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Phylogenetic Diversity of *Archaea* in Sediment Samples from a Coastal Salt Marsh

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The *Archaea* present in salt marsh sediment samples from a tidal creek and from an adjacent area of vegetative marshland, both of which showed active methanogenesis and sulfate reduction, were sampled by using 16S rRNA gene libraries created with *Archaea*-specific primers. None of the sequences were the same as reference sequences from cultured taxa, although some were closely related to sequences from methanogens previously isolated from marine sediments. A wide range of *Euryarchaeota* sequences were recovered, but no sequences from *Methanococcus*, *Methanobacterium*, or the *Crenarchaeota* were recovered. Clusters of closely related sequences were common and generally contained sequences from both sites, suggesting that some related organisms were present in both samples. Recovery of sequences closely related to those of methanogens such as *Methanococcoides* and *Methanolobus*, which can use substrates other than hydrogen, provides support for published hypotheses that such methanogens are probably important in sulfate-rich sediments and identifies some likely candidates. Sequences closely related to those of methanogens such as *Methanoculleus* and *Methanogenium*, which are capable of using hydrogen, were also discovered, in agreement with previous inhibitor and process measurements suggesting that these taxa are present at low levels of activity. More surprisingly, we recovered a variety of sequences closely related to those from different halophilic *Archaea* and a cluster of divergent sequences specifically related to the marine group II archaeal sequences recently shown by PCR and probing to have a cosmopolitan distribution in marine samples.

The microbial communities inhabiting anoxic habitats such as freshwater and marine sediments are important for biological carbon and sulfur cycling (30). In such situations the terminal steps in the mineralization of organic carbon to CH_4 or CO_2 are thought to be carried out by methanogenic *Archaea* (MA) or sulfate-reducing bacteria (SRB) acting in competition. The direction of the terminal step of mineralization has been hypothesized to be controlled by the amount of sulfate present in the environment (5, 26, 48). In sulfate-limited freshwater sediments, most of the organic carbon is converted by MA to methane. In sulfate-rich marine and salt marsh sediments, the balance is shifted toward the production of CO_2 by SRB (2, 39). However, even under sulfate-rich conditions, methane production can be detected in some salt marsh sediments (33, 41).

One possible explanation is that continued methanogenesis occurs because some MA can use noncompetitive substrates, i.e., those inaccessible to SRB, such as methanol, methylamines, and methionine (21, 33, 47). While many of the MA isolated into pure culture from marine sediments use acetate, formate, or CO_2 plus H_2 for methanogenesis, some can indeed use noncompetitive substrates (21, 46). Trimethylamine-utilizing organisms resembling *Methanoccoides methylutens* and *Methanolobus tindarius* have been isolated from Georgia salt marsh sediments (13). *Methanosarcina acetivorans* isolated from marine canyon sediments primarily uses acetate for methanogenesis but can also utilize methanol and methylamines (42).

The ability to investigate microbial community composition by using phylogenetic analysis of 16S rRNA sequences (15) offers another perspective in addition to the one gained from traditional culture isolation methods, particularly for *Archaea*, which are sometimes described as difficult to grow (46). Molecularly based studies have additional advantages in that they provide sequences which are directly comparable, via tree diagrams, between different studies and samples. These data can be reanalyzed as new sequences are discovered, providing an increasingly comprehensive picture of microbial relationships and environmental distributions. For example, hitherto unknown sequences from two presently uncultured monophyletic groups of *Archaea* (termed group I and group II) have been found in almost all marine samples so far analyzed (6, 14, 27). The ecological inference is that these *Archaea* are potentially important in ocean processes, an inference recently supported by probe data for Antarctic samples (7). In the present investigation we have used 16S rRNA gene sequences to identify members of the archaeal community in creek sediment and vegetative marsh top samples from a coastal salt marsh, which previously has been extensively studied at the process level (1, 3, 4, 31, 41).

