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Archaeal Diversity and the Prevalence of Crenarchaeota in Salt Marsh Sediments

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Archaeal Diversity and the Prevalence of *Crenarchaeota* in Salt Marsh Sediments[▽]

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Crenarchaeal 16S rRNA sequences constituted over 70% of the archaeal clones recovered from three salt marsh sites dominated by different grasses. Group I.1a *Crenarchaeota* dominated at two sites, while group I.3b *Crenarchaeota* sequences were most abundant at a third site. Abundances of 16S rRNA genes related to "*Candidatus* Nitrosopumilus maritimus" differed by site and sampling date.

Archaeal diversity in marine systems has been explored extensively in coastal and open ocean environments but has rarely been the subject of estuarine or salt marsh habitats, which are some of the most productive ecosystems in the world (14). In the only study of archaeal 16S rRNA gene diversity in a salt marsh, Munson et al. (16) reported the detection of sequences that were affiliated only with *Euryarchaeota* from vegetated marsh and unvegetated creek bottom sediments. Conversely, estuarine archaeal communities appear to be dominated by *Crenarchaeota* (1, 7). Given the importance of salt marshes and estuaries in maintaining healthy coastal environments and the recently elucidated role of archaea in nitrogen and carbon cycling (nitrification and anaerobic methane oxidation, for example), characterizing archaeal communities is critical to a better understanding of these coastal habitats, yet few studies have been conducted.

In this study, we have begun to address some of the knowledge gaps by characterizing archaeal diversity in a salt marsh in Long Island Sound. We used the distinctive vegetation patterns of New England salt marshes, which are dominated by *Spartina alterniflora* and *Spartina patens*, as proxies for different edaphic conditions. *S. alterniflora* is found in two phenotypically distinct but genetically identical forms (2). Tall *S. alterniflora* (SAT) grows to heights of 1 to 2 m and is typically found at the edges of the marsh and along creek banks, while short *S. alterniflora* (SAS) may reach heights of only 30 cm and is found slightly higher on the marsh, where soil drainage is limited and sediments are more reduced (6, 10, 17). *S. patens* typically grows on the high marsh in areas that receive less flooding, where salinity is lower and redox is higher than on the low marsh (6).

Surface sediment samples (0 to 2 cm) were collected on six dates from October 2005 to October 2006 from the Barn Island tidal marshes in southeastern Connecticut from sites representing SAT, SAS, and *S. patens* (see references 20 and 21 for more-complete site descriptions). Triplicate cores (6.5 cm) were collected on all dates except in April, when only duplicate cores were collected. SAS and *S. patens* samples contained dense roots and consisted of dark, sulfidic-smelling mud with high clay content. SAT samples consisted of coarse sandy sediment in which roots were easily removed during processing. Pore water was extracted from sediments by centrifugation. Salinity was measured using a hand-held refractometer. pH was measured with a YSI model 100 meter (Yellow Springs Instruments, Yellow Springs, OH). Pore water ammonium (NH_4^+) was measured using the phenol-hypochlorite method for seawater samples (19).

We extracted DNA from sediments by using a PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). DNA concentrations were measured using a Quant-iT double-stranded-DNA HS assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA). Archaeal 16S rRNA genes were amplified using the primers 21F (8) and 1492R (13) to produce nearly full-length (1,500-bp) sequences. Each 20-μl reaction mixture contained 10 μl iQ Supermix (Bio-Rad), 0.5 μM each primer, and 1 μl of a 1:10 dilution of DNA (approximately 2 to 10 ng). The cycle conditions were as follows: 95°C for 10 min, followed by 35 cycles at 95°C for 20 s, 53°C for 20 s, and 72°C for 2 min. PCR products were cloned into pSC vectors (Stratagene, Agilent Technologies, Santa Clara, CA). Inserts were amplified from selected clones by using T3 and T7 primers, and clones containing the correct-size insert were sequenced bidirectionally by High-Throughput Sequencing Solutions (Seattle, WA).

Analysis of 150 archaeal 16S rRNA gene sequences revealed distinct archaeal communities at each site (Fig. 1). Although sequences belonging to *Euryarchaeota* and *Crenarchaeota* were recovered from all three sites, *Crenarchaeota* sequences constituted at least 70% of the archaeal 16S rRNA sequences recovered from each site. Most sequences (60%) at the *S. patens* site were affiliated with group I.1a *Crenarchaeota* (CGI.1a) and shared ≥97% sequence identity with that of the aerobic ammonia-oxidizing "*Candidatus* Nitrosopumilus maritimus" isolate (12). At the SAT site, more than 25% of the clones clustered with CGI.1a, but no CGI.1a sequences were recovered at the SAS site. Sequences affiliated with *Crenarchaeota* group I.3b constituted the majority of clones at the SAS site.

