Stalking the Wild Crenarchaeote: A fluorescence in-situ hybridization (FISH) microscopic search

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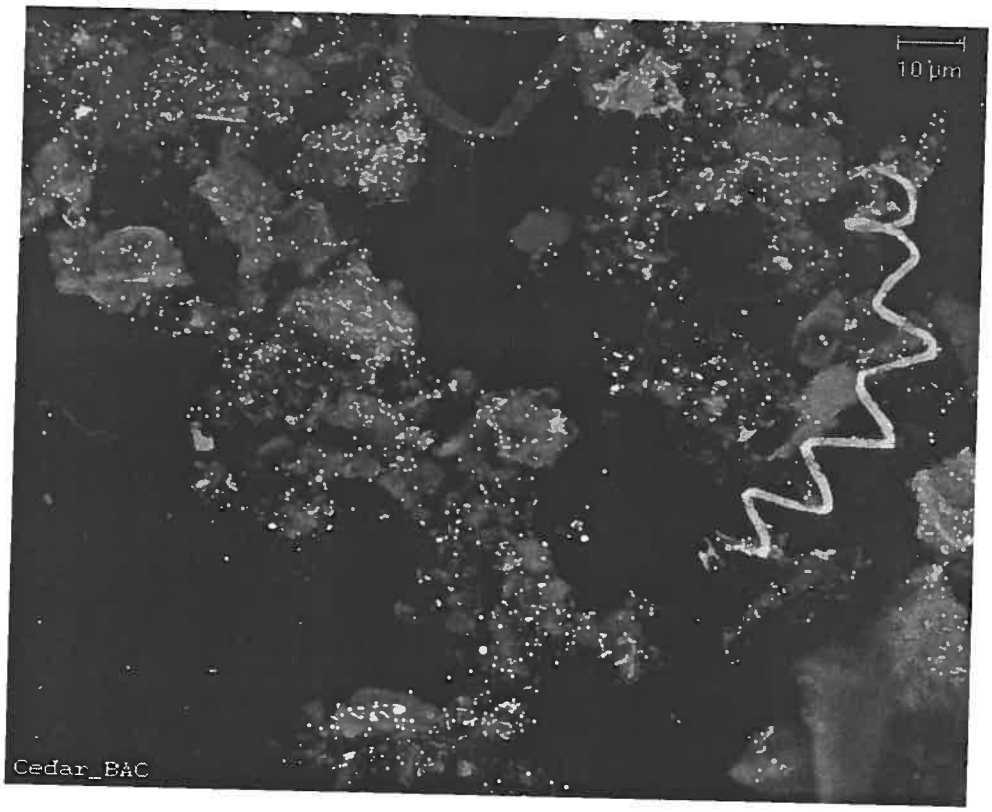

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Stalking the Wild Crenarchaeote: A fluorescence in-situ hybridization microscopic search for what PCR tells us is there, but we've never really seen, counted, or otherwise spied upon in soil, sediment, termites and beyond.

Abstract

Cold-dwelling, uncultivated Crenarchaeota commonly compose a significant fraction of archaeal 16S rRNA genes amplified from non-marine habitats. However, clone libraries tell us little about the abundance of Crenarchaeota compared with Bacteria, or total Archaea in these environments. Fluorescence in-situ hybridization (FISH) is a method that utilizes sequence information from uncultivated microorganisms to track their prevalence in the environment without the biases associated with PCR. In this study, FISH with single oligonucleotide probes was utilized to assess the relative abundance of uncultivated, mesophilic Crenarchaeota in a diverse set of terrestrial locales around Woods Hole, MA. Very few Crenarchaeota were found in this study. Crenarchaeota may make up a small fraction of the total microbial community, or may be inactive in samples tested, although methodological limitations prevent accurate determination of their abundance at this time. This study suggests that methods aimed at reducing autofluorescence and non-specific hybridization coupled with signal amplification (i.e. CARD-FISH) or polynucleotide probing are necessary for targeting small sets of specific soil or sediment Bacteria or Archaea.

Introduction

The discovery of ubiquitous, nonthermophilic Crenarchaeota is a particularly salient result of culture-independent molecular phylogenetic surveys. The cultivated Crenarchaeota (one of two main lineages comprising the domain Archaea) are exclusively isolated from extremely hot environments (DeLong and Pace, 2001). However, 16S rRNA gene sequences related to cultured Crenarchaeota are frequently encountered in "non-extreme" habitats. For example, it is proposed that approximately $1.0 * 10^{28}$ cells (roughly 20%) of all marine picoplankton in the deep ocean, Earth's largest biome, are members of a single Crenarchaeotal lineage (Karner et al., 2001). This finding strongly suggests that nonthermophilic Crenarchaeota play important, but yet unknown, roles in global biogeochemistry. Indeed, the Crenarchaeota may represent the most abundant genome type on the planet and a large branch on the tree of life for which no physiological, biochemical, or genetic information is currently available.

A goal of PCR-based culture-independent surveys is to generate hybridization probes targeted to microorganisms in their native habitat. Fluorescence in-situ hybridization (FISH) microscopy of deep ocean samples reveals that planktonic Crenarchaeota are intact, sometimes dividing, cells containing substantial amounts of rRNA (DeLong et al., 1999). In addition, FISH microscopy shows that Crenarchaeotal populations are spatially and seasonally structured (Murray et al., 1998). These findings indicate that Crenarchaeotes are active and dynamic members of the pelagic microbial community.

