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Archaeal Nucleic Acids in Picoplankton from Great Lakes on Three Continents

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ABSTRACT

Phylogenetic analysis of PCR-amplified 16S rRNA genes revealed the presence of archaea in picoplankton collected from the Laurentian Great Lakes in North America, Africa's Lake Victoria, and Lakes Ladoga and Onega in northeastern Eurasia. From 1 to 10% of the rRNA extracted from size-fractionated picoplankton (>0.2 μm but <1.2 μm) collected in the epilimnion and hypolimnion of these lakes was specific to the Archaea, whereas the majority of rRNA was derived from Bacteria. Analysis of the 16S rRNA genes cloned from these samples indicated they were closely related to crenarchaeal sequences that have been widely characterized from marine environments. The presence of nearly identical 16S rDNA clones in several of these geographically disparate lakes suggests a cosmopolitan distribution of specific subgroups of these Archaea in freshwater environments. Despite their abundance in the water column of freshwater lakes, we have no representatives of these crenarchaea in pure culture, and so their physiological characteristics and ecological role remain unknown.

Introduction

Numerous studies using molecular techniques have revealed a vast diversity of Archaea in natural environments. Most of the cultured and described members of the archaeal domain have until recently been conceptualized as either extremophiles, inhabiting hypersaline or hyperthermal environments, or strictly anoxic methanogens [8, 49]. However, recent observations suggest that archaeons from both kingdoms of the Archaea (the Crenarchaeota and the Euryarchaeota) are present in more moderate surface habitats [29]. Recent studies have detected nonthermophilic crenarchaeal groups in oceanic waters [10, 12, 15, 31], in freshwater sediments [28, 41] and lakes [21, 36], and in various soils [4, 5, 22]. Our current understanding is shifting toward the view that the some of the Archaea are globally distributed and inhabit a much broader range of environments than previously believed.

An emerging theme of the recent studies of archaeal diversity and distribution is that many archaeons in

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moderate environments are phylogenetically situated within the kingdom Crenarchaeota [4, 5, 10, 40]. Members of this kingdom were thought to be predominately thermophilic [49], but the recent discovery of nonthermophilic crenarchaeal groups has led to a reevaluation of physiological diversity among the Archaea [9, 29]. Unfortunately, no representatives of these putatively free-living moderate crenarchaea have yet been isolated in pure culture, and what we know about their abundance and distribution has been discovered using molecular techniques. Although a few such molecular studies have focused on lakes [17, 21, 27, 28, 36, 46], none have compared the archaeal nucleic acid component of picoplankton communities from geographically disparate large lakes. The purpose of this study was to characterize the archaea and to assess the relative abundance of archaeal nucleic acids in size-fractionated (>0.2 μm but <1.2 μm) picoplankton from great lakes on three continents using quantitative 16S rRNA-based oligonucleotide hybridizations targeting the domains Archaea, Bacteria, and Eucarya. We also performed a preliminary characterization of the detected archaeal nucleic acids using both a 16S rRNA-based phylogenetic analysis and a quantitative nested hybridization approach.

Methods

Site Locations and Descriptions

Picoplanktonic cells were collected from Lakes Erie, Huron, Michigan, Ontario, and Superior in North America (Laurentian Great Lakes), Lakes Ladoga and Onega in Russia, and Lake Victoria in Africa (Table 1). All lakes studied were sampled in August, with the exception of site SGM in Lake Superior during 1992, which was sampled in July, and site V96-4 in Lake Victoria during 1996 [48], which was sampled in May. To ensure that sampling captured only picoplanktonic microorganisms, all samples were taken at offshore sites to avoid the potentially confounding influence of terrestrial runoff, which could contribute microbial nucleic acids from surrounding soils.

CTD and transmissometer casts (SeaBird Sea Cat CTD and SeaTech transmissometer) were performed to determine the thermal profiles of each lake to aid the selection of water depths to sample. All lakes were thermally stratified at the time samples were taken. In lakes of this size, complete mixing between the upper, warmer epilimnion and the lower, colder hypolimnion is rare during summer, and thus the two layers can be regarded as physically separate habitats that may support different microbial assemblages. At most of the sites, one epilimnetic and one hypolimnetic depth was chosen to evaluate the potential difference in picoplanktonic nucleic acid composition between the epilimnion and hypolimnion. Epilimnetic samples were collected at 5 m in all lakes except Victoria (3 m). Hypolimnetic sampling depths ranged from 14 to 65 m, but typically were between 35 and 65 m in the deeper Laurentian and Russian great lakes.