MATERIALS AND METHODS

Sediment cores and characteristics. Sediment cores (25 cm in length and 10 cm in diameter) were taken in October 1994 from two sites in the Colne Point Salt Marsh located in northeast Essex, United Kingdom. The cores were taken from the side of the tidal Ray Creek (31) and from an area of raised, vegetative marsh top approximately 400 m north of the creek site. The sediment cores were returned to the laboratory, where triplicate subcores were taken at 5-cm depth intervals to a depth of 20 cm by using 5-ml syringes with the distal tips removed. One subcore from each depth slice was used to determine the salinities, pHs, and organic carbon contents of the sediments. Each depth slice was centrifuged at 5,000 × *g* for 5 min to extract pore water. Salinity was determined with an optical salinometer (Leica model 10419). The pore water samples were diluted 10 times with ultrapure water, and the pH was measured with a pH meter. The organic carbon contents of dried (60°C) sediment samples were measured in a CN analyzer (Perkin-Elmer CHNS/O analyzer 2400). The results of these analyses are given in Table 1.

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TABLE 1. Sediment characteristics of the marsh top and Ray Creek sites from the Colne Point salt marsh during October 1994

Site	Depth (cm)	Salinity (‰) (mean ± SD)	Organic carbon content (% of dry wt) (mean ± SD)	pH (mean ± SD)
Marsh top	0–5	49.3 ± 2.3	13.45 ± 1.74	6.86 ± 0.8
	5–10	39.3 ± 1.2	12.22 ± 0.62	6.07 ± 0.06
	10–15	48.0 ± 2.0	11.45 ± 0.49	6.30 ± 0.13
	15–20	53.3 ± 1.2	11.39 ± 1.25	6.72 ± 0.12
Creek	0–5	35.3 ± 1.2	3.66 ± 0.14	7.49 ± 0.06
	5–10	37.3 ± 2.3	5.59 ± 4.86	6.21 ± 0.18
	10–15	34.0 ± 2.0	3.15 ± 0.25	6.37 ± 0.41
	15–20	35.3 ± 4.2	3.49 ± 0.20	6.00 ± 0.11

Nucleic acid extraction and purification. Nucleic acids were extracted from triplicate samples at two depths (0 to 5 and 5 to 10 cm), sampling the region of most active methanogenesis (0 to 10 cm), for both creek and marsh top. To remove extracellular nucleic acids, each sample was washed by adding sodium phosphate buffer (30 ml of a 120 mM buffer [pH 8.0]) and shaking for 10 min at 150 rpm on an orbital shaker. The samples were centrifuged at 6,000 × *g* for 10 min, the supernatants were discarded, and the washing was repeated twice (44). The washed samples were stored at −20°C.

A mechanical method based on bead beating was used to break open cells in the samples (28). Initial replicated (three times) experiments indicated that while extended beating up to 6 min increased the DNA yield, it also increased shearing of DNA. Since shearing may increase PCR artifacts, we compromised on 1 min of beating, which yielded on average 15.9 ± 1.7 μg (creek) and 21.8 ± 1.4 μg (marsh top) of nucleic acid per g (wet weight) of sample. Most DNA was above 5 kb, with discernible bands at ca. 20 kb, and all samples contained small- and large-subunit rRNAs.

To each sediment sample (0.5 g [wet weight]) in a 2-ml screw-cap microcentrifuge tube were added 0.5 g of prebaked (overnight at 250°C) glass beads (0.1-mm diameter), 0.5 ml of extraction buffer (sodium phosphate buffer [120 mM, pH 8.0] plus 1% [wt/vol] acid-washed polyvinylpolypyrrolidone) (18), and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The samples were briefly vortexed to resuspend the glass beads and sediment, placed in a bead beater (Mikro-Dismembrator U.B.; Braun Biotech International, Melsungen, Germany), and beaten at 2,000 rpm. The samples were centrifuged for 15 min at 13,000 × *g*, and the aqueous phases were transferred to fresh 1.5-ml tubes.