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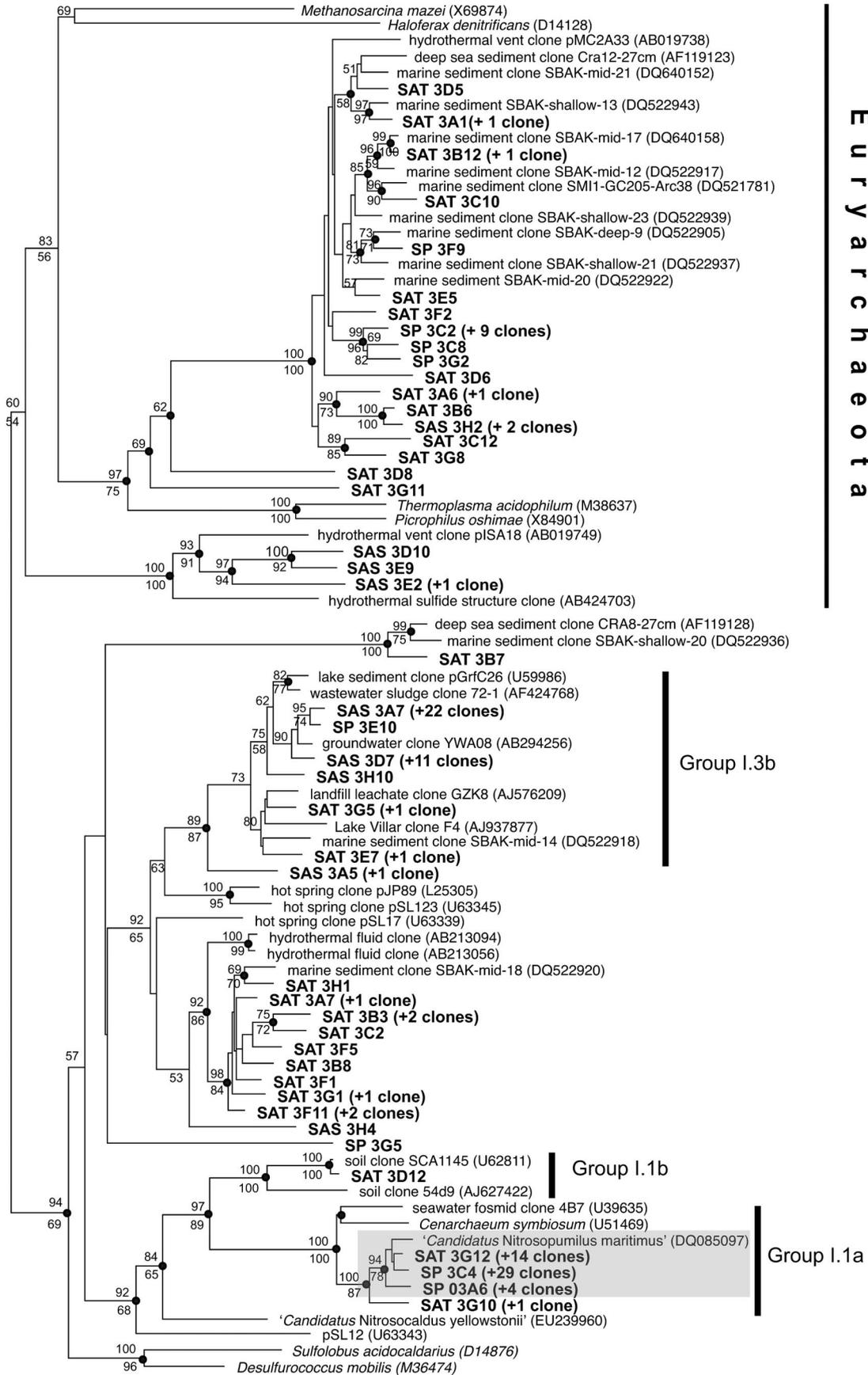
TABLE 1. Pore water salinities, pHs, and ammonium concentrations for sediment samples collected at three sites during the study period[a]

Mo	Salinity (psu)			pH			NH_4^+ concn (μM)		
	SAS	SAT	*S. patens*	SAS	SAT	*S. patens*	SAS	SAT	*S. patens*
March	32.0 ± 3.6	12.0 ± 0.0*	30.0**				37.0 ± 17.0	258.0 ± 66.5	97.3 ± 21.5
April	23.8 ± 1.1*	13.0 ± 1.4*	25.5 ± 6.4*	6.3 ± 0.2*	6.3 ± 0.1*	6.1 ± 0.1*	34.9 ± 18.4*	70.5 ± 5.5*	29.4 ± 3.2*
June	ND	18.0**	ND	6.4 ± 0.1	6.5 ± 0.3	5.7 ± 0.3	95.1 ± 62.73	235.7 ± 139.1	111.0 ± 30.2
July	25.3 ± 0.6	24.0 ± 1.0	24.0 ± 1.0	5.3 ± 0.4	6.2 ± 0.1	5.3 ± 0.3	33.5 ± 13.9	152.1 ± 112.8	27.0 ± 11.7
October	32.7 ± 1.2	31.3 ± 1.2	30.0 ± 0.0	6.4 ± 0.1	6.3 ± 0.1	6.3 ± 0.0	4.0 ± 3.8	25.1 ± 19.4	12.5 ± 18.6

[a] Values are means ± standard deviations of results from triplicate cores, except in cases where values represent duplicate cores (*) or a single measurement (**). ND, no data.