Nucleic Acid Sampling and Extraction

Picoplankton samples were collected from water in each lake studied using submersible pumps, Van Dorn, or Niskin bottles. A March pump (model 5C-MD; March Mfg., Glenview, IL) attached to polyvinyl tubing (1.9 cm ID) was used in Lakes Erie and Superior in 1992. A Town and Country Water Systems pump (model TC3222-00; Sta-Rite Industries, Delevan, WI) attached to nylon tubing (1.6 cm ID) was used to take samples in the other Laurentian Great Lakes during 1992 and all of 1993. A Van Dorn bottle was used to collect samples in Lakes Onega and Ladoga. Niskin bottles were used to collect samples in Lake Victoria. All sampling equipment was flushed or rinsed with two volumes of water from each depth prior to sample collection. In all lakes sampled except Lake Victoria, up to 40 L of lake water were prefiltered through 142 mm glass fiber filters (Gelman A/E, 1.2 μm nominal pore size) to remove larger eucaryotic cells [20]. Total direct counts of prokaryotic cells decreased slightly after prefiltration, but on average 86% (2% standard error, $n = 11$) of the total prokaryotic cells observed by epifluorescence microscopy passed this prefiltration in 1992 samples from the Laurentian Great Lakes [20]. Smaller cells in the remaining fraction were then captured on Durapore membrane filters (Millipore, 142 mm, 0.22-μm pore size), which were folded and stored at −20°C in Whirl-Pak bags until extraction. During 1992, at least 99% (0.1% standard error, $n = 14$) of the cells in these filtrates were captured on the Durapore extraction filters. Thus, about 85% of the picoplanktonic cells in the original water samples (0.22 to 1.2 μm size fraction) were captured on these filters [20]. Nucleic acids were usually extracted within a few months after sampling, but in no cases longer than 1 year. Lake Victoria samples were collected in the same manner, except smaller prefilters and extraction filters were used (47 mm) and total filtrate volume was typically about 14 L.

Nucleic acids were extracted by using a variation of the procedure developed by Fuhrman et al. [13] as modified by Hicks and Pascoe [20]. Briefly, picoplanktonic cells captured on membrane filters were lysed in 8.75 ml of STE buffer (pH 8.0) containing 0.2 mL of lysozyme solution (10 mg/mL, Sigma Chemical Co.), 50 μL of predigested proteinase K solution (20 μg/μL, Gibco BRL), and 1 mL of 10% SDS. Nucleic acids were ethanol precipitated at −20°C overnight, pelleted in a centrifuge (14,000*g*, 20 min, 4°C), rinsed, air dried, and resuspended in 0.5 mL TE buffer (pH 8.0). These crude extracts were purified by phenol:chloroform:isoamyl alcohol (24:6:1) and chloroform:isoamyl alcohol (24:1) extractions, precipitated with 5M NaCl and ice-cold ethanol, rinsed with 70% ethanol, dried, and then redissolved in 300 μL of TE buffer (pH 8.0) [20]. Samples from the North American Great Lakes were purified further using Sephadex G-200 spin columns to remove humic acid contaminants

Table 1. Sites in great lakes on three continents where picoplankton samples were obtained

Lake	Site[a]	Latitude and longitude	Depth (m)	Date
Erie	E15M	42°31.00′N, 79°53.36′W	5	8/7/92, 8/8/93
			45	8/7/92
Huron	H45M	45°08.12′N, 82°59.00′W	5	8/11/93
			30	8/11/93
Michigan	M18M	42°44.00′N, 87°00.00′W	5	8/22/92
			65	8/22/92
	M47M	45°10.42′N, 86°22.30′W	5	8/18/92, 8/25/93
			50	8/25/93
			65	8/18/92
Ontario	O33M	43°31.50′N, 76°55.00′W	5	8/9/92
			65	8/9/92
	O64	43°35.48′N, 78°48.06'W	5	8/10/92
			65	8/10/92
Superior	SGM	46°56.31′N, 91°31.92′W	5	7/14/92
	S101100	47°23.05′N, 89°32.22′W	5	8/17/93
			36	8/17/93
	S94260	48°05.52′N, 87°55.08′W	5	8/19/93
Ladoga	L14	60°59.07′N, 30°57.60′E	5	8/8/94
			50	8/8/94
	L17	61°24.75′N, 31°21.20′E	5	8/13/94
			50	8/13/94
Onega	ON1	61°18.52′N, 31°21.20′E	5	8/14/94
			50	8/14/94
Victoria	V96-4	1°34.8'S, 32°51.9′E	3	5/21/96
			14	5/21/96

[a] E15M, H45M, M47M, M18M, O33M, O64, S101100, and S94260 are all USEPA sampling stations. SGM, L14, L17, and ON1 are arbitrary site designations. V96-4 is identical to a site designated in a prior publication [48].

[45]. Nucleic acid concentration and purity were determined spectrophotometrically by measuring absorbance in the range 220–320 nm. Absorbance at 260 nm was used to estimate nucleic acid concentration and purity was monitored by A_{260}:A_{280} ratios (1.9–2.2 range) and A_{260}:A_{230} ratios (1.5–2.4 range). The purity of nucleic acid extracts with absorbance ratios within both of these ranges was sufficient for hybridizations with oligonucleotide probes. Samples with absorbance ratios outside these ranges were not chosen for further analysis [20].

Standard Cultures

Nucleic acids representative of all target taxa (when available) were used as standards to normalize raw hybridization signals to taxon-specific and nonspecific binding of probes (Table 2). These nucleic acids were extracted and purified from cultured cells using the same procedure as was used for natural picoplankton samples.

Preparation and Labeling of Oligonucleotide Probes

First, three 16S rRNA-based domain-level probes developed by other investigators were used to determine the relative abundances of bacterial (S-D-Bact-0338-a-A-18), archaeal (S-D-Arch-0915-a-A-20), and eucaryal (S-D-Euca-1195-a-A-16) nucleic acids in the picoplankton samples (Table 2). Afterwards, four other 16S rRNA-based probes (Table 2) targeting the Crenarchaeota, Euryarchaeota, Methanobacteriaceae, and the uncultured Group I marine crenarchaeota were used to help quantify the nucleic acid contributions of organisms from these groups using a nested approach [3]. Nested hybridizations continued until these four archaeal target groups were quantified or nucleic acid extracts were depleted, whichever came first.