More recently, culture-independent surveys of Archaea in non-marine environments have shown that, almost without exception, Crenarchaeota comprise a substantial fraction of the archaeal community. Crenarchaeota are detected in the following settings:

- gut of soil-feeding termite
- pine mycorhizospheres from boreal forest
- upland pasture rhizosphere soil
- bulk forest soil
- oxic basalt aquifer groundwater
- freshwater sediment
- ferromanganous micronodules
- anoxic hypersaline mat sediment
- alpine forest and tundra soil
- boreal forest lake water
- colonizing terrestrial plant rhizospheres
- agricultural soil
- coastal salt marsh
- contaminated soil
- rice paddy soil
- anaerobic digester

However, FISH microscopic studies in many non-marine environments have not been performed (although see Simon et al., 2000), and estimates of population size, spatial and temporal distribution, and observations of growth and morphology are completely unavailable.

Objectives

The purpose of this study was to utilize FISH to address the following questions about Crenarchaeota in non-marine environments:

1. What portion of Archaea are Crenarchaeota? (Additionally, what portion of cells are Bacteria or Archaea?)
2. Do Crenarchaeota appear to be dividing?
3. Do Crenarchaeota have spatial relationships to other kinds of cells? Do they form aggregates?

Methods

Probe selection

Probe CREN512 was designed by Jurgens et al. (2000) and is deposited in the probeBase database at http://www.microbial-ecology.de/probebase/index.html. Its specificity was tested and compared to other Crenarchaeotal probes against an ARB database using the probe match function. CREN probes were also compared based on a 16S rRNA in-situ accessibility map for the Crenarchaeote *Metallosphaera sedula* (Behrens et al., 2003).

Sample collection and preparation

Samples were collected from locations listed in Table 1. Wood-feeding termites were obtained by Jared Leadbetter using corrugated cardboard traps placed under logs overnight. Termites were maintained in plastic containers of wood and cardboard at room temperature. Termite hindguts were collected from specimens using forceps and

placed in PBS buffer. Hindgut tissue was disrupted via vortexing in the presence of glass beads until a turbid solution was achieved.

FISH was carried out according to Pernthaler et al., 2001. Briefly, all cultures and samples were fixed in 2-4% paraformaldehyde. Soil and sediment samples were washed in PBS, suspended in 1:1 PBS/ethanol, and sonicated for 30 seconds at low intensity using 1-second pulses. Cultures, water samples, and termite gut were filtered onto .2 μm polycarbonate membrane filters with cellulose nitrate support filters. Filters were dried and stored at -20°C. Diluted soil and sediment samples were spotted onto 6-well gelatin coated slides, dried and stored at -20°C. Turbid liquid cultures were occasionally spotted onto slides in this manner. In some cases, dried filters or slides were passed through a 70%, 80%, 95% ethanol dehydration series. Washed, fixed root samples were dried and stained directly in deep-well slides.

FISH

FISH was carried out with probes NON338, EUB338, ARCH915, and CREN512 (Table 2) using hybridization conditions listed in Table 3. Hybridizations were incubated at 46°C for 1.5-3 hours and washed for 15 minutes at 48°C. Preparations were counterstained with DAPI or SYBR green I. Visualization and photodocumentation was performed on a Zeiss Axioplan confocal laser-scanning microscope.

Controls

Probes were tested against the following cultures: 1) a methanogenic enrichment of a PCE degrading consortium (R. Daprato, Rice University and generous fellow student), 2) *Escherichia coli* transformed with Crenarchaeotal 16S rDNA recombinant plasmid 3) *Methanocaldococcus jannaschii* (S. Molyneaux, Woods Hole Oceanographic Institute), and 4) *Sulfolobus solfataricus* (K. Stedman, Portland State University).

Table 1. Woods Hole, MA vicinity sites sampled for this study

Location	Sample Type
National Academy of Sciences forest	soil, roots
Woods Hole golf course "rough" grassland	soil
Nobska pond	water, sediment (2 layers: black, sand)
Sippewissett salt marsh	microbial mat (4 layers: green, pink, black, sand)
Wood-feeding termite	gut
Woods Hole coastal waters	surface, bottom, 10 m
Cedar swamp	soil

Table 2. Fluorescence in-situ hybridization probes used for this study.

Probe	Target	Sequence 5' > 3'	Label	% Formamide
NON338		ACTCCTACGGGAGGCAGC	Cy3	35
EUB338	Domain Bacteria	GCTGCCTCCCGTAGGAGT	Cy3	35
ARCH915	Domain Archaea	GTGCTCCCCCGCCAATTCCT	Cy3	35
CREN512	Crenarchaeota	CGGCGGCTGACACCAG	Cy3	0,15

Table 3. Buffers used for FISH in this study for probes optimized to 0, 15, or 35% formamide.

