APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3112–3119 Vol. 60, No. 9
0099-2240/94/$04.00+0
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Identifying Members of the Domain *Archaea* with rRNA-Targeted Oligonucleotide Probes

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Received 22 February 1994/Accepted 5 July 1994

Two 16S rRNA-targeted oligonucleotide probes were designed for the archaeal kingdoms *Euryarchaeota* and *Crenarchaeota*. Probe specificities were evaluated by nonradioactive dot blot hybridization against selected reference organisms. The successful application of fluorescent-probe derivatives for whole-cell hybridization required organism-specific optimizations of fixation and hybridization conditions to assure probe penetration and morphological integrity of the cells. The probes allowed preliminary grouping of three new hyperthermophilic isolates. Together with other group-specific rRNA-targeted oligonucleotide probes, these probes will facilitate rapid in situ monitoring of the populations present in hydrothermal systems and support cultivation attempts.

Both in concept and conduct, microbial ecology today is a discipline much changed from the microbial ecology of only a decade ago. The experimental basis for microbial ecology throughout most of this century has been enrichment culturing. As powerful as this method is, it suffers from severe limitations; and these limitations have defined the scope and perspective of the field: only a fraction of the microbial types in most, if not all, niches has been isolated and grown in pure culture so far. Also, throughout most of this century microbiologists lacked the means to determine the natural relationships among microorganisms. Because of these limitations, the microbial ecologist's description of a niche was incomplete, uncertain at best, and totally lacking the all-important phylogenetic dimension (which macroecologists take for granted).

Technological advances and their application to microbial phylogeny now permit a fuller microbial ecology. It is possible merely to isolate genes directly from environmental samples and clone and sequence them in order to determine the phylogenetic groupings to which the genes (and corresponding organisms) belong (33, 34). An excellent example of the use of the new approach is the finding of several new archaeal species that represent not only major new taxa, but also possibly a novel archaeal biotope (10, 16). Knowing genetic sequences then permits the design of (labeled) oligonucleotide hybridization probes that can be used to identify and distinguish the corresponding organisms in samples taken from natural settings (1, 3, 11, 37, 47). Ultimately, the microbial ecologist should be able to take a phylogenetic census of any microbial niche using the proper inclusive set of specific, taxonomically nested whole-organism probes: at the highest level, probes would segregate all organisms in a niche into one of the three domains, while lower-level probes would identify the known families, genera, species, etc., within each domain (39). Any organisms identified by the highest-level probes but not detected by any of the lower-level probes would represent taxa for which cultured representatives do not yet exist. These probes, as well as assays, are useful in attempts to isolate a particular microorganism (27).

Hydrothermal ecosystems have become an exciting source of organisms for the microbiologist (e.g., see references 6, 14, 20, 21, 43–45, 55). To date, an unexpectedly large number of organisms with unique phenotypes, belonging almost exclusively to the members of the domain *Archaea*, have been isolated from these environments (41, 42), and it seems likely that the limitations of enrichment cultivation methods have prevented us from assessing the true extent of this number. Organisms from these systems are obvious candidates for definition in terms of the above-described type of probe set.

In the present communication, we begin to apply the above-described strategy to the *Archaea*. So far, a systematic study on whole-cell hybridization of *Archaea* has not been performed, and it cannot be taken for granted that methods optimized for members of the domain *Bacteria* will also be suitable for *Archaea*. It is necessary to evaluate and modify existing protocols to assure permeability of the cell periphery and maintenance of cell integrity.

Our current perception of the archaeal phylogeny is shown in Fig. 1. At present, no whole-cell probes exist for identifying members of the major archaeal taxa, the kingdoms *Euryarchaeota* and *Crenarchaeota* (51). The present communication describes the application of two new probes, based on well-defined 16S rRNA signatures separating the two archaeal kingdoms. These two group-specific probes then represent the first step in the in situ characterization of high-temperature biotopes.

MATERIALS AND METHODS

Organisms and growth conditions. The following microorganisms were used in this study (ATCC is the American Type Culture Collection [Rockville, Md.], and DSM is the Deutsche Sammlung von Mikroorganismen und Zellkulturen [Braunschweig, Germany]): *Acidianus infernus* DSM3191 (35), *Aquifex pyrophilus* DSM6858 (23), *Archaeoglobus fulgidus* DSM4303 (40), *Archaeoglobus profundus* DSM5631 (7), *Bacil-*