Each oligonucleotide was synthesized (Michigan State University) and purified by polyacrylamide gel electrophoresis and column chromatography (Tokyo Pearl TSK DEAE) before radiolabeling the 5′ end with [γ-^{32}P]ATP (NEN Radiochemicals) for 1 h at 37°C using T4 polynucleotide kinase (Promega Corp. [10, 42]). After radiolabeling, each oligonucleotide probe was again purified on TSK DEAE resin (250 μL, Supelco) mini-columns constructed from sterile polyethylene 1-mL pipette tips plugged with glass wool. Each column was rinsed with 1 mL of 50 mM ammonium acetate solution before the probes were added. Unlabeled probe and excess [γ-^{32}P]ATP were removed from these columns with two 1-mL rinses of ammonium acetate solutions (50 m*M* followed by 250 m*M*). The radiolabeled oligonucleotide probe was then eluted by rinsing the column with 1 mL of 500 m*M* ammonium acetate followed by two 1-mL rinses of 1 M ammonium acetate. These fractions were combined, dried, rinsed with 50 μL of 50% methanol, and dried again. Finally, each radiolabeled probe was resuspended in 200 μL of autoclaved Milli-Q water and the radioactivity was determined by liquid scintillation counting. The specific activity (CPM/μg) of the probe was then calculated assuming that any

Table 2. Oligodeoxynucleotide probes, sequences, target groups, wash temperatures in 1X SET, references, and standard cultures

Probe name[a]	Sequence (5′–3′)[b]	Target group	Wash (°C)	Reference	Standard cultures[c]
S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	Bacteria	45	[2]	*Pseudomonas fluorescens* (ATCC 13525)
S-D-Arch-0915-a-A-20	GTGCTCCCCCGCCAATTCCT	Archaea	56	[42]	*Haloarcula marismortui* [34]
S-D-Euca-1195-a-A-16	GGGCATCACAGACCTG	Eucarya	40	[16]	*Saccharomyces cerevisiae* (MSU-DMCC SAC-1)
S-K-Cren-0499-a-A-18	CCAGRCTTGCCCCCGCT	Crenarchaeota	65	[6]	*Sulfolobus solftaricus* (ATCC 35091)
S-K-Eury-0498-a-A-14	CTTGCCCRGCCCTT	Euryarchaeota	51	[6]	*Thermoplasma acidophilum* (ATCC 25905)
S-*-Cren-0667-a-A-15	CCGAGTACCGTCTAC	Group I marine Crenarchaeota	35	[10]	Uncultured group, none available
S-F-Mbac-0310-a-A-22	CTTGTCTCAGGTTCCATCTCCG	Methanobacteriaceae	40	[37]	*Methanobrevibacter* spp., strain RFM-3 (MSU-DMCC RFM-3)

[a] These are names of the probes suggested by Alm et al. [1]. The taxonomic level of specificity for S-*-Cren-0667-a-A-15 is left undesignated here, after MacGregor et al. [28].
[b] R = A or G.
[c] MSU-DMCC = Michigan State University — Department of Microbiology Culture Collection.

loss of probe following purification on TSK DEAE columns was consistent for each labeling reaction.

Quantitative Oligonucleotide Hybridizations

A variation of Stahl and Amann's [42] procedure developed by DeLong et al. [10] was used for quantitative hybridizations. Nucleic acid samples were aliquoted into 2.4-μg portions for environmental extracts and 0.6-μg portions for nucleic acid standards. Nucleic acids were denatured in glutaraldehyde (0.5% final concentration) for 15 min at room temperature and then diluted with a polyadenylic acid solution (3 μL of 10 mg Poly A/mL, 1 μL 0.2% bromphenol blue, 302 μL 50% glutaraldehyde, and 29.7 mL Milli-Q water). The nucleic acids were blotted on a MagnaCharge hybridization membrane (Micron Separations, Inc.) in a six-dot concentration gradient (50 to 800 ng). Nucleic acid standards (positive and negative controls) from appropriate taxa were blotted in different six-dot concentration gradients (12.5 to 200 ng) on each membrane containing samples (Table 2). The loaded hybridization membranes were then air-dried and baked at 80°C for 1 h. Membranes were prehybridized at 45°C for 30 min in hybridization buffer (6×SET, 0.5% SDS, 1×Denhardt's solution, Poly A at 100 μg/ml) prior to adding a radiolabeled probe [10]. The prehybridization buffer was discarded and the appropriate radiolabeled probe (10^7 CPM/mL) and fresh hybridization solution (10 mL) were added. The membranes were hybridized overnight (12–22 h) at 45°C. Hybridized membranes were sequentially washed (1×SET, 0.5% SDS) for 2 min at 30°C, at 30°C for 30 min, and then at a stringent wash temperature specific to each probe for 30 min (Table 2). For all domain-level probes and for the group I marine crenarchaeota probe, the final wash temperature was determined empirically in other studies. For all other probes, the final wash temperature was calculated using the theoretical dissociation temperature of the probe. This temperature was determined using the equation $T_d = 81.5 + 16.6 \log M + 0.41[\%(G + C)] - 820/n$, where M is the molar concentration of monovalent cations and n is the length of the probe in nucleotides [25].