Nonthermophilic *Crenarchaeota* have been reported as common constituents of marine communities in open ocean and estuarine habitats (e.g., references 7 and 11), but their presence in salt marshes has not been reported. Although Munson et al. (16) found archaeal communities in a United Kingdom salt marsh consisting exclusively of *Euryarchaeota*, the primers used in their study lacked the specificity to detect CGI.1a sequences. We also did not recover any sequences related to known methanogens and halophiles, which were quite prevalent in the United Kingdom study. Although community differences between the two marshes are at least partially due to differences in primer specificity, differences may also reflect sediment conditions. For example, the salinities reported to occur in samples from the United Kingdom study ranged from 49 to 53 practical salinity units (psu), which are considerably higher than the salinity values recorded at our sites (12 to 32 psu) (Table 1) and could explain the differences in archaeal communities.

Most *Euryarchaeota* clones in our study were closely related to sequences recovered from other marine environments, particularly sediments from Skan Bay, AK (Fig. 1). *Euryarchaeota* sequences were more frequently recovered from the SAT and *S. patens* sites (30% and 26% of all sequences, respectively) than from the SAS site (15%). Unfortunately, since there are no cultured representatives related to our *Euryarchaeota* clones, their physiology remains unknown.

Archaeal 16S rRNA gene richness was more than 3 times greater at the SAT site than at the SAS or *S. patens* site (Fig. 2). The SAT site is a coarse, sandy sediment along a creek bank that is well flushed and has ammonium concentrations that are typically 2 to 3 times higher than those at the SAS or *S. patens* site (Table 1). The increased diversity at the SAT site may be related to increased drainage and tidal flushing, due to its position along a creek bank, compared to the levels for the SAS and *S. patens* sites, which are higher on the marsh. SAT also experiences a greater range of salinity than SAS or *S. patens*. Other studies have reported lower levels of diversity of bacterial 16S rRNA genes (3), denitrifying bacteria (22), and ammonia oxidizing bacteria (4) associated with higher salinity,

but the relationship between salinity and archaeal diversity has not been explored adequately.

Based on the unexpected prevalence of CGI.1a sequences in two of three clone libraries, we quantified the abundance of 16S rRNA genes related to "*Ca.* Nitrosopumilus maritimus" (designated the Nm cluster) at all three sites over 1 year by using quantitative PCR. Nm cluster 16S rRNA genes (ca. 415 bp) were amplified using the method of Könneke et al. (12), but we modified the forward primer (5′-CTGGAATGGTTT RTGTTYC) to target additional sequences recovered in our study. The specificity of Nm cluster primers was confirmed by routine melting curve analysis (95°C for 1 min, 57°C for 1 min, and then a 0.5°C increase every 10 s) and sequencing of over 50 clones generated from the three sites in our study. All sequences were related to the Nm cluster (data not shown). Standard curves were generated in each run by using standards ranging from 18 to 1.8×10^5 gene copies/μl of DNA and used to estimate gene abundance in the sediment samples. Standards were plasmid DNAs purified from archaeal 16S rRNA clones recovered from the same marsh samples. We also tested for inhibitory effects by calculating the slopes of each sample at different dilutions (ranging from 1:5 to 1:15). Dilutions ranging from 1:8 to 1:12 gave similar slopes (coefficient of variation = 11.6%), so we used 1:10 dilutions for final analysis. Additionally, slopes were not significantly different among samples from different sites (analysis of covariance; $P = 0.46$).

