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Pacific Northwest Marine Sediments Contain Ammonia-Oxidizing Bacteria in the β Subdivision of the *Proteobacteria*

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The diversity of ammonia-oxidizing bacteria in aquatic sediments was studied by retrieving ammonia monooxygenase and methane monooxygenase gene sequences. Methanotrophs dominated freshwater sediments, while β-proteobacterial ammonia oxidizers dominated marine sediments. These results suggest that γ-proteobacteria such as *Nitrosococcus oceani* are minor members of marine sediment ammonia-oxidizing communities.

Recent studies of ammonia-oxidizing bacteria have used PCR primers that target β-proteobacteria such as *Nitrosomonas* and *Nitrosospira* (17). This approach ignores the ammonia oxidizing bacteria in the γ subdivision of the *Proteobacteria*. This is unfortunate since γ-proteobacteria such as *Nitrosococcus oceani* may be important ammonia oxidizers, especially in marine habitats (18, 19), where β-proteobacteria are rarely found (3, 13).

To test the hypothesis that γ-proteobacteria dominate marine sediment ammonia-oxidizing communities, we used a conserved primer set to amplify the ammonia monooxygenase (*amoA*) and methane monooxygenase (*pmoA*) genes from all known ammonia oxidizers and methanotrophs. These primers anneal to conserved regions of these genes (9) and have been applied to soil and freshwater habitats (e.g., see references 2, 6, and 10). Since the topology of the *amoA-pmoA* phylogenetic tree is in good agreement with the corresponding 16S ribosomal DNA (rDNA) tree (14), we can tentatively identify the dominant ammonia oxidizers using this approach. We constructed clone libraries of *amoA* and *pmoA* genes from freshwater and marine sediments. Our marine clone libraries were dominated by β-proteobacterial *amoA* genes, leading us to reject the hypothesis that γ-proteobacteria dominate ammonia-oxidizing communities in marine sediments.

Sites and sampling. Marine sediments were collected from three well-characterized Pacific Northwest sampling sites. Two were located along the Washington continental margin (Pacific Ocean) and one was located in Puget Sound, Wash. As a control, freshwater sediment was collected from Wintergreen Lake in Michigan. Sample designations (in parentheses) and specific locations are as follows: Washington continental margin site 301 (WC301), 46°48.60′N, 124°37.20′W, 119-m water depth; Washington continental margin site 306 (WC306), 48°29.60′N, 126°43.22′W, 630-m water depth; Puget Sound (PS), 47°43.50′N, 122°23.90′W, 182-m water depth; Wintergreen Lake (FW), 42°23′58″N, 85°23′00″W, 3-m water depth. The report of Braker et al. (1) contains more-detailed information about the sampling sites. Surface (depth, 0 to 2 cm) sediment samples were collected and stored frozen until DNA extraction. Oxygen was available for ammonia oxidation at the freshwater sampling site (by direct measurement), and ammonia oxidation rates at sites WC301, WC306, and PS were 1.78, 0.29, and 2.24 mmol $NH_4^+ \cdot m^2 \cdot day^{-1}$, respectively (5).

Community analysis. DNA was extracted from sediments using the procedure of Gray and Herwig (4). We amplified *amoA* and *pmoA* functional genes using PCR primers that were slightly modified from those described by Holmes et al. (9) to favor γ-proteobacteria (Table 1). PCR reaction conditions were similar to those used by Holmes et al. (9), with the exception of adding 200 ng of bovine serum albumin (Roche Molecular Biochemicals, Indianapolis, Ind.) per μl to the reaction mixture. After amplification, the 530-bp product was excised from a 2% agarose gel and purified using the Qiaquick gel extraction kit (Qiagen, Valencia, Calif.) to ensure a properly sized insert. Clone libraries of the PCR products were constructed for each sediment using the TA cloning kit (Invitrogen, Carlsbad, Calif.). Clones containing a 530-bp insert were screened by restriction fragment length polymorphism (RFLP) analysis as previously described (20) using the restriction enzymes *Hha*I (GCG ↓ G; Gibco BRL, Rockville, Md.) and *Msp*I (C ↓ CGG; Gibco BRL). Unique RFLP patterns were enumerated, and nucleotide sequence data were obtained from the 10 most abundant restriction fragment types in each sediment sample. Sequences were initially compared to all GenBank database sequences using the BLAST algorithm found at the GenBank web site (http://www.ncbi.nlm.nih.gov/BLAST/). Those sequences similar to *amoA* or *pmoA* were aligned and compiled in the Genetic Data Environment software package. A neighbor-joining phylogenetic tree was constructed from derived amino acid sequences using the programs SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE of Phylip (version 3.5) found at the Phylip web site (http://evolution.genetics.washington.edu/phylip.html).

