitseva.** 1975. A new thermophilic species, *Bacillus thermocatenulatus* nov. spec. Mikrobiologiya **44:**265–268.
17. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, and A. J. M. Stams.** 1995. Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichment cultures. Syst. Appl. Microbiol. **18:**67–73.
18. **Harmsen, H. J. M., D. Prieur, and C. Jeanthon.** 1997. Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichments of thermophilic subpopulations. Appl. Environ. Microbiol. **63:**2876–2883.
19. **Heinen, U. J., and W. Heinen.** 1972. Characteristics and properties of a caldoactive bacterium producing extracellular enzymes and two related strains. Arch. Mikrobiol. **82:**1–23.
20. **Huber, R., T. A. Langworthy, H. Konig, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter.** 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144:**324–333.
21. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rickinger, H. Fricke, and K. O. Stetter.** 1992. *Aquifex pyrophilus* gen. nov. sp. nov. represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacterium. Syst. Appl. Microbiol. **15:**340–351.
22. **Huber, R., C. R. Woese, T. A. Langworthy, H. Fricke, and K. O. Stetter.** 1989. *Thermosipho africanus* gen. nov. represents a new genus of thermophilic eubacteria within the "*Thermotogales*". Syst. Appl. Microbiol. **12:**32–37.
23. **Huber, R., C. R. Woese, T. A. Langworthy, J. K. Kristjansson, and K. O. Stetter.** 1990. *Fervidobacterium islandicum* sp. nov., a new extremely ther-

mophilic eubacterium belonging to the "*Thermotogales*". Arch. Microbiol. **154:**105–111.

24. **Jannasch, H. W.** 1995. Microbial interactions with hydrothermal fluids, p. 273–296. *In* S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. Geophysical monograph 91. American Geophysical Union, Washington, D.C.
25. **Jannasch, H. W., R. Huber, S. Belkin, and K. O. Stetter.** 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch. Microbiol. **150:**103–104.

25a. **Jeanthon, C., and S. L'Haridon.** Unpublished data.

26. **Jeanthon, C., A.-L, Reysenbach, S. L'Haridon, A. Gambacorta, P. Glénat, N. R. Pace, and D. Prieur.** 1995. *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a oil reservoir. Arch. Microbiol. **164:** 91–97.
27. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii*, sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136:**254–261.
28. **Karl, D. M.** 1995. Ecology of free-hydrothermal vent microbial communities, p. 35–124. *In* D. M. Karl (ed.), The microbiology of deep-sea hydrothermal vents. CRC Press, Inc., Boca Raton, Fla.
29. **Kawasumi, T., Y. Igarashi, T. Kodama, and Y. Minoda.** 1984. *Hydrogenobacter thermophilus* gen. nov. sp. nov., an extremely thermophilic aerobic, hydrogen-oxidizing bacterium. Int. J. Syst. Bacteriol. **34:**5–10.
30. **Kristjánsson, J. K., S. Hjörleifsdöttir, V. T. Marteinsson, and G. A. Alfredsson.** 1994. *Thermus scotoductus*, sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. X-1. Syst. Appl. Microbiol. **17:**44–50.
31. **Kryukov, V. R., N. D. Savelyeva, and M. A. Pusheva.** 1983. *Calderobacterium hydrogenophilum* nov. gen., nov. sp., an extreme thermophilic bacterium and its hydrogenase activity. Mikrobiologiya **52:**781–788.
32. **Larionov, V., K. Kouprina, and T. Karpova.** 1984. Stability of recombinant plasmids containing the *ars* sequence of yeast extrachromosomal rDNA in several strains of *Saccharomyces cerevisiae*. Gene **28:**229–235.

32a. **L'Haridon, S., V. Cilia, and C. Jeanthon.** Unpublished data.

33. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1996. The Ribosomal Database Project. Nucleic Acids Res. **24:**82–85.
34. **Manaia, C. M., B. Hoste, M. C. Gutierrez, M. Gillis, A. Ventosa, K. Kersters, and M. S. Da Costa.** 1994. Halotolerant *Thermus* strains from marine and terrestrial hot springs belong to *Thermus thermophilus* (ex Oshima and Imahori, 1974) nom. rev. emend. Syst. Appl. Microbiol. **17:**526–532.