The supernatants were extracted once each with buffered phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and finally, chloroform alone. The nucleic acids were precipitated with 2 volumes of 30% polyethylene glycol 8000 and 1/10 volume of 5 M sodium chloride solution (40). Samples were gently mixed and placed on ice for 30 min. The precipitated nucleic acids were pelleted at 13,000 × *g* for 15 min; the supernatant was carefully removed, and the pellets were washed once with ice-cold ethanol (70%, vol/vol). The nucleic acid pellets were dried and resuspended in 50 μl of sterile double-distilled water. Nucleic acid concentrations were determined by measuring absorbance at 260 nm, and sizes were measured by gel electrophoresis. The DNA preparations had A_{267}/A_{280} ratios of 1.7 to 1.8.

DNA extracts were further purified on a 1% Tris-acetate-EDTA agarose gel. Gel slices containing DNA fragments of >5,000 bp were excised and extracted by using SpinBind gel (Flowgen, Staffordshire, England). Each extract was dialyzed twice against 400 μl of Tris-EDTA with MicroCon30 cartridges (Amicon).

PCR amplification of archaeal 16S rDNA fragments. The replicate DNA extracts from each sample depth were pooled. Two PCR protocols were necessary to obtain sufficient product for making gene libraries. For samples taken at depths of 5 to 10 cm, primary amplifications used *Archaea*-specific primers 1A (forward) (5′-TCYGKTTGATCCYGSCRGAG-3′) and 1100A (reverse) (5′-TGGGTCTCGCTCGTTG-3′) (10). For each sample five replicate reaction mixtures (45) of 50 μl contained 5 μl of 10× PCR buffer (670 mM Tris-HCl, 20 mM $MgCl_2$), 0.5 μl of deoxynucleoside triphosphate mix (25 mM each), 1 μl of primer 1A (20 pmol), 1 μl of 1100A (20 pmol), 2 μl of bovine serum albumin (100 μg/ml), 1 μl of template DNA, 1 U of AmpliTaq polymerase (Perkin-Elmer), and Millipure water to the 50-μl volume. PCR incorporated a hot start of 95°C for 10 min and 80°C for 2 min before addition of *Taq* polymerase, followed by 10 primary cycles (94°C for 1 min, 50°C for 30 s, and 72°C for 2 min), and then 25 secondary cycles (92°C for 30 s; 55°C for 30 s, and 72°C for 2.5 min), with a final extension at 72°C for 5 min, with an HTR-1 thermal cycler (Hybaid Instruments, Teddington, Middlesex, United Kingdom).

For the samples taken at depths of 0 to 5 cm, a two-stage heminested PCR procedure was necessary to obtain sufficient material for cloning. Primary amplifications (three replicates for each sample) used the reverse primer 1404R (5′-CGGTGTGTGCAAGGRGC-3′) and 1AF. The annealing temperature initially was 63°C and was lowered 2°C every other cycle for the first 10 cycles to a final annealing temperature of 53°C. For the first 10 cycles, samples were denatured at 94°C for 1 min and extended at 72°C for 2 min. Following this "touchdown" (8), 25 further cycles of 92°C (30 s), 53°C (30 s), and 72°C (2.5 min) were carried out, along with a 5-min 72°C terminal extension. The very weak products of primary amplifications were pooled and concentrated to 10 μl with MicroCon30 cartridges. Aliquots (2 μl) were used as templates for replicate (five times) secondary amplifications with the 1A and 1100R primers and the PCR conditions described for the 5- to 10-cm depth.