With the exception of hybridization membranes with Laurentian Great Lakes samples taken in 1992, all membranes were wrapped in plastic wrap to expose a phosphoimaging screen (BI screen, Bio-Rad) for 12–18 h. A set of ^{14}C standards (Type ARC-146F, American Radiolabeled Chemicals, Inc.) was imaged concomitantly with all hybridization membranes to normalize sample signal intensities to known radioactivity units. The exposed screen was scanned using a Bio-Rad GS525 Molecular Imager, and densitometric analysis was performed on the resulting images with Molecular Analyst v2.1 software (Bio-Rad). The 1992 Laurentian Great Lakes sample membranes were analyzed by gas proportional counting using an Ambis Systems counter (Ambis Systems, Scanalytics, Inc.).

Calculations and Statistical Tests

The slope of nmol probe bound per unit mass rRNA was used to calculate taxon-specific percentages using the sum-over-domains

method as described by DeLong et al. [10]. Each raw hybridization response was corrected for the specific activity of the probe, the binding of probe to nucleic acid standards (where possible), and nonspecific binding. The slope (nmol probe bound per ng nucleic acid) was determined from 3 to 6 points in the linear portion of the binding curve using a simple linear regression model. Percentages were calculated by dividing the slope of sample binding (minus nonspecific binding to negative controls) by the slope of positive control binding (minus nonspecific binding to negative controls).

Unpaired, one-sided *t*-tests were performed (95% CL) to determine if all archaeal nucleic acid percentages were significantly different from zero. One-way ANOVAS were used to determine if the mean archaeal nucleic acid percentages were different at different depths in each lake. Unpaired, two-sided *t*-tests were performed (95% CL) on the mean archaeal nucleic acid percentages to determine if any consistent patterns of archaeal abundance or distribution were revealed. We compared the overall archaeal percentages (a pooled average inclusive of all sites and depths within each lake) of each lake to all other lakes. We then compared the archaeal mean nucleic acid percentages in each site to all other sites and at each depth within each site.

PCR Amplification and Cloning of Archaeal 16S rDNA

We attempted to amplify crenarchaeal rDNA in samples from all lakes with some exceptions. After the hybridization analyses were completed, samples from the Laurentian Great Lakes taken in 1992 were depleted of nucleic acid and were therefore not analyzed using PCR. PCR was performed only on samples from Lakes Erie, Huron, Michigan, Superior, Ladoga, Onega, and Victoria. We used Buckley et al.'s [5] primers 89Fb (5′-ACGGCTCAGTAACRC-3′) and S-D-Arch-0915-a-A-20. These primers mainly amplify nonthermophilic crenarchaeal 16S rRNA genes, but will also amplify the 16S rDNA from other archaea such as some members of the euryarchaeal orders Thermoplasmatales, Methanobacteriales, Methanococcales, and Thermococcales.

PCR amplification of nucleic acid extracts followed Buckley et al.'s [5] procedure with the following modifications. All nucleic acid samples and standards were predigested with ribonuclease (RNase One, Promega Corp.) for 30 min at 37°C prior to the addition of PCR reagents and *Taq* polymerase (Gibco BRL). Deoxynucleotide triphosphates were obtained from GeneAmp, and the thermocycler employed was a PTC-100, model 96-U (MJ Research, Inc.). The archaeal positive control was a nucleic acid extract of *Thermoplasma acidophilum* (ATCC 25905). In addition to blanks (containing no template), nucleic acid extracts from bacterial and eucaryotic microorganisms were selected as negative controls. These negative control nucleic acids were the same extracts used for hybridizations from *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*, respectively (Table 2). PCR amplification of crenarchaeal 16S rDNA was observed in samples from all lakes except Lakes Erie and Huron.

PCR reaction mixtures containing archaeal rDNA products of the expected size of ~850 base pairs (visualized on 1.5% agarose gels) were chosen for cloning using a TOPO-TA cloning kit (Invitrogen). Cloning was performed as recommended by the manufacturer. A small subset of clones (less than 10% of total clones in the library) from samples in all lakes except Lakes Erie and Huron was screened for archaeal 16S rDNA transformants. Rapid screening of transformants (using an inoculating loopful of colony as template) was performed as directed by the manufacturer, using the primer pair M13F (included in the kit, 5′-GTAAAACGACGGCCAG-3′) and S-D-Arch-0915-a-A-20 in a PCR reaction. Twelve clones from Lakes Michigan ($n = 2$), Superior ($n = 3$), Ladoga ($n = 2$), Onega ($n = 1$), and Victoria ($n = 4$) were randomly selected for sequencing from the hundreds of transformant colonies in different samples. These clones were chosen for a preliminary phylogenetic analysis but also to verify the quantitative hybridization results that archaeal nucleic acids were indeed present in lakes on the continents we studied. Colonies of these 12 transformants were grown overnight in LB broth amended with 75 μg/mL ampicillin at room temperature. Cells from these cultures were frozen at −80°C in glycerol stocks as recommended by the manufacturer until sequencing of the cloned rDNA fragment.