Community profiles of ammonia oxidizing bacteria. The Puget Sound clone library was representative of the other marine libraries. This library was dominated by three restriction fragment types but also contained diverse, rare fragments (Fig. 1A). Compared to the sequences in the GenBank database, most of these clones (75%) were *amoA* sequences most similar to β-proteobacterial ammonia oxidizers (Fig. 1A). Three of these sequences, PS-5, PS-8, and PS-47, accounted for 70% of the identified clones. Nine additional clones (16%) were associated with *pmoA* sequences similar to γ-proteobacterial methanotrophs. The five remaining marine clones (9%) displayed no similarity to known *amoA* or *pmoA* sequences. In contrast, the freshwater clone library (48 total clones) was composed entirely of γ-proteobacterial *pmoA* sequences from methanotrophs (Fig. 1B). Interestingly, one *pmoA* sequence

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TABLE 1. Primer and target *amoA* and *pmoA* gene sequences

Primer pair or target	No. of reps[a]	Primer or target sequence	
		Forward	Reverse
Primer pairs			
Original primer pair[b]		5′ GGN GAC TGG GAC TTC TGG 3′	5′ GAA SGC NGA GAA GAA SGC 3′
Modified primer pair[c]		5′ GGN GAC TGG GAC TTC TGG 3′	5′ –AA VGC VGA GAA GAA WGC 3′
Targets[d]			
Consensus γ-*amoA*	2	5′ GGG GAC TGG GAY TTC TGG 3′	5′ AAA ACC CGM RAA RAA RGC 3′
Consensus β-*amoA*	32 F, 68 R	5′ GGH GAC TGG GAY TTC TGG 3′	5′ RAA NCC GGM GAA GAA BGC 3′
Consensus γ-*pmoA*	5	5′ GGG GAC TGG GAC TTC TGG 3′	5′ GAA GGC GGA GAA GAA BGC 3′

[a] Number of available representatives (reps) from each major subdivision that were compared to create the primer sequence. F, forward (corresponding to *Nitrosomonas europaea amoA* positions 172 to 189); R, reverse (corresponding to *N. europaea amoA* positions 665 to 681).
[b] For a description, see reference 9.
[c] Primer pair used in this study.
[d] Consensus sequences were constructed by comparing all available representatives from each proteobacterial subdivision. If 80% or more of the sequences had an individual nucleotide at a position, that base is displayed. Otherwise, the ambiguous base is displayed. No α-proteobacterial *pmoA* sequences are available for these regions. B, cytosine, thymine, or guanine; H, adenine, cytosine, or thymine; M, adenine or cytosine; N, any nucleotide; R, adenine or guanine; S, cytosine or guanine; V, adenine, cytosine, or guanine; W, adenine or thymine; Y, cytosine or thymine.