Hybridization Buffer (2 ml)

% Formamide	0	15	35	
Stock Solution				Final Concentration
5M NaCl	360 ul	360 ul	360 ul	900 mM
1M Tris-HCl pH 7.3	40 ul	40 ul	40 ul	20 mM
Formamide	-	300 ul	700 ul	depends on probe
10 mg/ml BSA*	10 ul	10 ul	10 ul	5%
H_2O	add to 2 ml	add to 2 ml	add to 2 ml	
10% SDS (add last)	2 ul	2 ul	2 ul	0.01%

Washing Buffer (50 ml)

% Formamide	0	15	35	
Stock Solution				Final Concentration
5M NaCl	9.0 ml	3.2 ml	800 ul	depends on probe
1M Tris-HCl pH 7.3	1.0 ml	1.0 ml	1.0 ml	20 mM
0.5M EDTA	500 ul	500 ul	500 ul	5 mM
10 mg/ml BSA*	10 ul	10 ul	10 ul	5%
H_2O	add to 50 ml	add to 50 ml	add to 50 ml	
10% SDS (add last)	50 ul	50 ul	50 ul	0.01%

* Blocking reagent

Results and Discussion

Probe selection and optimization

CREN512 was analyzed in ARB using the probe match function and was found to "hit" members of all main lineages of environmental Crenarchaeota as well as those cultivated. CREN512 was the most inclusive probe found in the database. Position 512 of the *M. sedula* 16S rRNA in-situ accessibility map shows overlapping probes tested in this region with mid-range probe access. Although CREN512 was the best choice of tested probes available in the database for this experiment, although the lack of brightness detected for the controls and samples indicate that a better probe could be designed, possibly by selecting a region of the *M. sedula* 16S rRNA molecule with a higher degree of accessibility such as the 800-870 region.

Hybridizations with control cultures

A summary of hybridizations with control cultures is given in Table 4. There was no hybridization with probe CREN512 in the methanogenic enrichment culture, although probes BAC338 and ARCH915 distinguished rod shaped Bacteria from long spiral shaped Archaea, which are most likely *Methanospirillum hungatei* (R. Daprato, personal communication) (Figure 1). The cells hybridized with the ARCH915 probe were very faint with the DAPI stain, and would be overlooked if only DAPI counts were made. This could be because in longer cells, the DNA is not concentrated such that a DNA stain

This could be because in longer cells, the DNA is not concentrated such that a DNA stain like DAPI does not effectively light up the cell. These Archaea were one of the brightest hybridizations made in this study. The active growth phase of this well-fed culture at the time of sampling may be the cause.

Table 4. Fluorescence intensities for control hybridizations. (-) indicates no hybridization, (+) is dimmest, (++++) is brightest.

PROBE	NON338	EUB338	ARCH915	CREN512	expected hybridization
Control Cultures					
methanogenic enrichment*	-	+++ rods	++++ long spirilla	-	EUB, ARCH
Escherichia coli transformed with Crenarchaeote 16S rDNA^	-	++++ rods, long rods	+++ rods, long rods	+++ long rods	EUB, ARCH, CREN
Methanocaldococcus jannaschii#	++ cocci	++ cocci	+++ cocci	++ cocci	ARCH
Sulfolobus solfataricus~	-	-	++++ cocci	+++ cocci	CREN

* enrichment of methanogens (most likely *Methanospirillum hungatei*) from PCE degrading consortium (R. Daprato)

^ possibly a mixed culture of various *E. coli* transformants with Archaeal 16S rDNA, most of which are Crens; 96 well plate mixed in transit from Colorado

grown at 82°C under anaerobic conditions, then mercilessly exposed to oxygen and room temps for several days