Cloned 16S rDNA fragments from the 12 transformants selected for further study were re-amplified prior to sequencing using the same procedure for screening. Clones from Lakes Ladoga, Onega, and Victoria were reamplified using the primers 89Fb [5] and M13R (5′-CAGGAAACAGCTATGAC-3′) and a Gene Amp model 9600 thermal cycler (PerkinElmer). All other clones were reamplified using the primers M13F and M13R and a model 96-U PTC-100 thermal cycler (MJ Research, Inc.). A fraction of each PCR mixture (80 μL) was diluted to 400 μL with sterile Milli-Q water and the 16S rDNA inserts were purified and concentrated using UltraFree MC spin columns (30,000 NMWL, Millipore Corp.). Purified inserts were reconstituted in sterile Milli-Q water (50 μL) and their concentrations were quantified by measuring absorbance at 260 nm.

Inserts from Lakes Ladoga, Onega, and Victoria clones were sequenced using a Big Dye Terminator DNA Sequencing Kit (Applied Biosystems Inc.) and cloned fragments from Lakes Michigan and Superior were sequenced at the University of Minnesota Advanced Genetic Analysis Center (St. Paul, MN). Inserts were initially sequenced with M13F and 519R (5′-GWATTACCGCGGCKGCTG-3′) to verify that the amplicons were 16S rDNA-Iike sequences. Initial sequences were compared to sequences in the public database GenBank using the BLASTn tool (www.ncbi.nlm.nih.gov/BLAST/). Amplicons whose initial sequences were clearly similar to 16S rRNA genes were then fully sequenced. Final sequences were recovered using the primers M13F, 519R, 515F (5′-GTGCCAGCAGCCGCGGTAA-3′), and M13R. A total of 12 archaeal 16S rRNA gene sequences were recovered.

Phylogenetic Analysis

Phylogenetic analyses were performed using the sequence analysis program ARB (www.mikro.biologie.tu-muenchen.de).

Table 3. Percentages of domain-specific nucleic acid in size-fractionated picoplankton (0.2–1.2 μm) from great lakes on three continents[a]

Lake	Site	Depth (m)	Sample date	*n*	Bacteria (%)[b]	Eucarya (%)	Archaea (%)
Erie	E15M	5	8/7/92	2	97.1 (0.5)	2.2 (0.5)	0.7 (0.03)
		45	8/7/92	1	97.8	1.5	0.7
		5	8/8/93	4	89.9 (4.8)	6.7 (3.9)	3.9 (1.6)
Huron	H45M	5	8/11/93	1	81.5	16.1	3.3
		30	8/11/93	2	89.7	6.5 (4.9)[x]	4.3 (4.0)[x]
Michigan	M18M	5	8/22/92	1	97.9	0.5	1.6
		65	8/22/92	1	97.5	1.9	0.7
	M47M	5	8/18/92	2	95.5 (0.3)	3.1 (0.4)	1.5 (0.1)
		65	8/18/92	2	95.6 (1.1)	2.8 (0.2)	1.6 (0.03)
		5	8/25/93	4	91.5 (4.0)	5.2 (2.8)	3.8 (1.7)
		50	8/25/93	3	90.5 (3.7)	5.1 (2.6)	5.0 (1.6)
Ontario	O33M	5	8/9/92	2	98.0 (0.7)	1.1 (0.5)[‡]	1.0 (0.1)
		65	8/9/92	2	97.3 (0.6)	1.4 (0.6)[‡]	1.3 (0.0)
	O64	5	8/10/92	2	97.9 (0.1)	2.0 (0.1)	0.1 (0.02)
		65	8/10/92	2	97.8 (0.02)	1.4 (0.01)	0.8 (0.01)
Superior	SGM	5	7/14/92	1	90.9	7.3	1.9
	S101100	5	8/17/93	3	86.6 (7.6)	11.4 (6.4)	2.2 (1.7)[‡]
		36	8/17/93	3	84.9 (8.0)	11.1 (7.8)[‡]	4.6 (1.9)
	S94260	5	8/19/93	2	77.6 (21.6)	17.0 (17.3)[x]	5.7 (4.8)[x]
		36	8/19/93	1	95.0	2.2	2.8
Ladoga	L14	5	8/8/94	2	91.1 (7.8)	3.0 (2.1)[x]	5.9 (5.8)[x]
		50	8/8/94	1	84.4	5.5	10.1
	L17	5	8/13/94	2	95.0 (0.8)	2.9 (0.4)	2.1 (0.3)
		50	8/13/94	2	92.3 (1.2)	2.0 (0.3)	5.7 (0.9)
Onega	ON1	5	8/14/94	2	93.0 (1.6)	4.9 (0.8)	2.0 (0.8)
		50	8/14/94	2	92.9 (1.0)	2.8 (0.4)	4.0 (0.1)
Victoria	V96-4	3	5/21/96	1	94.1	0.7	5.2
		14	5/21/96	1	93.7	0.4	5.9

[a] Results shown are significantly different from zero at the 95% confidence level in an unpaired one-sided *t*-test. Numbers in parentheses represent the standard deviation from the mean.
[b] Quantitative hybridization results for the domain Bacteria in samples taken from the Laurentian Great Lakes in 1992 were reported previously [20]
[x] Not significantly different from zero.
[‡] Significantly different from zero at the 90% confidence level.