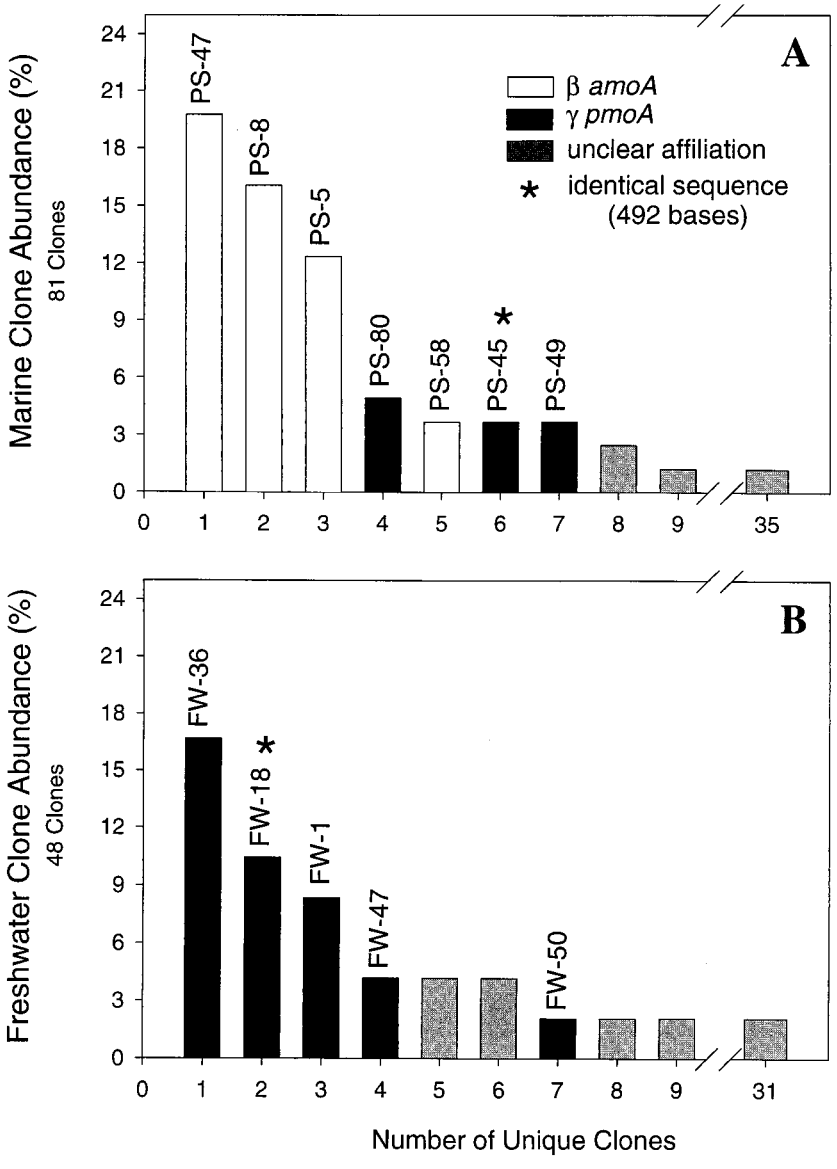

FIG. 1. Abundance of RFLP types in Puget Sound (A) and Wintergreen Lake (B) sediment clone libraries. Representative sequence types appear above each bar, and the phylogenetic affiliations and functional gene types are shown.