~stationary phase culture sent overnight from Portland, OR

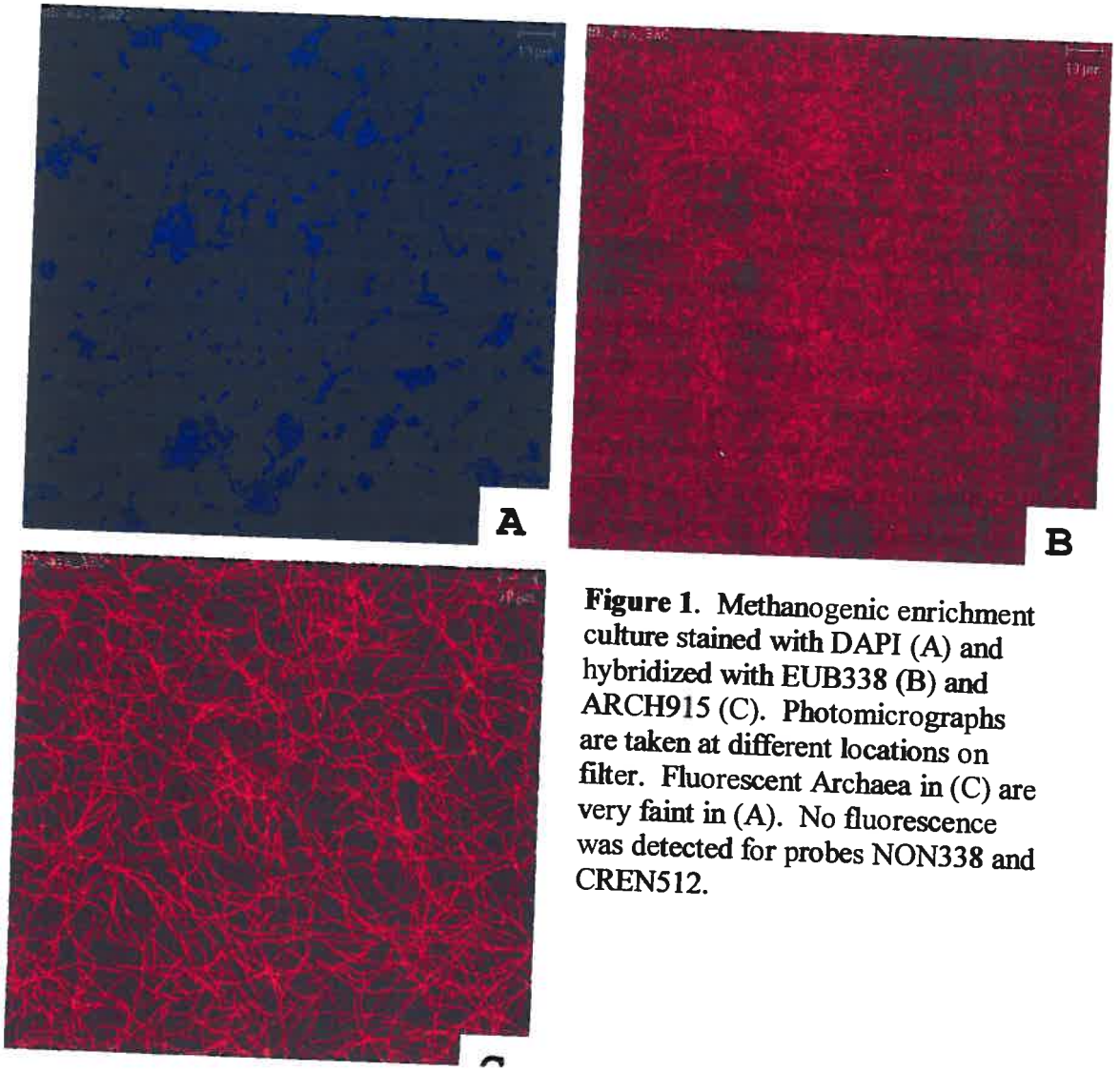

Figure 1. Methanogenic enrichment culture stained with DAPI (A) and hybridized with EUB338 (B) and ARCH915 (C). Photomicrographs are taken at different locations on filter. Fluorescent Archaea in (C) are very faint in (A). No fluorescence was detected for probes NON338 and CREN512.

S. solfataricus fluoresced with ARCH915 and CREN512, although the signal intensity was lower with the later probe (Figure 2). These hybridizations were dimmer than with ARCH915 with the enriched methanogens. Differences in membrane chemistry between the two Archaea may explain this. However, *S. solfataricus* was in stationary phase when the culture arrived in the lab. Therefore fewer ribosomes may have been present at the time of fixation, a more likely explanation.