Recently published archaeal 16S rDNA sequences were obtained from GenBank (www.ncbi.nlm.nih.gov) and inserted into the ARB environment along with our cloned sequences. Initial sequence alignments were performed using the ARB automatic alignment tool and then were adjusted manually to account for elements of secondary structure (e.g., hairpins and loops) and regions of primary sequence conservation. Sequences were aligned between positions 124 and 914 *(Escherichia coli* numbering) of the 16S rRNA; a majority of the approximately 750 positions available were used in subsequent analyses. Phylogenetic trees were constructed using fastDNAml [33], a maximum likelihood method. Outgroup sequences and sequences from nonthermophilic crenarchaea were varied during tree construction. Sequences of 16S rDNA from *Halorubrum lacusprofundi* and *Haloferax volcanii* (both from the kingdom Euryarchaeota) were used as outgroups to root the final tree and put the relative distances of all sequences in a larger, more generally inclusive archaeal context. Bootstrap values were based on 1000 trees computed by neighbor joining [39].

Results

Quantitative hybridizations revealed that the percentage of domain-level (i.e., total) archaeal nucleic acid was between 0.7% and 10.1% in all lakes studied (Table 3). One-sided, unpaired *t*-tests indicated that most of the relative percentages calculated for each domain-level probe were significantly different from zero ($P < 0.05$). Unpaired two-sided *t*-tests were used to compare archaeal percentages to the variables of lake, site, and depth. The following types of comparisons were performed: overall pooled averages from lake to lake, from site to site (both within and between lakes), and epilimnion to hypolimnion (both within and between lakes and sites). No consistent trends were revealed by these tests. The only significant difference was the percentage of total archaeal nucleic acid was higher during 1993 than in 1992 in the Laurentian Great Lakes ($P < 0.01$, Table 3).

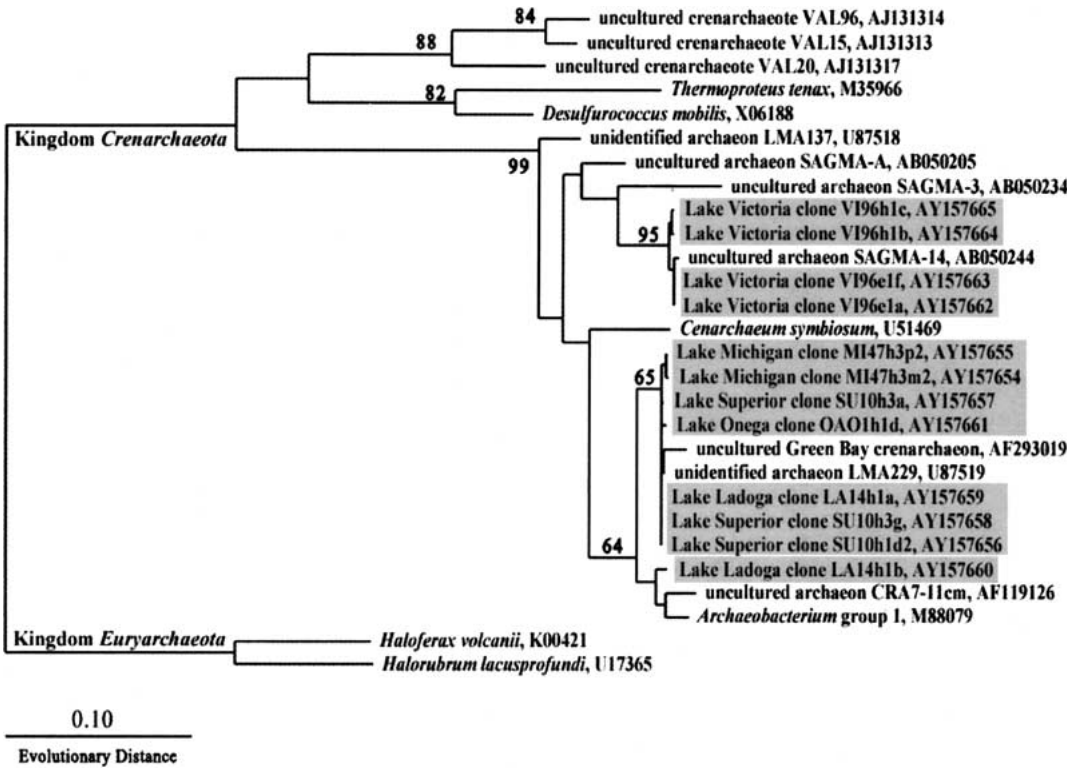

Fig. 1. Phylogenetic tree of archaeal 16S rRNA gene fragments inferred using maximum likelihood analysis for approximately 740 nucleotide positions between *E. coli* positions 1 and 915.Sequences recovered from lakes in this study appear in shaded boxes. Accession numbers for sequences included in the analysis appear after the sequence prefix(es). Scale bar distance indicates a 10% difference between nucleotide sequences. Numbers at each node are bootstrap values.

As expected, members of the domain Bacteria dominated the picoplankton community, although the overall percentage of total bacterial nucleic acid for the Laurentian Great Lakes was less in 1993 than in 1992 ($p < 0.01$, Table 3). Nucleic acids from the domain Eucarya were found in all lakes, but these percentages were never a high proportion of the total nucleic acid, indicating that prefiltration removed most of the eukaryotic cells. The percentage of eukaryotic nucleic acid detected in the Laurentian Great Lakes in 1992 was lower than in 1993 ($P < 0.01$, Table 3).