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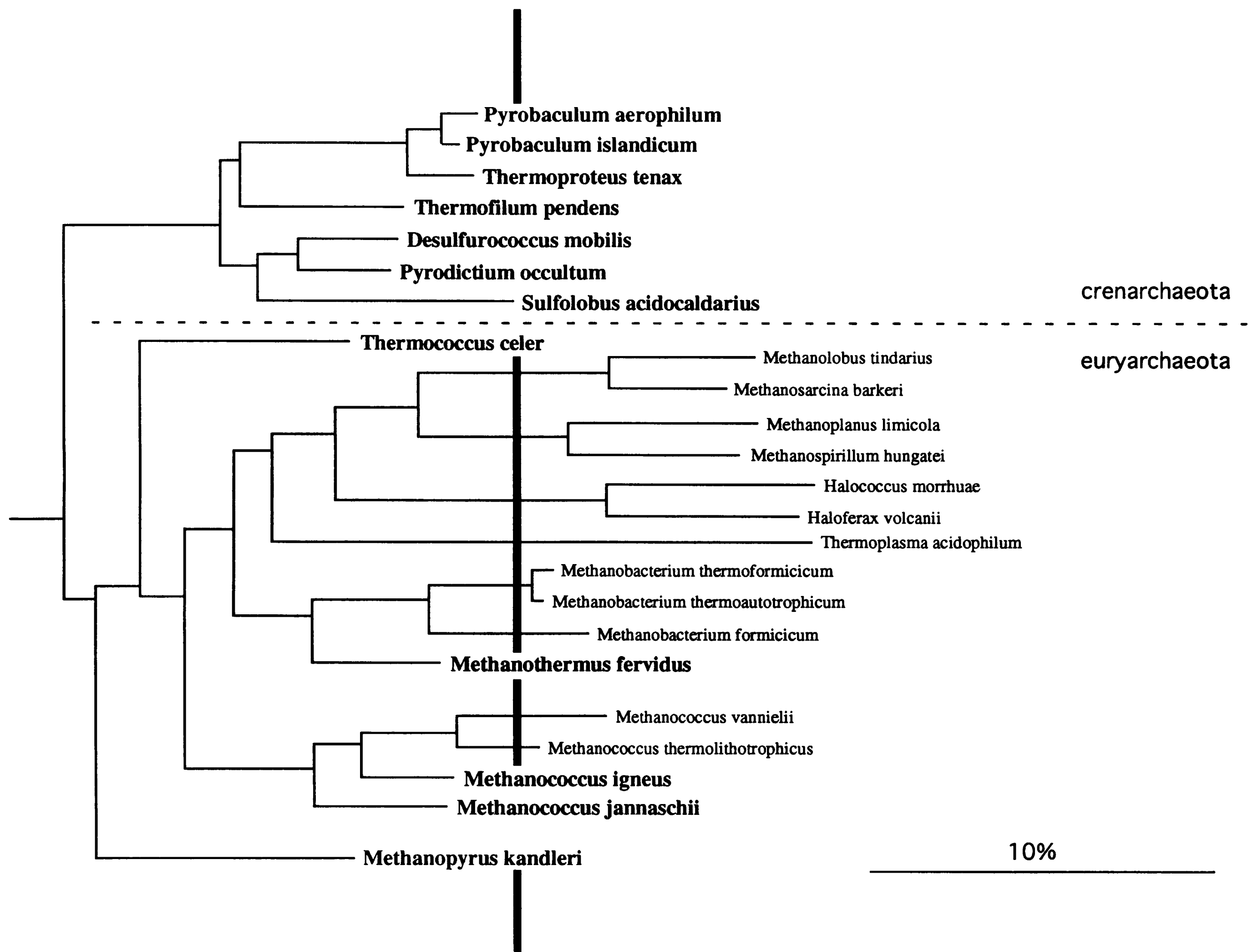

FIG. 1. Phylogenetic tree based on 16S rRNA sequence comparison. The dendrogram was constructed from evolutionary-distance matrices by the method of De Soete (12). Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities by using the Jukes-Cantor correction as modified by G. J. Olsen (26, 48) from an alignment of 16S rRNA sequences (Ribosomal Database Project, University of Illinois [29]). The root was determined by using *Aquifex pyrophilus* and *Thermotoga maritima* as outgroup sequences. All organisms within a given phylogenetic distance from the root of the tree, to the indicated bold line, are hyperthermophiles. These organisms are represented in boldface type. Scale bar, 10% evolutionary distance.

lus subtilis ATCC 6633, *Desulfurococcus mobilis* DSM2161 (54), *Escherichia coli* DSM30083, *Fervidobacterium islandicum* DSM5733 (25), *Halobacterium halobium* DSM670, *Halococcus morrhuae* DSM1307, *Methanobacterium thermoautotrophicum* DSM1053 (53), *Methanococcus igneus* DSM5666 (6), *Methanococcus thermolithotrophicus* DSM2095 (19), *Methanococcus vannielii* DSM1224 (38), *Methanoplanus limicola* DSM2279 (49), *Methanopyrus kandleri* DSM6324 (28), *Methanospirillum hungatei* DSM864 (13), *Methanothermus fervidus* DSM2088 (45), *Pseudomonas cepacia* DSM50181, *Pyrobaculum aerophilum* DSM7523 (46), *Pyrobaculum islandicum* DSM4184 (20), *Pyrobaculum organotrophum* DSM4185 (20), *Pyrococcus furiosus* DSM3638 (14), *Pyrodictium occultum* DSM2708 (43), *Staphylothermus marinus* DSM3639 (15), *Stygiolobus azoricus* DSM6296 (36), *Sulfolobus acidocaldarius* DSM639 (4), *Thermococcus litoralis* DSM5473 (32), *Thermoproteus tenax* DSM2078 (55), *Thermosipho africanus* DSM5309 (24), and *Thermotoga maritima* DSM3109 (22).