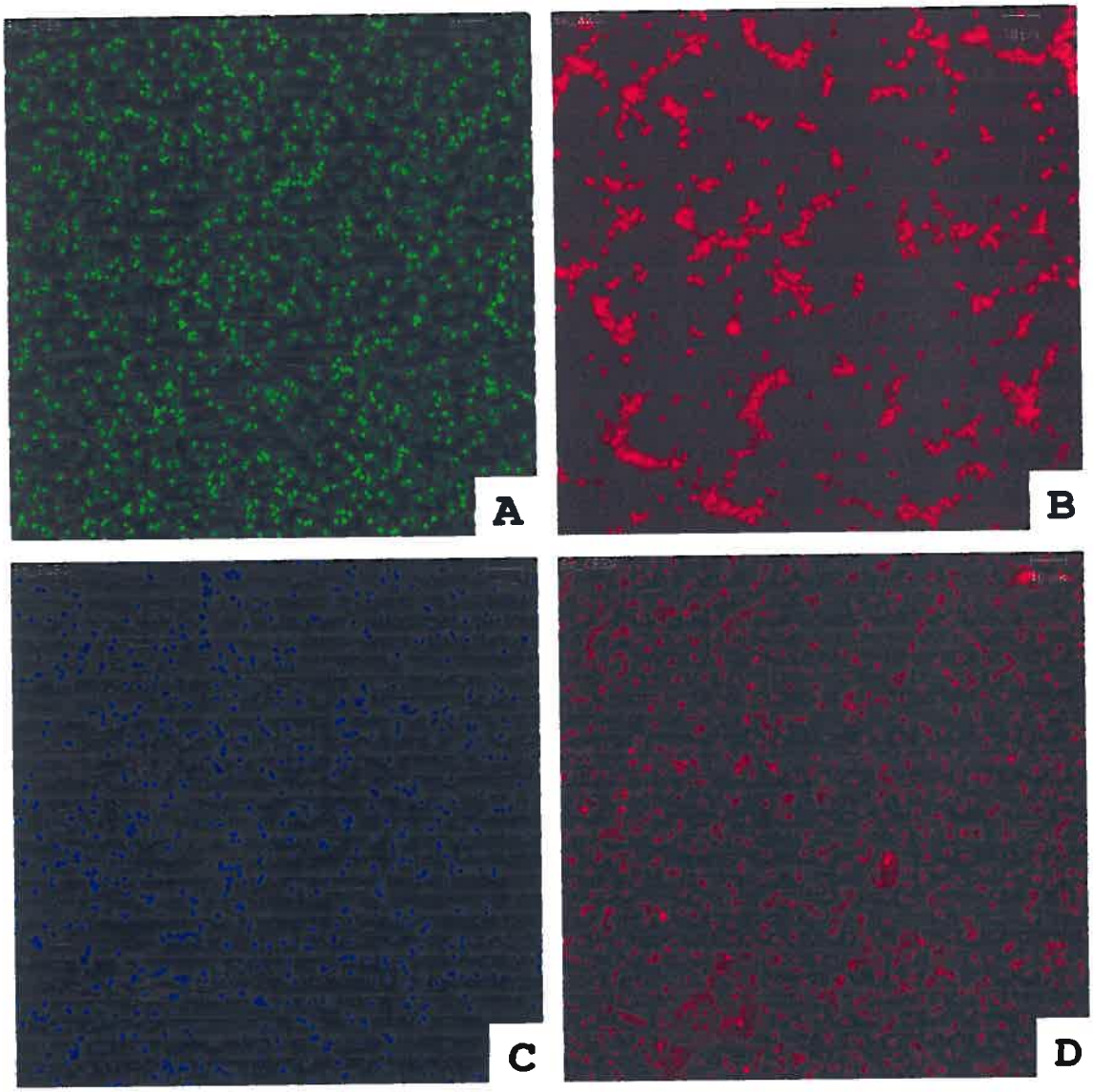

Figure 2. ***Sulfolobus solfataricus*** **cells stained with SYBR (A) and DAPI (C) and Cy3** fluorescence of hybridized ARCH915 (B) and CREN512 (D). No fluorescence was detected with NON338 and EUB338.

E. coli hybridized with BAC338, and a subset of these cells (often longer cells) hybridized with ARCH915 and CREN512 (Figure 3). It is possible that longer cells are older and contain a higher concentration of plasmids carrying the archaeal target DNA sequence. It was also found that 15% formamide in the hybridization buffer increased the hybridization efficiency with this culture (data not shown). 15% formamide was used in subsequent hybridizations.

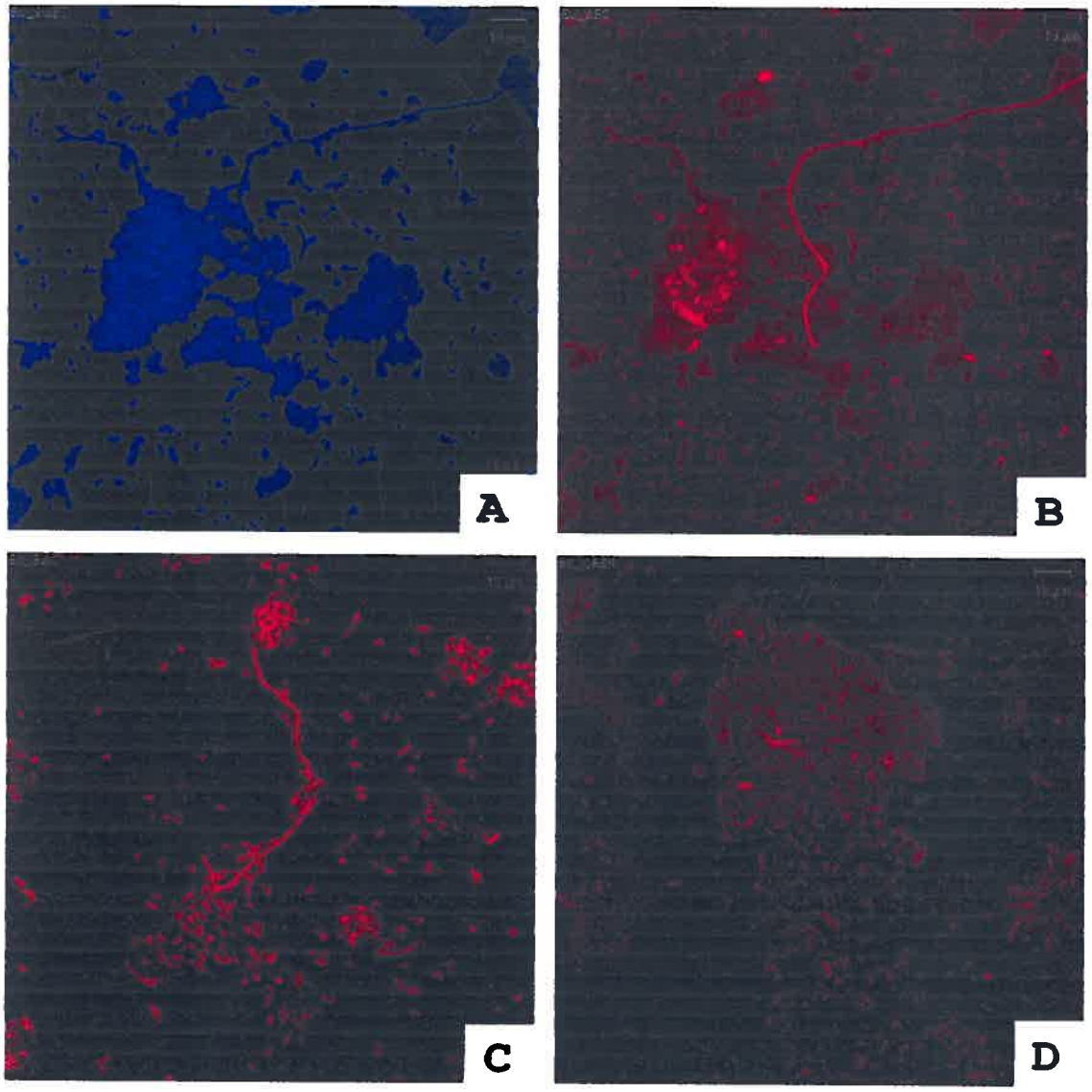

Figure 3. Cells are *E. coli* transformed with recombinant plasmid carrying Crenarchaeotal 16S rDNA stained with DAPI (A) and hybridized with Cy3-labeled EUB338 (C), ARCH915 (B), and CREN512 (D). A and B are photomicrographs of the same cells. Note that the longer cells are stained preferentially with the archaeal probes and are also not as bright as shorter cells in the DAPI stain.