Cloning of environmental PCR products. The PCR products for each sample were combined, concentrated with a MicroCon30 cartridge, and purified on a 1% Tris-acetate-EDTA agarose gel. The PCR band with the expected 1.1-kb size was excised and purified by using the QiaexII gel purification kit (Qiagen). PCR products were ligated into pGEM-T (Promega) and transformed into XL1-Blue MFR′ cells (Stratagene) on medium containing ampicillin (20 μg/ml), methicillin (80 μg/ml), and tetracycline (4 μg/ml). The resulting clones were screened for ribosomal DNA inserts by a PCR miniprep method as follows. Two hundred putative recombinants each from the creek and marsh top 5- to 10-cm libraries and 100 recombinants each from the 0- to 5-cm libraries (i.e., 600 clones in total) were inoculated onto fresh antibiotic-containing agar and incubated overnight at 37°C. Just-visible smears of each colony were transferred to the bottoms of individual wells in a 96-well microtiter plate (Costar) by using a sterile pipette tip. A master PCR mix was made up containing, for each reaction, 2.5 μl of 10× PCR buffer, 0.125 μl of deoxynucleoside triphosphates (25 mM each), 0.25 μl of primer SP6 (20 pmol/μl), 0.25 μl of primer T7 (20 pmol/μl), 0.5 μl of bovine serum albumin (100 μg/μl), 0.5 U of AmpliTaq, and 16 μl of sterile water. Primers SP6 and T7 bracket the multiple cloning site of the pGEM-T vector and produce a PCR product of 1.1 kb for recombinant clones. Aliquots (25 μl) of the master mix were added to each well and overlaid with 25 μl of sterile mineral oil, and PCR was carried out under the conditions described above (MJ Research Instruments PTC-100).

Subsamples (5 μl) of each PCR mixture were loaded onto a 1% agarose gel in a large gel tray (Anachem MaxiGel with three 48-well combs). The remaining 20 μl of each positive PCR mixture was transferred to another 0.5-ml tube, and the PCR product was precipitated by addition of 0.6 volume (12 μl) of 20% polyethylene glycol 8000–2.5 M NaCl followed by incubation at 37°C for 10 min (22). The DNA was pelleted by centrifugation at 13,000 × *g* for 10 min, washed with ice-cold ethanol (80%, vol/vol), and dried in a vacuum centrifuge. Resuspension of the PCR product in 7 μl of sterile double-distilled water provided sufficient PCR product for two sequencing reactions (9).

Sequencing of environmental clones. A preliminary screening to identify unique environmental clones was carried out by using a linear PCR (9) modified for single-lane sequencing (38) with ddT as the terminating base. An aliquot (3 μl) of the PCR product from 440 randomly chosen positive clones (100 from each of three libraries and 140 from the creek 5- to 10-cm sample) was sequenced by using the universal sequencing primer 519R (5′-GWATTACCGCGGCKGCTG-3′), which covers a highly variable region of 16S rRNA sequence (23). The resulting banding patterns were compared, and 133 unique clones, comprising all of the observed single-lane variation in each library (representing 28 to 60% of single lanes sampled, depending on the library), were sequenced for four bases by using the 519R primer and the remaining 4 μl of PCR product from the initial colony PCR. The resulting sequences, each comprising about 223 bp, were aligned by eye with archaeal sequences obtained from the Ribosomal Database Project (RDP) (25) by using the Genetic Data Environment version 2.2 program distributed by the RDP.

Following alignment, the sequences were analyzed by using programs in Phylip 3.5c (12) within the Genetic Data Environment version 2.2 program. A phylogenetic tree (not shown) based on 223 bases was generated by neighbor-joining analysis (37) of distances estimated by using the Jukes-Cantor correction (20). Thirty-four clones representative of the clusters detected were completely sequenced (ca. 1,034 bases in length) by using primers SP6 and T7 and sequencing primers 894RA (reverse) (5′-CRYACTYCCCAGGYGGY-3′), 869RA (reverse) (5′-CAACTTCTCTCGGACACTA-3′) (for clones 2P1, 2P8, 2C25, and 2C84), and 348FA (forward) (5′-GGYGCRGCDGGCGCGMAA-3′).

The full-length clone sequences (accession numbers AFO15964 to AFO993, inclusive) were aligned against reference archaeal sequences and analyzed for 783 aligned positions common to all sequences, using neighbor-joining analysis as described above. Bootstrapping (100 replicates) was used to assess support for particular nodes in the resulting tree (11, 12). Sequences were investigated for the presence of chimeric sequences by using the CHECK_CHIMERA program available through the RDP (25) and by comparison of neighbor-joining (37) trees based on either the 5′ or 3′ 300 bases of sequence, looking for taxa that radically changed position between trees. Three of 34 sequences (ca. 9%) were recognized as potentially chimeric and were excluded from further analysis.