Cells were grown as described in the references given above. GC74 was isolated from samples from the deep-sea hydrothermal system at Guaymas, Mexico. This hyperthermophilic, coccoid microorganism grows heterotrophically on chitin (17). Isolate KC4 is a rod-shaped nitrate-reducing extreme thermophile from low-salt-concentration hydrothermal areas in Indonesia (18). Preliminary 16S rRNA sequence data indicate that KC4 could represent a new phylogenetic lineage within the bacterial domain (50a). 1A comprises disc-shaped nitrate-reducing hyperthermophilic microorganisms isolated from deep-sea hydrothermal vents at the Mid-Pacific Rise (41b). The isolate Kol8 grows chemolithoautotrophically by reduction of elemental sulfur. This coccoid hyperthermophile was isolated from the Kolbeinsey Ridge north of Iceland (6, 41a). Cell masses for extraction of total RNA were obtained by cultivation in a pressurized enamel-protected fermentor (100-liter volume; Bioengineering, Wald, Switzerland).

Cell fixation. For whole-cell hybridization experiments, cells were fixed by the addition of 3 volumes of a paraformaldehyde solution (4% [wt/vol] in phosphate-buffered saline [PBS])

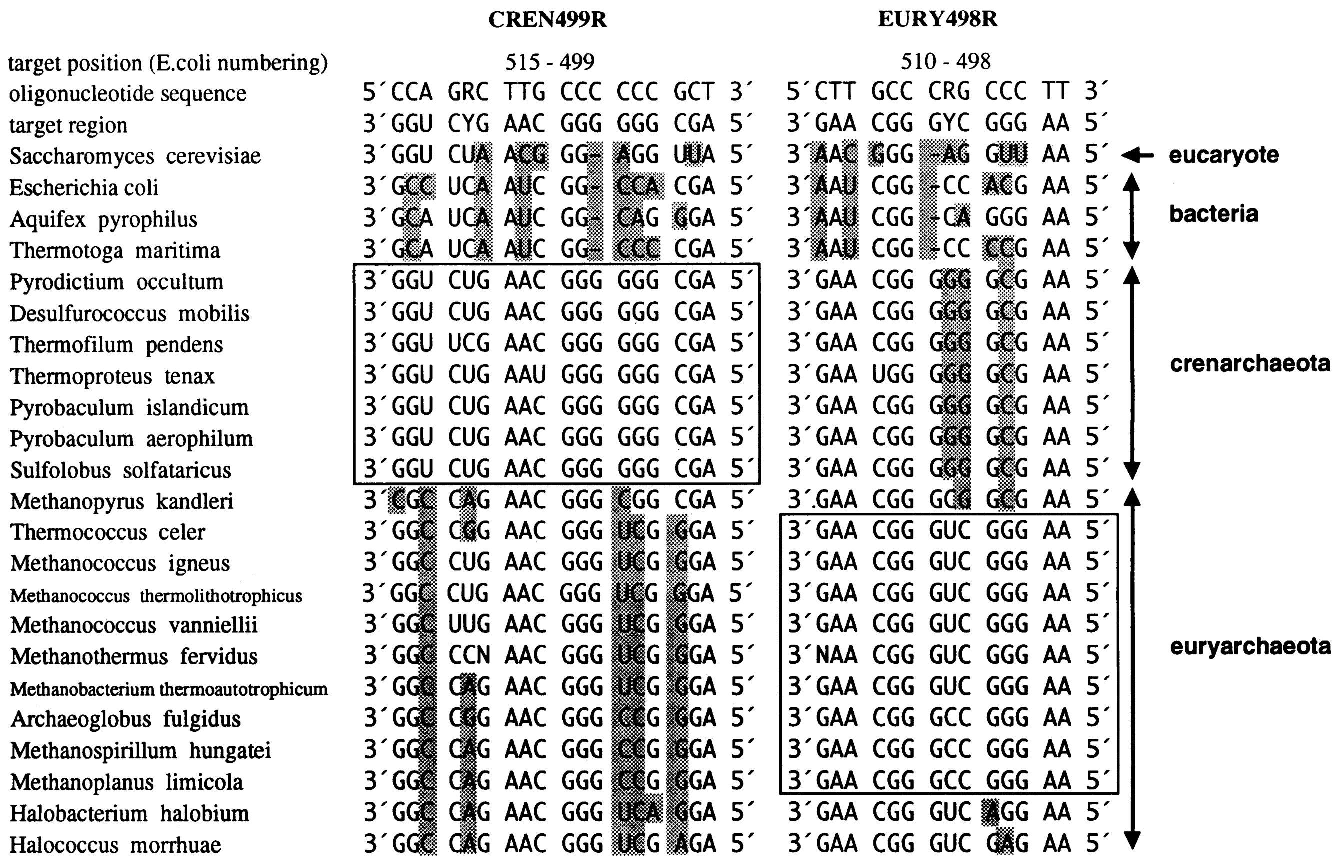

FIG. 2. Alignment of the probes CREN499 and EURY498 with the complementary 16S rRNA region from organisms characteristic of the three domains. Mismatches are shaded, and sequences showing no mismatch are boxed.