M. jannaschii cells hybridized with all probes, including NON338 (Figure 4). These strictly anaerobic, hyperthermophiles were inadvertently exposed to O_2 and room temperature for several days after stationary phase was achieved. A post-hoc rationale is that neglect simulates the effect of sampling an anaerobic community. I have no idea why this would cause all probes to hybridize. This demonstrates the importance of fixing and hybridizing cells immediately upon sampling from their natural environment.

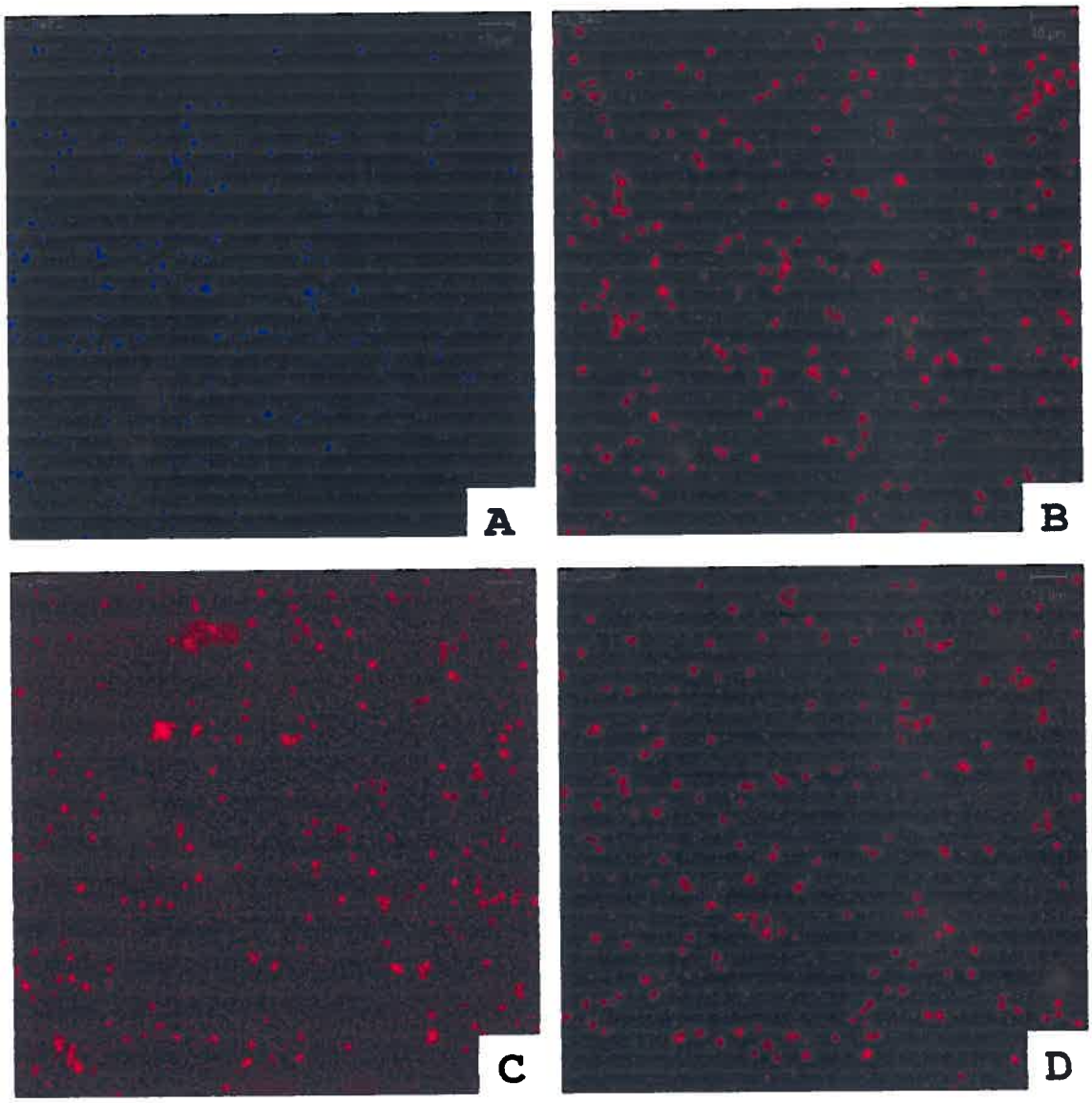

Figure 4. *Methanocaldococcus jannaschii* stained with DAPI (A) and hybridized with Cy3-labeled EUB338 (B), ARCH915 (C), and CREN512 (D). Non-specific hybridization related to multi-day exposure to O_2 and room temperatures possible.

Hybridizations with environmental samples

The termite gut microbial community is shown here and exemplifies results typical for most samples (Figure 5). DAPI and the EUB probe show a morphologically diverse set

of bacteria. The ARCH probe hybridizes with several types of filamentous cells, some of which seem to be coated with bacteria (DAPI) (not shown). In this sample it appears that the CREN probe hybridized with one filamentous cell. This cell is not autofluorescent, that is, it is not visible under blue excitation (all surrounding debris was visible under blue excitation). It is also not DAPI stained (although see results for *M. hungatei*), and not autofluorescent under UV excitation. Only one cell was found. Figure 6 shows hybridizations with CREN512 for roots collected from forest soil where very similar results were obtained, only the putative CREN hybridized cells are small rods, and there are more than one. In addition, these cells stain with the DNA stain SYBR green I.