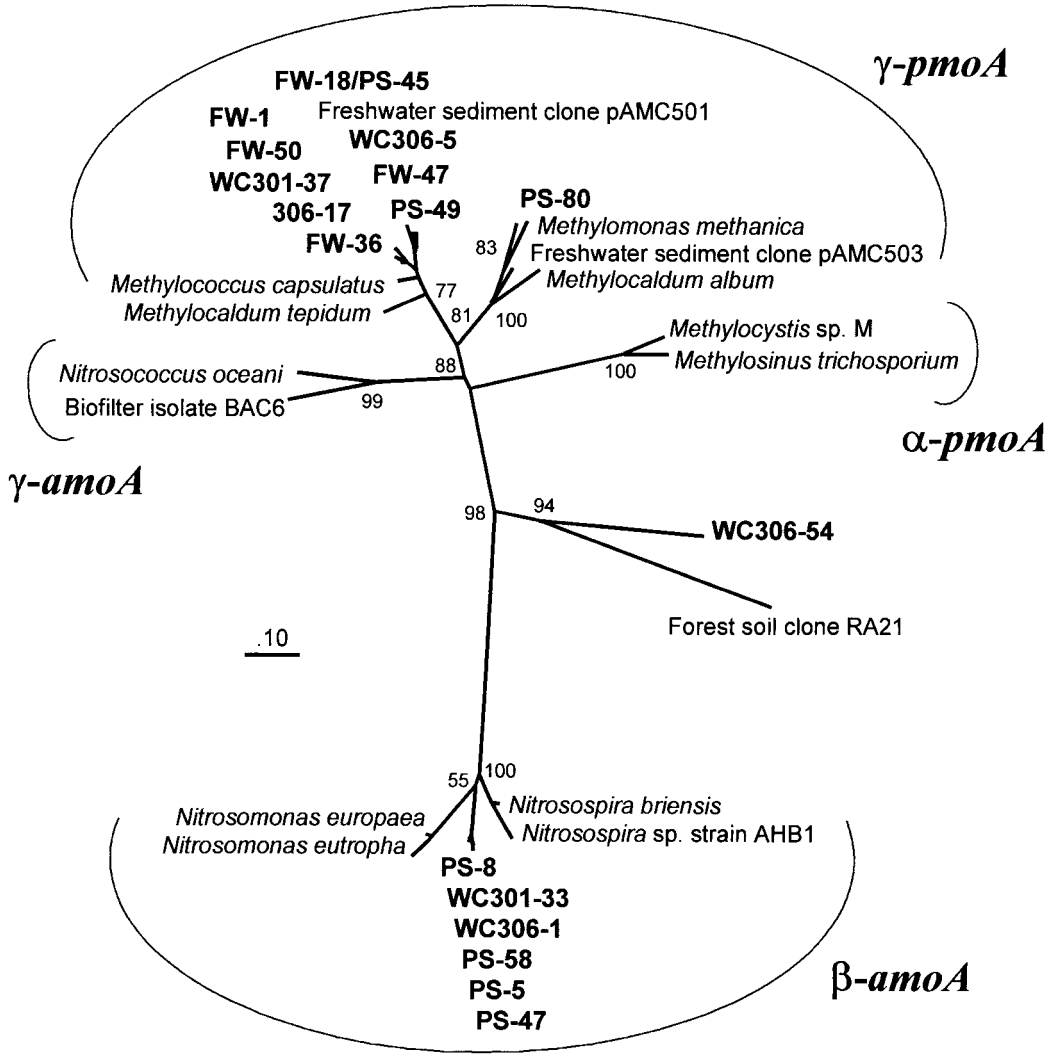

FIG. 2. Neighbor-joining phylogenetic tree of derived *amoA* and *pmoA* amino acid sequences. Major proteobacterial lineages are denoted. Trees are based on comparison of 163 amino acids. This figure also contains sequences from clone libraries constructed from sediment samples collected from the Washington continental margin. Bootstrap values from 100 resamplings are shown, except for those nodes with values below 50 or where branching orders between the consensus tree and this tree were inconsistent.

type was found in both freshwater and marine habitats (FW-18 and PS-45, respectively). The sequences of FW-18 and PS-45 were identical over the 490 available nucleotides. We failed to detect γ-proteobacterial *amoA* sequences similar to the *Nitrosococcus oceani* sequence in either habitat.

The evolutionary relationships among our retrieved sequences are explored in Fig. 2. This phylogenetic tree contains all marine sequences, including those we retrieved from the Washington continental margin sediments. Marine *amoA* sequences clustered together, but distinct from *Nitrosomonas* and *Nitrosospira* sequences. Most marine *pmoA* sequences were similar to *Methylococcus capsulatus*, a type I methanotroph. This cluster includes freshwater sediment sequences retrieved from Lake Washington in Seattle, Wash. (represented by clone pAMC501 [2]). One clone (WC306-54) showed no clear affiliation to *amoA* or *pmoA* sequences, indicating a possibly novel lineage. Although this clone appears to cluster with a sequence retrieved from a beech forest soil in Denmark (clone RA21) (10), the nucleotide and amino acid sequence similarities are very low (58.0 and 55.7%, respectively). Freshwater clones clustered with type I methanotrophs. Again, no γ-proteobacterial *amoA* sequences were retrieved.

Marine sediments contain β-proteobacterial ammonia oxidizers. We failed to detect any γ-proteobacterial *amoA* sequences, leading us to reject the hypothesis that γ-proteobacteria dominate ammonia-oxidizing communities in marine sediments. Our clone libraries were instead populated by β-proteobacterial *amoA* sequences. These sequences form a distinct branch, evolutionarily separate from the *Nitrosomonas* and *Nitrosospira* lineages (Fig. 2). Stephen et al. (16) found a similar pattern of ammonia oxidizer diversity in polluted marine sediments. Using PCR primers specific to the β-proteobacterial ammonia oxidizers, these authors discovered two clusters of environmental 16S rDNA sequences (clusters 1 and 5) distinct from the *Nitrosomonas* and *Nitrosospira* lineages. These novel clusters contain no cultivated representatives. The functional *amoA* genes we retrieved from marine sediments may come from the same organisms as the 16S rDNA sequences found in cluster 1 or cluster 5 ammonia oxidizers.

Aquatic sediments contain type I (12) or type II (11) meth-

anotrophs, or both (2, 6). Our *pmoA* sequences were exclusively type I (γ-proteobacterial). We were surprised to discover remarkable similarity between freshwater and marine *pmoA* sequences, including an identical sequence in both libraries (clones PS-45 and FW-18). Salinity is a strong adaptive barrier. Microorganisms in equivalent marine and freshwater habitats are generally very different (3, 13), even though they perform similar functions (8). We expected to find phylogenetically distinct clusters of freshwater and marine sequences, similar to those observed in environmental 16S rDNA surveys (7, 15, 21). Instead, *pmoA* sequences of freshwater and marine habitats display no evolutionary divergence.

This study should not be used to prove the importance of the β-proteobacterial ammonia oxidizers. It is likely that we are missing important community members by using selective techniques such as probing, PCR amplification, and culturing. By limiting studies to the β-proteobacterial ammonia oxidizers, we may still be ignoring important components of native bacterial communities.

Nucleotide sequence accession numbers. The *amoA* and *pmoA* gene sequences presented in this paper were submitted to GenBank under the following accession numbers: PS-5, AF211883; PS-8, AF211884; PS-45, AF211873; PS-47, AF211885; PS-49, AF211874; PS-58, AF211886; PS-80, AF211872; WC301-33, AF211887; WC301-37, AF211877; WC306-1, AF211888; WC306-5, AF211875; WC306-17, AF211876; WC306-54, AF211889; FW-1, AF211878; FW-18, AF211879; FW-36, AF211881; FW-47, AF211880; FW-50, AF211882.

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