Nm cluster 16S rRNA gene abundance ranged from below the detection level in some samples at SAS to 1.4×10^9 copies g (dry weight) sediment^{-1} at the *S. patens* site and were significantly lower at the SAS site than at the SAT or *S. patens* site (Kruskal-Wallis test; $P < 0.0001$) (Fig. 3). Although Nm cluster 16S rRNA gene abundance was slightly lower at all sites in October, abundance remained relatively high from March to July at the SAT and *S. patens* sites, and we did not detect any significant seasonal variation during the months that we sampled. Finding numbers as high as 10^9 was surprising, particularly given that previous reports of total cell counts in marine sediments typically range from 10^8 to 10^{10} cells per gram (9, 18). Although we measured only the gene copy number, there

FIG. 1. Phylogenetic relationships among representative archaeal 16S rRNA gene sequences recovered from sites in the salt marsh dominated by different grasses. The neighbor-joining tree is based on 1,078 positions and was inferred with the Kimura two-parameter correction method. Parsimony analysis was performed using a full heuristic search with a random addition sequence. Maximum-likelihood analyses were performed using the HKY 85 model. Bootstrap values (based on 100 replicates) greater than 50% are indicated at each node for neighbor-joining (above) and parsimony (below) analyses. Nodes supported by maximum-likelihood analysis are indicated by closed circles. The shaded box represents sequences targeted by Nm cluster primers. SP, *S. patens*.
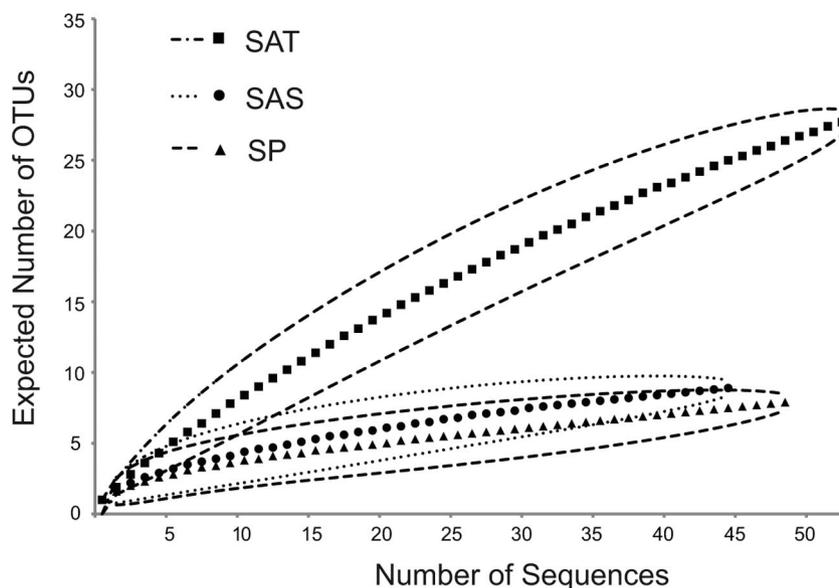
FIG. 2. Rarefaction analysis of archaeal 16S rRNA genes at the three sites. Operational taxonomic units (OTU) were defined by 97% nucleotide identity. The sites represent areas dominated by the SAS, SAT, and *S. patens* (SP) marsh grasses.

is only one 16S rRNA gene copy according to the genome of "*Ca.* Nitrosopumilus maritimus." If this is true for all Nm cluster archaea, then gene copy number should approximate cell numbers, suggesting that Nm cluster archaea could account for a significant component of the microbial community in some samples. The lack of Nm cluster sequences in the clone library from SAS is likely due to the low abundance of these archaea in the samples. However, using Nm cluster primers to generate clones, we were subsequently able to confirm Nm cluster 16S rRNA sequences at the SAS site (data not shown).

The largest differences in Nm cluster 16S rRNA gene abundance were found between samples at the SAS and *S. patens* sites, yet there were few differences in salinity, pH, or NH_4^+ concentration between these sites (Table 1). Differences in Nm cluster 16S rRNA gene abundance may be related to differences in alternative edaphic conditions. For example, significant differences in sulfide and redox levels have been reported previously for these two sites, with SAS sites showing higher sulfide levels and lower redox levels than *S. patens* sites (21). Additionally, factors that influence the oxygenation of the sediments, such as bioturbation (5) and oxygen from the roots (15), were not measured in this study but may be of particular importance when the Nm cluster archaea are also aerobic ammonia oxidizers, like "*Ca.* Nitrosopumilus maritimus."

Our results indicate that nonthermophilic *Crenarchaeota* are prevalent members of microbial communities in salt marsh sediments in Long Island Sound. To our knowledge, this is the first report of group I *Crenarchaeota* in salt marshes, and our data indicate that these crenarchaea constitute a stable component of archaeal communities in some areas of the marsh. The differential distribution of Nm cluster 16S rRNA genes also suggests that alternative environmental variables (e.g., sulfide, redox, or plant root exudates from different grasses) may be important in regulating their distribution, but future studies are needed to address these hypotheses.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under accession numbers FJ655622 to FJ655771.

FIG. 3. Mean abundances of 16S rRNA genes related to "*Candidatus* Nitrosopumilus maritimus" at sites representing areas dominated by the SAT, SAS, and *S. patens* (SP) marsh grasses. Error bars represent the standard errors of the means for three replicates, except for samples collected in April, in which case the values represent two replicates.

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