directly to the culture at the mid-logarithmic growth phase. After the fixation (3 h at room temperature), cells were washed with PBS and stored in a 1:1 mixture of PBS and 98% ethanol at −20°C (2). Fixed cells were spotted on precleaned, gelatin-coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] microscopic slides (Paul Marienfeld KG, Bad Mergentheim, Germany), dried at 46°C for 30 min, and dehydrated in 50, 80, and 98% (vol/vol) ethanol (3 min each).

Oligonucleotide probes. An alignment of archaeal 16S rRNA sequences (Ribosomal Database Project, University of Illinois [29]) was screened for signature positions distinguishing the two major branches within the archaeal domain, the kingdoms *Euryarchaeota* and *Crenarchaeota* (9). Two oligonucleotides (CREN499 and EURY498) complementary to a region characteristic for each archaeal kingdom were designed. The oligonucleotides were synthesized with a C-6–TFA aminolink [6-(trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite] at the 5′ end by MWG Biotech (Ebersberg, Germany). The sequences of the probes and the target sites are shown in Fig. 2. In addition, the following 16S rRNA-targeted oligonucleotides were used: UNIV1392, a probe complementary to a region conserved in all three domains of life (33), and EUB338 (2) and ARCH915 (39), complementary to conserved regions within the bacterial and archaeal domains, respectively. Labeling of the oligonucleotides with tetramethylrhodamine-5-isothiocyanate (TRITC; Research Organics, Cleveland, Ohio) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide-ester (FLUOS; Boehringer, Mannheim, Germany) and purification were performed as described elsewhere (3). For labeling with digoxigenin, 50 μl of the oligonucleotide (1 μg/μl in 100 mM sodium borate, pH 8.5) was mixed with 50 μl of digoxigenin-3-*O*-methylcarbonyl-ε-aminocapronic acid-*N*-hydroxysuccinimide-ester (5 mg/ml in *N,N*-dimethylformamide; Boehringer) and incubated at room temperature for 10 h. The labeled oligonucleotide was purified by high-pressure liquid chromatography (HPLC) (column, Kontron RP 18 Partisil 10 ODS 3; flow rate, 1 ml/min; gradient, from 100% buffer A [100 mM triethylammonium acetate, pH 7.0] to 80% buffer B [1:1 mixture of buffer A and acetonitrile] in 30 min).

Whole-cell hybridization. An 8-μl volume of hybridization solution was spotted in each well of the microscopic slide, and slides were incubated for 2 h at 45°C in a moisture chamber equilibrated isotonically to the hybridization solution. After hybridization, the slide was rinsed with approximately 2 ml of washing solution and immersed in 50 ml of washing solution at 48°C for 20 min. The sodium dodecyl sulfate (SDS) concentrations in the hybridization and washing solutions were varied in order to optimize probe penetration and morphological integrity of the cells. Optimal stringencies of hybridizations were empirically determined by gradually increasing the formamide concentration and keeping the hybridization temperature at 45°C (30). The following hybridization and washing solutions were used for the different probes and organisms. The hybridization solution contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01 to 1% SDS, and 50 ng of probe. For