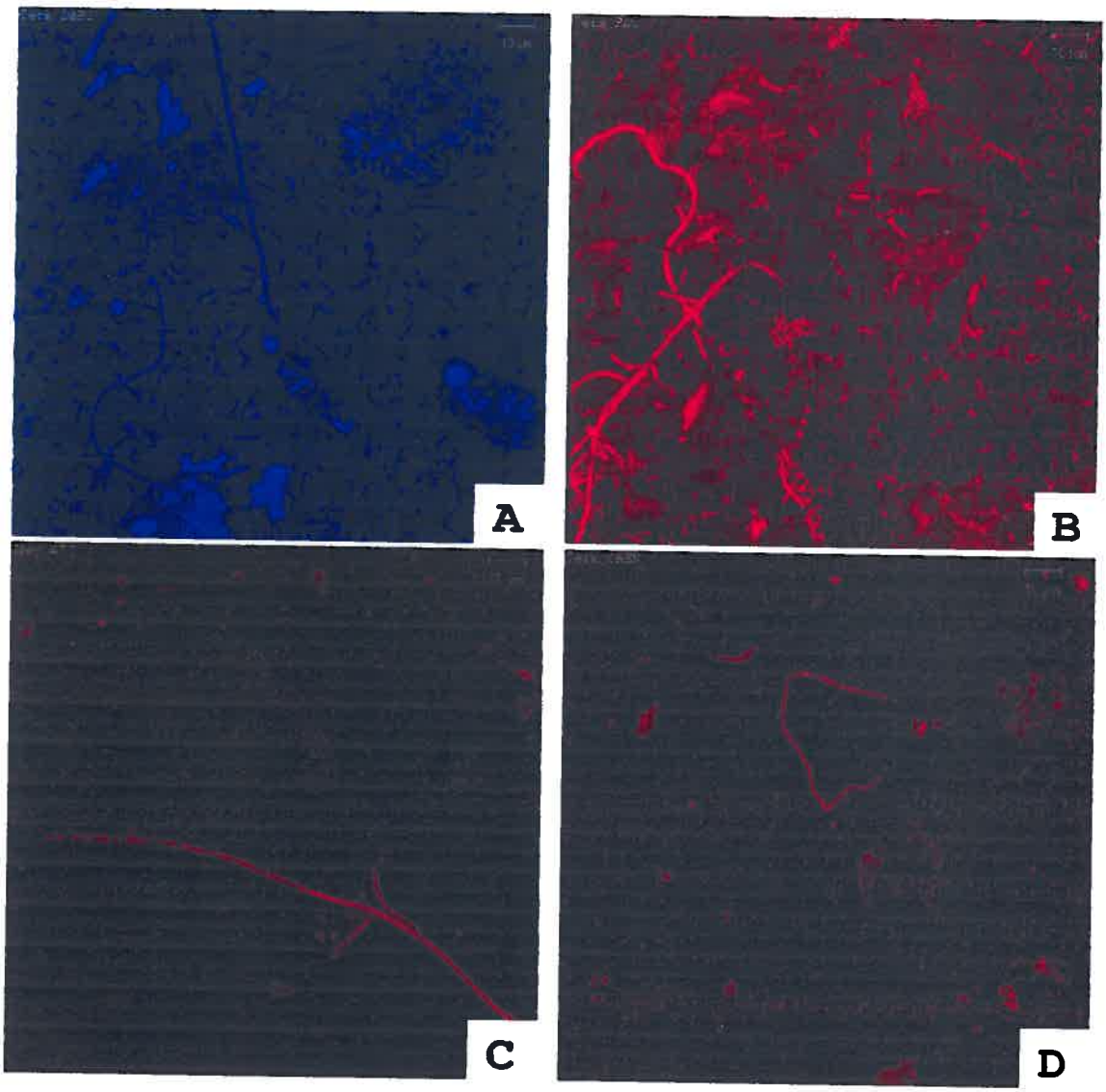

Figure 5. Termite gut microbial community stained with DAPI (A) and Cy3-labeled probes EUB338 (B), ARCH915 (C), and CREN512 (D). The putative Crenarchaeote (D) was not autofluorescent, but was also not stained with DAPI. Note reduction in background in C and D with blocking buffer compared with B.