RESULTS AND DISCUSSION

Analysis of 16S rDNA sequences from Ray Creek sediments and vegetative marsh top samples 400 m apart revealed a diversity of *Euryarchaeota* sequences (Fig. 1). However, no clones related to *Methanococcus* or *Methanobacterium* were

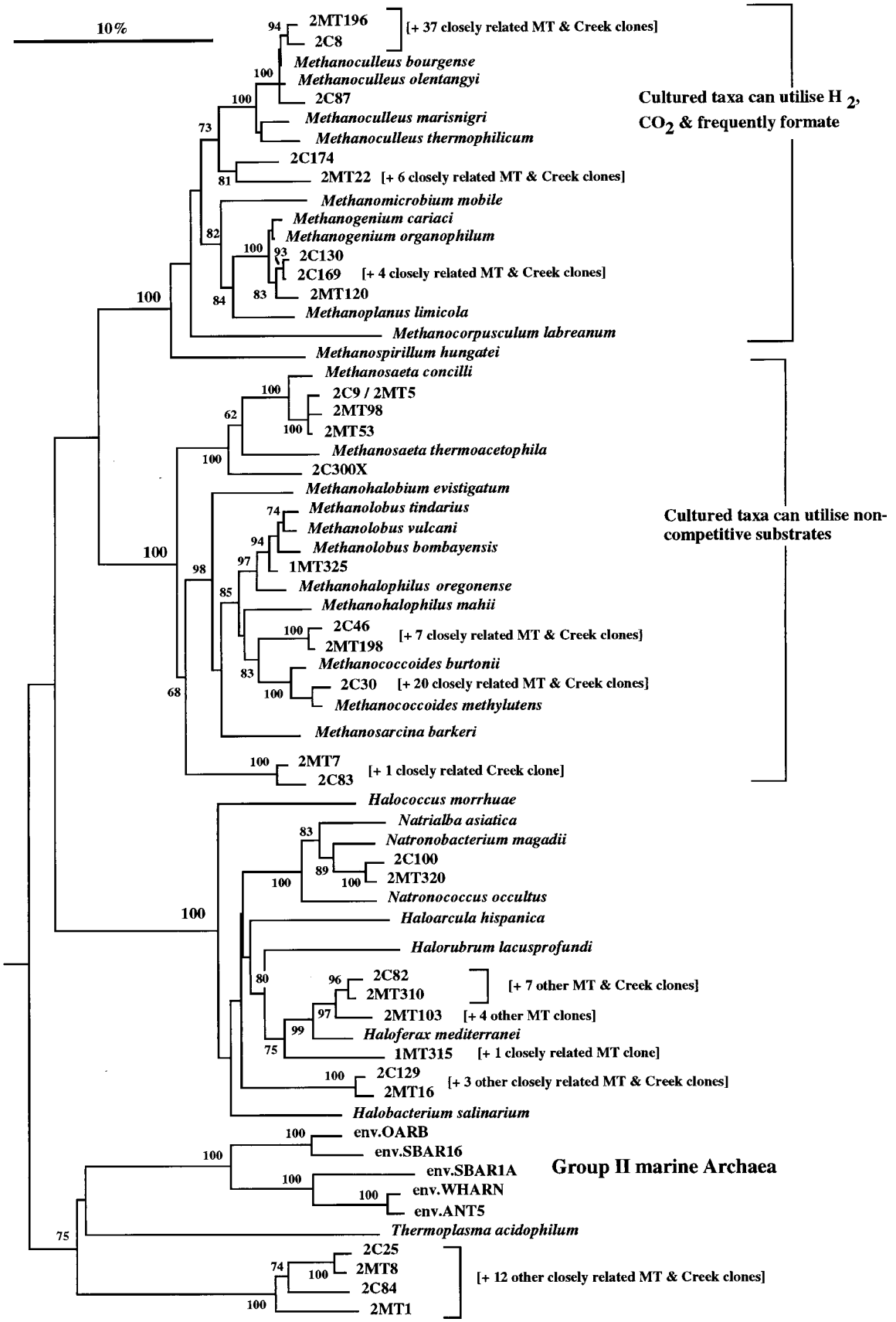

FIG. 1. Neighbor-joining tree showing relationships between environmental sequences and reference *Euryarchaeota* based of 783 bases upon aligned 16S rDNA sequence. Abbreviations are exemplified as follows: 2MT196, clone 196 from marsh top 5- to 10-cm library; 1MT325, clone 325 from marsh top 0- to 5-cm library; 2C8, clone 8 from Ray Creek 5- to 10-cm library. The bootstrap support values above 70% are shown at nodes. The tree was pruned, to save space, from one containing sequences from *Methanococcus*, *Methanobacterium*, and *Sulfolobus.*

recovered, although the primers also amplify 16S rRNA genes from cultured representatives of these genera (not shown). Nor did we recover sequences related to *Methanosarcina*, although *Methanosarcina mazei* (24) was previously isolated from enrichments prepared from Ray Creek sediment samples (2). We did not detect *Crenarchaeota* sequences in our library sampling, although preliminary experiments demonstrated amplification products from DNA of *Sulfolobus* (not shown). To estimate how thoroughly the different gene libraries were sampled for the diversity they contained, we estimated the coverage (16) for each by using a cutoff value of 97% sequence similarity to determine if sequences were considered to be different or the same (29). This cutoff is based upon published comparisons of full-length sequences, which have suggested that between recognized prokaryote species, 16S rRNA sequence similarity is normally less than 97% (43). With this value the coverage for the different libraries always exceeded 80%, suggesting that they were reasonably well sampled for clone diversity.

Clusters of closely related sequences were common among sampled clones (Fig. 1). These clusters generally contained representatives from the marsh top and Ray Creek sediment samples (Fig. 1), suggesting that some related organisms were present in both samples. As often observed in 16S rRNA-based surveys of natural samples (6, 14, 27), none of the sequences were the same as reference sequences from cultured taxa, although some were closely related to sequences from cultured methanogens previously isolated from marine sediments. The methanogen-like sequences fell into two strongly supported clusters containing sequences from methanogens for which phenotypes have been described (Fig. 1). It is sometimes suggested that 16S rRNA sequences provide little information