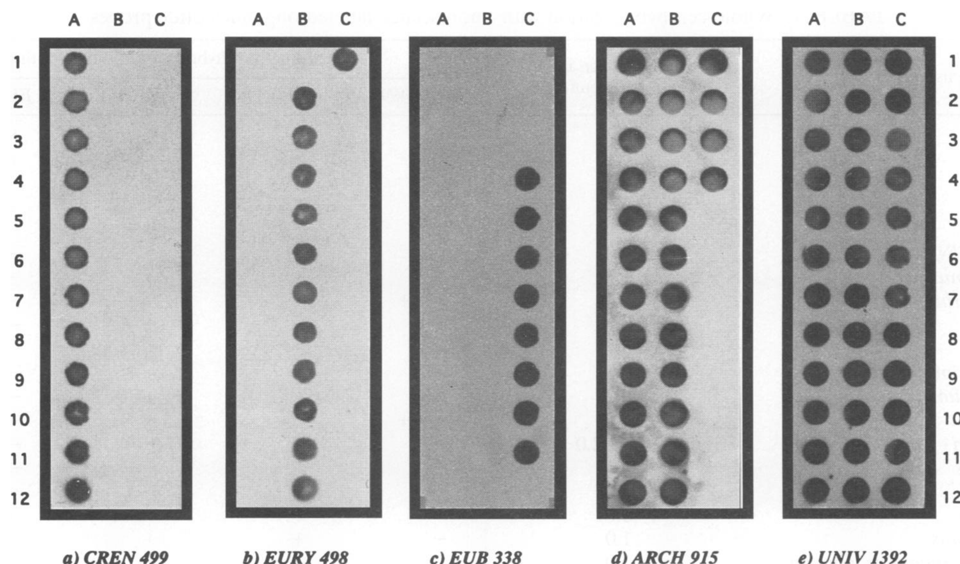

FIG. 3. Dot blot hybridization of filter-bound RNA with the digoxigenin-labeled oligonucleotide probes CREN499 (a), EURY498 (b), EUB338 (c), ARCH915 (d), and UNIV1392 (e). Lanes A show members of the kingdom *Crenarchaeota* only. Rows: 1, *Pyrodictium occultum*; 2, isolate 1A; 3, isolate Kol8; 4, *Thermoproteus tenax*; 5, *Pyrobaculum islandicum*; 6, *Pyrobaculum organotrophum*; 7, *Pyrobaculum aerophilum*; 8, *D. mobilis*; 9, *Staphylothermus marinus*; 10, *Sulfolobus acidocaldarius*; 11, *Acidianus infernus*; 12, *Stygiolobus azoricus*. Lanes B show members of the kingdom *Euryarchaeota* only. Rows: 1, *Methanopyrus kandleri*; 2, *Thermococcus litoralis*; 3, isolate GC74; 4, *Pyrococcus furiosus*; 5, *Methanococcus igneus*; 6, *Methanococcus thermolithotrophicus*; 7, *Methanococcus vannielii*; 8, *Methanothermus fervidus*; 9, *Methanobacterium thermoautotrophicum*; 10, *Archaeoglobus fulgidus*; 11, *Archaeoglobus profundus*; 12, *Methanoplanus limicola*. Lanes C show members of the kingdom *Euryarchaeota* (rows 1 through 3) and of the domain *Bacteria* (rows 4 through 11). Rows: 1, *Methanospirillum hungatei*; 2, *Halococcus morrhuae*; 3, *Halobacterium halobium*; 4, *Aquifex pyrophilus*; 5, *Thermotoga maritima*; 6, *Thermosipho africanus*; 7, *F. islandicum*; 8, isolate KC4; 9, *E. coli*; 10, *B. subtilis*; 11, *Pseudomonas cepacia*; 12, *S. cerevisiae*.

hybridizations with probes EUB338 and ARCH915, the solution contained 20% (wt/vol) formamide. For simultaneous hybridization with probes CREN499 and EURY498, no addition of formamide was necessary to achieve optimal stringency. The washing solution for UNIV1392, EUB338, and ARCH915 contained 180 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, and 0.01 to 1% SDS, and that for CREN499-EURY498 contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, and 0.01 to 1% SDS. After the washing step, the slides were rinsed with distilled water, dried, and mounted in Citifluor AF1 (Citifluor Ltd., London, United Kingdom).

Fluorescence microscopy. For microscopy of the hybridized cells, a Nikon Microphot EPI-FL microscope equipped with a UV lamp and the filter sets G-1B and B-2A (Nikon) was used. The results were recorded on Scotch Chrome P800/3200 color reversal film. Exposure times were 2 to 10 s for epifluorescence and 0.02 to 0.05 s for phase-contrast photomicrographs.

Total RNA extraction. Extraction of total RNA from cell pellets was performed by a conventional procedure (31, 52).

Dot blot hybridization. Hybridization of immobilized RNA with digoxigenin-labeled oligonucleotide probes was done according to the procedure described by Manz et al. (30). The following hybridization and washing solutions were used for the different probes. The hybridization solution contained 0.9 M NaCl, 0.01% SDS, 0.1% *N*-lauroylsarcosine, 4% blocking reagent (Boehringer), and 10 pmol of digoxigenin-labeled oligonucleotide probe; for hybridizations with the probes EUB338, ARCH915, and UNIV1392, a solution containing 20% (wt/vol) formamide was used. Hybridizations with the probes CREN499 and EURY498 were performed with 15 and 30% (wt/vol) formamide respectively. The washing solution for EUB338, ARCH915, and UNIV1392 contained 240 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, and 0.01% SDS; that for EURY498 contained 120 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, and 0.01% SDS; and that for CREN499 contained 330 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, and 0.01% SDS.