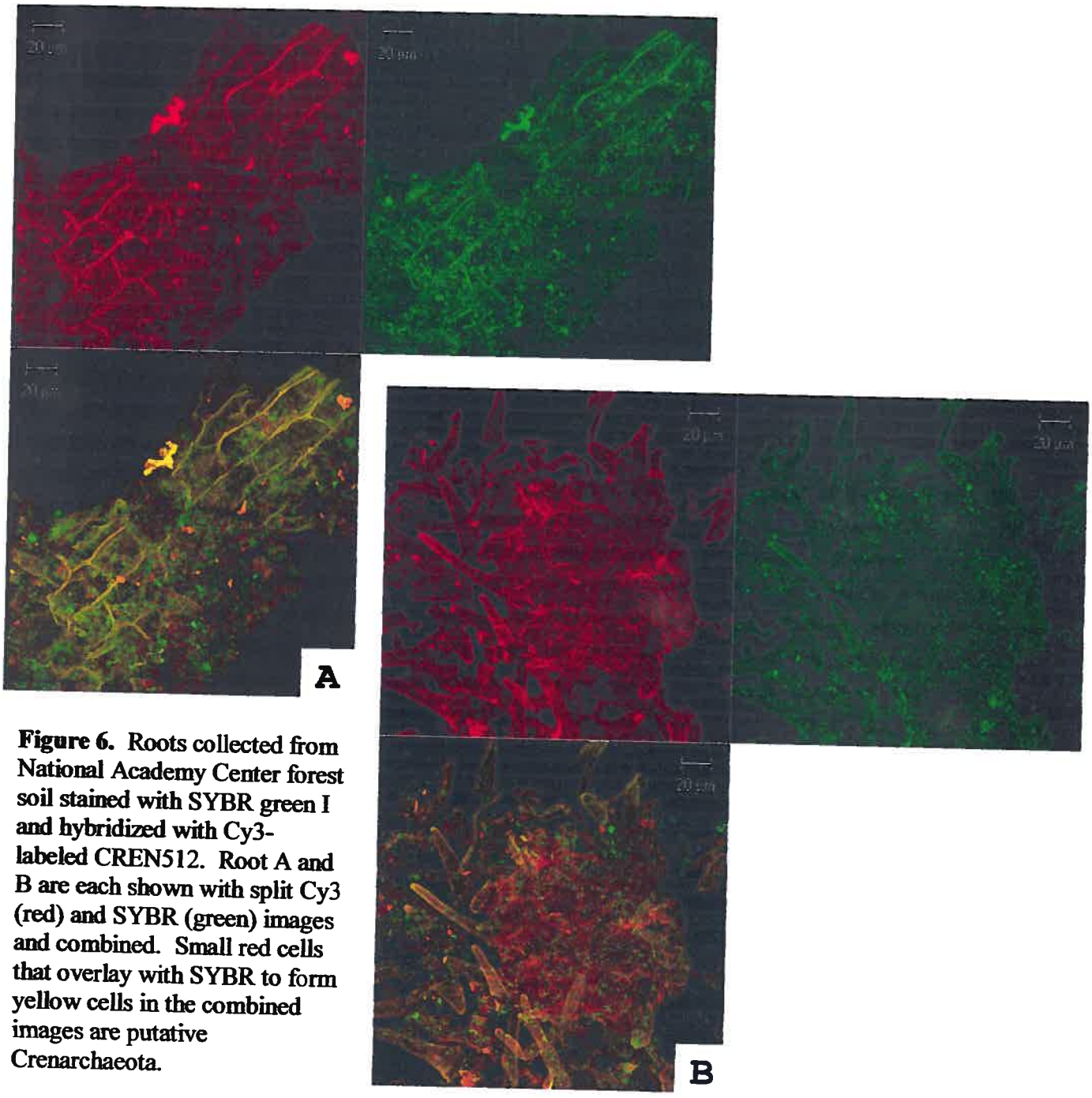

Figure 6. Roots collected from National Academy Center forest soil stained with SYBR green I and hybridized with Cy3-labeled CREN512. Root A and B are each shown with split Cy3 (red) and SYBR (green) images and combined. Small red cells that overlay with SYBR to form yellow cells in the combined images are putative Crenarchaeota.

Conclusions

The obstacles to FISH in soil and sediment are as follows: 1) non-specific hybridization to organic matter and other particles is high, 2) auto-fluorescence by minerals and pigments is also high, 3) cells in these environments are often stuck to the aforementioned organic matter and minerals, 4) cells in these environments are often very small, slow growing, or starving so ribosome content is low. It may be the case as well that Archaea in soil and sediment are about 100X less abundant than Bacteria. In most of

the soil and sediment tested here the EUB probe hybridized to the vast majority of DAPI stained cells. Only a few ARCH or CREN hybridized cells were ever observed, and in these cases it was difficult to tell if they were really cells, as the DAPI stains were often faint (although see results for *M. hungatei*) or obscured by background fluorescence. One strategy that seemed to improve the search was to use a blocking buffer (5% BSA) in the hybridization and washing buffer to reduce the amount of non-specific background noise. Also, passing the samples through an ethanol dehydration series seemed to improve the signal intensity. It is also tempting to further sonicate the samples to separate the cells from the substratum. However, it turned out that a little more is too much, and many of the samples were cell free after a second round of sonication. The following modifications in FISH for soils and sediments may improve this situation: 1) use a signal amplification reaction such as CARD-FISH (Pernthaler et al., 2002), 2) use polyribonucleotide probes (DeLong et al., 1999), 3) design probes with better access to the ribosome (Behrens et al., 2003), 4) further optimize the hybridization conditions, 5) use reagents aimed at permeabalizing the membranes.

The results of this study, although confounded by methodological limitations, indicate that Crenarchaeotal populations are very small in terrestrial habitats. How do we reconcile this with their widespread appearance in clone libraries and 16S rDNA diversity? Could it be that these sequences represent DNA from non-active cells, input from other environments like the deep ocean where they are abundant? This seems unlikely, especially given the number of clades unique to terrestrial environments. I think it more likely that the populations are indeed small compared with Bacteria, but that they are active, unique cells whose fundamental biology we do not understand such that FISH may not yet be compatible with their ribosome content, membrane chemistry, location, and size.

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