concerning phenotype. This partly reflects observations that even closely related prokaryotes may exhibit phenotypic differences. However, if all studied members of a strongly supported monophyletic group behave in a particular way, then it is most parsimonious to hypothesize that this feature is ancestral and that new members will also express it, unless there is good reason to believe otherwise. We have followed this logic to infer phenotypes for some of the sequences ("organisms") which cluster strongly with sequences from cultured taxa, while recognizing that such inferences are only provisional on obtaining further data.

Process measurements with Ray Creek sediment samples have previously suggested that methanogenesis and sulfate reduction are not separated either spatially or temporally but occur within the same layer of sediment at the same time of year (41). Methane emission from the surface of sediments was much greater than could be accounted for by bicarbonate methanogenesis (41). These data were interpreted to suggest that methanogens capable of using noncompetitive substrates, i.e., those inaccessible to SRB, were primarily responsible for the observed methane efflux (41). The same hypothesis has also been proposed to explain methane emissions in high-sulfate Californian salt marsh sediments (33). A number of the methanogen sequences isolated from samples of Ray Creek sediments and vegetative marsh top were indeed closely related to sequences from cultured taxa which can disproportionate C_1 compounds such as methylamines, methanol, and other methyl-containing compounds. For example, some of the most common sequence types encountered in our libraries were closely related to sequences from *Methanococcoides methylutens* and *Methanococcoides burtonii*, both of which are obligate methylotrophs. Other sequences were related to *Methanolobus*, which utilizes a similar range of substrates. Our data thus support the hypothesis that such methanogens may be important in situ and also identify some of the groups which may be active. Preliminary experiments (29a) using sediment slurry microcosms also suggest that the addition of methylated amines increases the amount of signal detected by an oligonucleotide probe designed to detect the *Methanococcoides*-like 16S rRNA sequences. It was interesting that sequences which were related to those from obligate aceticlastic methanogens from the genus *Methanosaeta* were detected (Fig. 1). Acetate in Ray Creek sediments is utilized mainly by SRB rather than methanogens (3), but aceticlastic methanogens may survive at low population sizes even if they are sequestering only a small part of the total acetate flux (17).