RESULTS

Design of kingdom-specific oligonucleotides. Within the 16S rRNA molecule is a class of positions whose compositions vary little if at all within the various phylogenetic groupings (50). Such positions can be used as signatures to differentiate various phylogenetic groupings of organisms. In order to find signatures that define and distinguish the two archaeal kingdoms, an alignment of 16S rRNA sequences (Ribosomal Database Project, University of Illinois [29]) was screened for various archaeal consensus sequences. The alignment contained 46 euryarchaeal, 8 crenarchaeal, and about 600 bacterial sequences. The region from positions 500 to 513 exhibits an accumulation of signature positions specific for the two major branches of the archaeal domain (9). Figure 2 shows an alignment of two probes (CREN499 and EURY498) complementary to this region in the 16S rRNA together with several sequences characteristic of the three domains. The probe CREN499 shows no mismatches with all known crenarchaeal sequences, but four or more mismatches with sequences of the kingdom *Euryarchaeota* and the domains *Bacteria* and *Eucarya*. The oligonucleotide EURY498 is complementary to almost all euryarchaeal sequences in the region between positions 498 and 510 (*E. coli* numbering [5]). The only exceptions are *Methanopyrus kandleri* (two mismatches), the extreme halophiles (one mismatch), and a few members of the orders

TABLE 1. Whole-cell hybridization with fluorescence-labeled oligonucleotide probes

Organism	SDS concn in hybridization buffer (%)	Strength of hybridization signal[a] with probe:				
		CREN499	EURY498	ARCH915	EUB338	UNIV1392
Pseudomonas cepacia	0.01	−	−	−	+++	+++
Thermotoga maritima	0.1	−	−	−	++	++
Aquifex pyrophilus	0.5	−	−	+	++	++
Isolate KC4	0.05	−	−	−	+++	+++
Pyrodictium occultum	0.01	++	−	++	−	++
Desulfurococcus mobilis	0.01	++	−	++	−	+++
Staphylothermus marinus	0.01	++	−	++	−	++
Sulfolobus acidocaldarius	0.01	+++	−	+++	−	+++
Acidianus infernus	0.1	++	−	++	−	++
Thermoproteus tenax	0.1	+	−	+	−	+
Pyrobaculum islandicum	0.1	++	−	++	−	++
Pyrobaculum aerophilum	0.1	++	−	++	−	++
Isolate Kol8	0.01	+++	−	+++	−	+++
Methanopyrus kandleri	0.01–2.0	−	−	−	−	−
Thermococcus litoralis	0.1	−	++	++	−	++
Pyrococcus furiosus	0.05	−	++	++	−	++
Methanococcus igneus	0.01	−	+++	+++	−	+++
Methanothermus fervidus	1.0	−	+	+	−	+
Methanobacterium thermoautotrophicum	1.0	−	+	+	−	+
Archaeoglobus fulgidus	0.01	−	++	++	−	++
Archaeoglobus profundus	0.01	−	++	+++	−	+++
Methanospirillum hungatei	0.01	−	+++	+++	−	+++
Methanoplanus limicola	0.01	−	+++	+++	−	+++
Halococcus morrhuae	1.0	−	−	+	−	+
Isolate GC74	0.1	−	++	++	−	++

[a] +++, very good hybridization; ++, good hybridization; +, weak hybridization; −, no hybridization.

Methanomicrobiales and *Methanobacteriales* (one mismatch for *Methanocorpusculum parvum*, *Methanocorpusculum labreanum*, *Methanococcoides methylutens*, *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanosphaera stadtmanae*, and *Methanobrevibacter arboriphilicus*).

Dot blot hybridizations. Whole-cell hybridization raises problems of probe penetration into the cell and of cellular morphological integrity. In order to check the probe specificity independently of those difficulties, hybridizations of digoxigenin-labeled oligonucleotide probes with isolated, filter-immobilized RNA were performed. These hybridizations were also necessary for those organisms for which 16S rRNA sequences are not available (i.e., *Acidianus infernus, Stygiolobus azoricus, Staphylothermus marinus*, and *Thermococcus litoralis*). The results of the dot blot hybridizations are presented in Fig. 3.

It is evident that even at a low stringency of hybridization (15% formamide) probe CREN499 has good specificity for all the crenarchaeal RNAs used (Fig. 3a). This probe also bound to RNA isolated from strains 1A and Kol8. There was no hybridization signal with members of the kingdom *Euryarchaeota*, with the *Bacteria*, or with *Saccharomyces cerevisiae*. The hybridization of the same filter with the probe EURY498 and a hybridization buffer containing 30% formamide is shown in Fig. 3b. A positive signal was seen for most of the euryarchaeal RNAs and for the isolate GC74. However, as expected from the sequence comparison, there was no hybridization with *Methanopyrus kandleri* and the extreme halophiles. At a lower stringency (10% formamide), a hybridization with the latter organisms could also be achieved; but in this case, there was also a weak hybridization with the crenarchaeal RNAs (data not shown). As a control, hybridizations of the same filter were performed with the probes EUB338, ARCH915, and UNIV1392 (Fig. 3c, d, and e, respectively). As expected, the probe ARCH915 shows a hybridization signal with all *Archaea*. However, there was also a positive signal with *Aquifex pyrophilus*, a hyperthermophilic oxygen reducer representing the deepest known branching on the bacterial tree (8). Probe EUB338 exhibited good specificity for all the bacterial RNAs used. The probe UNIV1392, which is complementary to a region in the small subunit rRNA identical in all known organisms, was used to establish that all dots on the filter contained equivalent amounts of RNA.