The nested quantitative hybridizations used to detect four archaeal taxa yielded only small percentages of nucleic acids specific to the kingdom Crenarchaeota and the group I marine crenarchaeota (data not shown). Based on hybridizations using Burggraf et al.'s Crenarchaeota probe ([6], Table 2), crenarchaeal nucleic acid was only detected in samples from the epilimnia and hypolimnia of Lakes Ladoga and Onega accounting for 1.0% to 2.6% of the total archaeal nucleic acid. These percentages were significantly different from zero. No nucleic acids from the kingdom Euryarchaeota [6] or family Methanobacteriaceae [37] were detected in these samples using oligonucleotide probes that target these taxa.

Nucleic acid from the group I marine crenarchaeota was detected in picoplankton samples from Lakes Erie, Huron, Superior, Ladoga, Onega, and Victoria (data not shown) and contributed from 0.1% to 1.5% of the total archaeal nucleic acid depending on the lake. However, the small percentages calculated were only significantly different from zero for samples from the epilimnion and the hypolimnion of Lake Ladoga (L14) and the hypolimnion of Lake Onega ($P < 0.05$ in all cases). The hybridization signals from DeLong et al.'s group I probe ([10]; S-*-Cren-0667-a-A-15) were not normalized to a positive control, and so the percentages reported here only represent estimates of the relative abundance of group I crenarchaeal rRNA in these great lakes picoplankton communities.

Phylogenetic analysis consistently placed the archaeal clones recovered in this study among the nonthermophilic clusters within the kingdom Crenarchaeota (Fig. 1). The majority of clones from the temperate lakes (Ladoga, Onega, Michigan, and Superior) were most closely related to a clone (LMA229) recovered from Lake Michigan

sediment [28] and uncultured archaea associated with ferromanganous nodules in Lake Michigan [43]. As a group, these crenarchaeal sequences appear more closely related to marine nonthermophilic crenarchaea than they do to other freshwater clones previously described such as the crenarchaeal VAL clones [21]. The clones from Lake Victoria clustered together (Fig. 1) and were most closely related to the SAGMA-14 clone recovered from an African gold mine [44]. Although the Victoria sequences grouped in a cluster distinct from the other clones in this study, they were also placed among the marine clusters of nonthermophilic crenarchaea.

Discussion

Detecting archaeal nucleic acids in the size-fractionated picoplankton of all the great lakes examined was a striking feature of this study. The amounts of archaeal nucleic acids observed (Table 3) were similar to abundances measured in coastal marine environments [7], but less than the amounts observed in the Santa Barbara Channel or in picoplankton from the Pacific, Antarctic, and Arctic Oceans ([10, 24, 30]; J.T. Hollibaugh, personal communication). Estimates of the abundance of archaea in some small freshwater lakes (as detected by FISH) were similar to our estimates insofar as they rarely exceed 10% [17, 36]. It is worth noting that prefiltration removed larger eukaryotic picoplankton cells, and so our estimates of relative archaeal abundance are based on hybridization of probes with nucleic acid extracts from primarily prokaryotic microorganisms. The relative abundances we report here are probably higher than if we had not filtered out the larger picoplanktonic eukaryotes. Nevertheless, archaeal nucleic acids were detected in all lakes studied.

Although there was a difference between the amounts of archaeal nucleic acids in the epilimnion and hypolimnion at two sites we investigated, this relationship was neither as consistent nor as pronounced as it is in marine environments [30]. Karner et al. [24] found that the fraction of crenarchaeota in picoplankton increased with depth in the Pacific Ocean, reaching 39% of all picoplankton below the euphotic zone. Unlike the Pacific Ocean, the temperate great lakes we studied are not permanently stratified. Periodic mixing of lakes in temperate zones may minimize differences in picoplankton communities in different water masses.

Nested hybridizations were largely unsuccessful in revealing the phylogenetic sources of the archaeal nucleic acids detected at the domain level. Jurgens et al. [21] recently reported being unable to detect crenarchaeal cells in lakes with Burggraf et al.'s probe [6] that targets the kingdom Crenarchaeota (Table 2), but they still were able to amplify and identify crenarchaeal sequences. Our results were similar. We could amplify and sequence crenarchaeal nucleic acids from Lake Michigan and Superior samples, but this crenarchaeal probe (i.e., S-K-Cren-0499-a-A-18) failed to hybridize with the same samples in either of these lakes. After further examination of its specificity, we found Burggraf et al.'s [6] probe matched less than half of the crenarchaeal sequences in the most recent 16S rRNA database and failed to match most uncultivated crenarchaeal clone sequences isolated from different environments including the 16S rRNA gene fragments we amplified. This probe's narrow specificity and the types of crenarchaeal sequences in our samples may have been responsible for the small amount of crenarchaeal nucleic acids we detected by hybridizations.

Very small amounts of nucleic acid from the group I marine crenarchaeota were detected by probing samples from all lakes we studied except Lakes Michigan and Victoria. Amplifying and sequencing crenarchaeal nucleic acids (Fig. 1) verified that nucleic acids from microorganisms within this environmental archaeal group were present in Lakes Michigan, Superior, Ladoga, Onega, and Victoria as they are in many oceanic regions.