The other methanogen-like sequences in our gene libraries were recovered in a strongly supported monophyletic group also containing sequences from cultured taxa which can reduce CO_2 by using hydrogen, or sometimes formate, as an electron donor (46). The most common sequences detected overall in our libraries were a cluster of sequences closely related to reference sequences from *Methanoculleus olentangyi* and *Methanoculleus bourgenase* (Fig. 1). These two taxa were isolated from freshwater sediment and a bioreactor inoculated with sewage sludge, but the closely related (Fig. 1) *Methanoculleus marisnigri* and *Methanoculleus thermophilicum* were isolated from marine sediments (46). A cluster of deeply branching sequences, represented by 2C174 and 2MT22 (Fig. 1), formed a moderately strongly supported sister group to the *Methanoculleus* group. Sequences closely related to *Methanogenium cariaci* and *Methanogenium organophilum*, species which were isolated from marine sediments, were also detected. Methanogenesis from hydrogen has not been considered the major source of methane in Ray Creek as judged by process measurements (41) and inhibitor studies on slurries (4, 32). However, although out-competed for hydrogen, methanogens may persist by sequestering even a small proportion of the hydrogen available in the environment (17), and hydrogen methanogenesis can be detected in salt marsh sediments when SRB have been inhibited (32). Our present data support the occurrence of MA in the salt marsh sediments which are related to species capable of hydrogen methanogenesis.

A variety of sequences related to different halophilic *Archaea* were detected in gene libraries from both sites. This was unexpected, since cultured halophilic *Archaea* are reported to require at least 1.5 M NaCl for growth (46). There is one report of the isolation of *Halococcus* from seawater (36), but strains of this genus may resist osmolysis and are postulated to remain viable but not active at seawater salinity (34). At present we have not detected sequences specifically related to *Halococcus* in our library sampling. It has been suggested that in saline soils there may be microsites with sufficiently high salt concentrations to permit the growth of halophilic *Archaea* (35), and this may partially explain the occurrence of halophilic sequences in our samples. While the creek sediments are inundated with seawater twice daily during normal tidal cycles, the measured salinities of marsh top samples in October were significantly higher (ca. 50‰ [~0.8 M NaCl]) than that of seawater (ca. 30‰), and it is conceivable that surface evaporation could further increase salt levels. Intriguingly we also detected sequences related to alkaliphiles, specifically *Natronobacterium magadi*, cultured strains of which grow only in the presence of extremely high salt levels and high pH (34). Further work, including direct probing of rRNA to estimate relative abundances (6, 15) and selective isolation to try to recover viable cultures, is needed to further investigate if halophilic *Archaea* play a significant role in the Colne Point salt marsh.

Another unexpected discovery was of sequences related to a

clade comprising sequences from the uncultured marine group II *Archaea* (6) and *Thermoplasma acidophilum*. Representatives of the group II marine *Archaea*, for which no phenotype is currently known, have been detected in samples from the Pacific and Atlantic Oceans and in the Antarctic, where they may be important components of the picoplankton at certain times of the year (6, 7, 14). A relationship between *Thermoplasma* isolated from a burning coal refuse pile (19) and the group II marine *Archaea* was previously reported in these studies. Our data demonstrate that highly divergent members of this clade are also present in a coastal salt marsh and reinforce the notion that these particular *Archaea* may be ecologically important in marine systems.

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