Whole-cell hybridizations. Fixed cells from characteristic *Archaea*, four bacterial species, and three new isolates were hybridized with the probes CREN499 and EURY498 as well as with EUB338, ARCH915, and UNIV1392 (as controls); the results are summarized in Table 1. These results agree with those obtained from the dot blot hybridization experiments: no probe-conferred fluorescence was detected for *Methanopyrus kandleri*. *Methanothermus fervidus*, *Methanobacterium thermoautotrophicum*, and *Halococcus morrhuae* gave only very weak hybridization signals, which was undoubtedly due at least in part to failure of the probe to penetrate the rigid cell walls. Cells of *Halobacterium halobium* showed complete lysis during the fixation process. The SDS concentration which provided optimal probe penetration and morphological integrity of cells is given in Table 1. Epifluorescence photomicrographs from mixtures of a few selected pure cultures of bacteria and members of the two archaeal kingdoms are shown in Fig. 4.

DISCUSSION

The deep and short branchings in both the archaeal and bacterial trees comprise exclusively hyperthermophilic species (42, 50). With the exception of the two bacterial genera *Aquifex* and *Thermotoga*, all belong to the *Archaea*. (Fig. 1). The present study demonstrated that the method of whole-cell hybridization with fluorescence-labeled oligonucleotide probes is well suited for use with the archaeal domain. The new probes EURY498 and CREN499 were designed to be complementary