34a. **Marteinsson, V. T., and J.-L. Birrien.** Unpublished data.

35. **Martiensson, V. T., J.-L. Birrien, C. Jeanthon, and D. Prieur.** 1996. Numerical taxonomic study of thermophilic *Bacillus* isolated from three geographically separated deep-sea hydrothermal vents. FEMS Microbiol. Ecol. **21:** 255–266.
36. **Martiensson, V. T., J.-L. Birrien, J. K. Kristjansson, and D. Prieur.** 1995. First isolation of thermophilic aerobic non-sporulating heterotrophic bacteria from deep-sea hydrothermal vents. FEMS Microbiol. Ecol. **18:**163–174.
37. **Meunier, J.-R.** 1994. Biodiversité et systématique des populations de microorganismes thermophiles isolés d'écosystèmes hydrothermaux abyssaux. Diplôme de thèse. Institut National Agronomique Paris—Grignon, Paris, France.
38. **Neuner, A., H. W. Jannasch, S. Belkin, and K. O. Stetter.** 1990. *Thermococcus litoralis* sp. nov.: a new species of extremely marine archaebacteria. Arch. Microbiol. **153:**205–207.
39. **Nobre, M. F., H. G. Trüper, and M. S. Da Costa.** 1996. Transfer of *Thermus ruber* (Loginova et al. 1984), *Thermus silvanus* (Tenreiro et al. 1995), and *Thermus chliarophilus* (Teinreiro et al. 1995) to *Meiothermus* gen. nov. as *Meiothermus ruber* comb. nov., *Meiothermus silvanus* comb. nov., and *Meiothermus chliarophilus* comb. nov., respectively, and emendation of the genus *Thermus*. Int. J. Syst. Bacteriol. **46:**604–606.
40. **Oshima, T., and K. Imahori.** 1974. Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. Int. J. Syst. Bacteriol. **24:**104–112.
41. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microbiol. Ecol. **9:**1–55.
42. **Patel, B. K. C., H. W. Morgan, and R. M. Daniel.** 1985. *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. **141:**63–69.
43. **Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl.** 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. **60:**1232–1240.
44. **Reysenbach, A.-L., G. S. Wickham, and N. R. Pace.** 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. **60:**2113–2119.
45. **Saiki, T., R. Kunura, and K. Arima.** 1972. Isolation and characterization of extremely thermophilic bacteria from hot springs. Agric. Biol. Chem. **36:** 2357–2366.
46. **Sambrook, J, E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and E. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, England.
48. **Stetter, K. O.** 1988. *Archaeoglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. Syst. Appl. Microbiol. **10:**172–173.
49. **Stetter, K. O., M. Thomm, J. Winter, G. Wildgruber, H. Huber, W. Zillig, D. Janecovic, H. König, and P. Palm.** 1981. *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zentralbl. Bakteriol. Hyg. I Abt. Orig. C **2:**166–178.
50. **Suzuki, Y., T. Kishigami, K. Inoue, Y. Mizoguchi, N. Eto, M. Tagaki, and S. Abe.** 1983. *Bacillus thermoglucosidasius* sp. nov., a new species of obligately thermophilic bacilli. Syst. Appl. Microbiol. **4:**487–495.
51. **Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts.** 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. **12:**219–286.
52. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
53. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M3 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
54. **Zarilla, K. A., and J. J. Perry.** 1987. *Bacillus thermoleovorans* sp. nov., a specis of obligately thermophilic hydrocarbon utilizing endospore-forming bacteria. Syst. Appl. Microbiol. **9:**258–263.
55. **Zillig, W., I. Holz, D. Janekovic, W. Schäfer, and W. D. Reiter.** 1983. The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. Syst. Appl. Microbiol. **4:**88–94.
56. **Zillig, W., K. O. Stetter, D. Prangishvilli, W. Schäfer, S. Wunderl, D. Janekovic, I. Holz, and P. Palm.** 1982. *Desulfurococcaceae*, the second family of the extremely thermophilic, anaerobic, sulfur-respiring *Thermoproteales.* Zentralbl. Bakteriol. Hyg. I Abt. Orig. C **3:**304–307.