We did not detect euryarchaeal nucleic acids in any great lakes with Burggraf et al.'s probe that targets the kingdom Euryarchaeota [6]. This probe (S-K-Eury-0498-a-A-14, Table 2) was previously thought to be specific to most euryarcheaota. However, it now appears that this probe is unable to detect more than 50% of the euryarchaeal sequences in the 16S rRNA database [21]. Considering that the robustness of the kingdom-level archaeal probes we used has been questioned and that only one primer set was used to amplify archaeal nucleic acids in this study, it is too early to speculate about true diversity of archaeons in these great lakes. For example, crenarchaeal nucleic acids were successfully amplified in most lakes, but no archaeal 16S rDNA was amplified in samples from Lakes Huron and Erie. This failure indicates that other archaeal groups, possibly members of the Euryarchaeota, were responsible for the archaeal nucleic acids detected by the domain-level hybridizations in these lakes. Thus, it would not be surprising if members of other archaeal groups were found in the picoplankton of these lakes in the future.

It is significant that no methanobacterial nucleic acids were detected by hybridization in any of the great lakes samples using Raskin et al.'s probe ([37], Table 2). This observation hints that the archaeal nucleic acids detected in the water columns were not simply resuspended from the deeper, anoxic portions of sediments in these great lakes. However, the absence of nucleic acids from other methanogens in the water column of these lakes should be confirmed before this idea is accepted.

Overall, nested hybridizations failed to account for the majority of archaeal nucleic acid detected at the domain level, a discrepancy that prompted the use of PCR and Buckley et al.'s [5] crenarchaeal primer set to amplify archaeal 16S rDNA genes as an independent verification of the presence of archaeal nucleic acids in these lakes. These PCR primers successfully amplified 16S rRNA genes of nonthermophilic crenarchaeota from template DNA prepared from picoplankton in great lakes on all three continents. Although the percentages of group I marine crenarchaeotal nucleic acid in our samples revealed by hybridization with DeLong et al.'s probe were very small (i.e., 0.1–1.5% of the total archaeal nucleic acid), the presence of this nonthermophilic group of archaea was established by PCR and cloning as it has been in surficial sediments from Lake Michigan [28].

All of the 16S rDNA amplicons from the great lakes picoplankton clustered with sequences previously identified as nonthermophilic crenarchaea (Fig. 1). With the exception of one clone from Lake Ladoga (LA14h1b), all of the clones in this study fell cleanly into two groups that clustered among the group I marine sequences currently available. The few clones selected for sequencing were very closely related to some crenarchaeal sequences recovered from Lake Michigan sediments [28, 43]. Interestingly, these great lakes sequences appeared more closely affiliated with the group I marine crenarchaea than with other freshwater nonthermophilic crenarchaeal sequences from smaller inland lakes such as some of Jurgens et al.'s [21] VAL clones (Fig. 1), Schleper et al.'s [41] pLAW sequences, and Hershberger et al.'s [18] pGrfB and pGrfC sequences. However, a more complete description of archaeal diversity is needed in great lakes before we can draw any firm conclusions about phylogenetic affiliations.

Although it is conceivable that archaea were introduced into the great lakes we studied from nearby marine or terrestrial environments, the presence of nearly identical 16S rDNA sequences from lakes in North America and Russia suggests that a phylogenetically related group of crenarchaea inhabit freshwater environments. Crenarchaea in marine environments are metabolically active and compete for trace level substrates in the environment, suggesting they are self-sustaining populations [26, 35]. The same may well be true for the crenarchaea in freshwater environments, but further work is necessary to confirm that archaea in the water columns of these great lakes are self-sustaining populations.

Although it is tempting to speculate about the differences between the archaeal sequences recovered from these great lakes, it is too early to conclude what factors might cause these differences. Other studies of lakes and the aquatic organisms in them have demonstrated that alone or in combination landscape geomorphology [19, 38], lake age [11], and trophic status [23] among other factors control or constrain biological diversity as well as productivity. It is also evident from previous studies that the breadth of the diversity of some microbial communities including picoplankton is surprising and sometimes completely unexpected [7, 21, 47]. Future studies should focus on what controls the diversity of archaea in picoplankton communities of different lakes. Some factors that control the diversity of these picoplankton communities may become more apparent after more archaeal and bacterial sequences from different lakes become available [32].

Although cultured archaeons reproduce optimally in environments characterized by high-temperature, near-saturating salt concentrations, or the absence of molecular oxygen, many uncultured archaeons apparently persist in relatively moderate environments of oceanic waters [7, 14], small freshwater lakes [17, 21, 36, 41], and various soils [4, 5, 22]. The results of this study provide clear evidence for the existence of populations of moderate crenarchaea in the picoplankton of great lakes on three different continents, extending their known habitat range. Archaeal nucleic acids detected in the picoplankton of these great lakes accounted for 1% to 10% of the total nucleic acids. Unrecognized crenarchaeons in the group I nonthermophilic archaeal cluster contributed some of these nucleic acids. The presence of nearly identical 16S rRNA sequences from great lakes in North America and Russia suggests a cosmopolitan distribution of freshwater crenarchaeons that inhabit freshwater environments. Identifying the full extent of archaeal diversity in the water columns of these lakes and recognizing factors that control the diversity and distribution of archaeal communities will require further investigations.

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