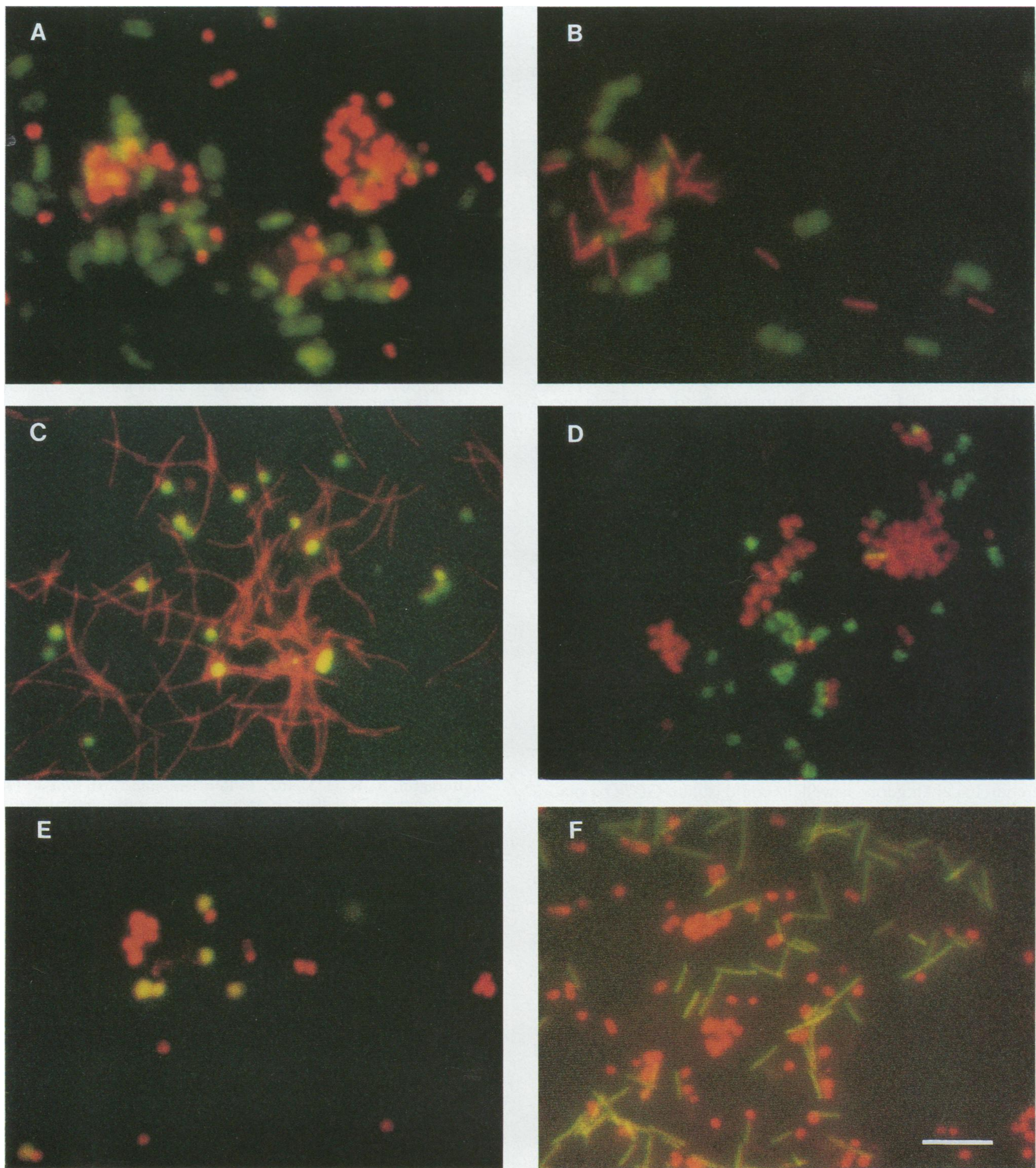

FIG. 4. Simultaneous whole-cell hybridizations of mixtures of different *Archaea* and *Bacteria* with fluorescein-labeled (green fluorescence) and tetramethylrhodamine-labeled (red fluorescence) probes. Epifluorescence photomicrographs (double exposures) were done at a magnification of ×1,250 (scale bar [panel F], 5 μm). Aggregation of cells is an artefact of the concentration and fixation process. For panels A and B, probe CREN499 (red fluorescence) and probe EURY498 (green fluorescence) were used. For panels C, D, and E, probe CREN499 (green fluorescence) and probe EURY498 (red fluorescence) were used. For panel F, probe EUB338 (green fluorescence) and probe EURY498 (red fluorescence) were used. Panels: A, *Sulfolobus acidocaldarius-Methanoplanus limicola*; B, *Pyrobaculum aerophilum-Methanoplanus limicola*; C, isolate Kol8-*Methanospirillum hungatei*; D, *Pyrodictium occultum-Archaeoglobus profundus*; E, *Desulfurococcus mobilis-Pyrococcus furiosus*; F, isolate KC4-isolate GC74.

to essentially the same target region within the 16S rRNA molecule. Therefore, even under low-stringency conditions the probes show a high degree of specificity for each of the archaeal kingdoms because of competitive effects when the two probes are used simultaneously in whole-cell hybridization experiments (30). Both probes also discriminate against *Bacteria* very well. Under the low-stringency conditions used in this study (20% formamide), this is not the case for the archaeal probe ARCH915 (39), however. Our results show hybridization of this oligonucleotide with rRNA of the bacterium *Aquifex pyrophilus*. The explanation for this is that 15 positions of the 20-mer probe ARCH915 are complementary to the target region in *Aquifex pyrophilus* 16S rRNA, including a stretch of 8 guanine or cytosine residues in the middle of the oligonucleotide, the only mismatches being located at the 3′ end (all other *Bacteria* show at least one additional mismatch in the middle region of the probe). CREN499 and EURY498 exhibit more than four mismatches with all members of the *Bacteria*, including the deeply branching genera *Thermotoga* and *Aquifex*. CREN499 has no mismatch to all known members of the kingdom *Crenarchaeota* but is well separated from members of the kingdom *Euryarchaeota* by at least four mismatches. It was shown that EURY498 can be used for most members of the kingdom *Euryarchaeota*. However, because of the greater phylogenetic diversity in this archaeal kingdom, there are a few exceptions, which tend to be members of highly diverged lineages (the extreme halophiles and seven members of the orders *Methanomicrobiales* and *Methanobacteriales*). By introducing degeneracies in the sequence of probe EURY498, those exceptions could be included without decreasing the probe's specificity. Consistent with its deeply branching position within the kingdom *Euryarchaeota* (9), *Methanopyrus kandleri* also exhibits two mismatches, which prevent hybridization of the probe EURY498.

By using the new probes CREN499 and EURY498 in whole-cell and dot blot hybridization experiments, the hyperthermophilic isolates 1A and Kol8 could be placed within the crenarchaeal kingdom as well, as could isolate GC74 be placed within the kingdom *Euryarchaeota*. These placements have been confirmed by partial sequencing of the 16S rRNA genes (5a).

Some problems remain to be solved; e.g., failure of probes to penetrate cell envelopes of *Methanopyrus kandleri*, *Methanothermus fervidus*, *Methanobacterium thermoautotrophicum*, and *Halococcus morrhuae*. Protocols to render their thick and rigid pseudomurein or heteropolysaccharide cell walls permeable, without destroying their morphological integrity, need to be developed. Variations of the fixation and hybridization protocol for members of the genus *Halobacterium* will also be necessary to prevent lysis at the low salt concentrations used during fixation and whole-cell hybridization. These problems, due to the cell wall heterogeneities, may require more than one single protocol to perform whole-cell hybridization experiments with natural samples from any given microbial habitat.

Together with the known domain-specific oligonucleotide probes, the two probes EURY498 and CREN499 can be used to get a fuller appreciation of the occurrence, abundance, and distribution of members of the two archaeal kingdoms, especially in hydrothermal systems. Of course, a spectrum of probes specific for gradually lesser phylogenetic rank have to be designed to define the ecology of hot springs in any detail. This will require the availability of additional sequences, either new isolates or 16S rRNA genes PCR amplified directly from the biotope.

ACKNOWLEDGMENTS

We thank Regina Gröbner-Ferreira and Christine Lemche for excellent technical assistance and Robert Huber for providing cell masses and cultures. We appreciate the help of Martin Keller with HPLC purification of oligonucleotides.

This study was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to K.O.S. and by a grant from the EEC (HRAMI project, contract BIOT-CT91-0294) to R.A.

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