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Diversity of Oxygenase Genes from Methane- and Ammonia-Oxidizing Bacteria in the Eastern Snake River Plain Aquifer

Daniel P. Erwin,[1] Issac K. Erickson,[2] Mark E. Delwiche,[3] Frederick S. Colwell,[3] Janice L. Strap,[1] and Ronald L. Crawford[1]*

Environmental Biotechnology Institute[1] and Biology Department,[2] University of Idaho, Moscow, and Idaho National Engineering and Environmental Laboratory, Idaho Falls,[3] Idaho

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PCR amplification, restriction fragment length polymorphism, and phylogenetic analysis of oxygenase genes were used for the characterization of in situ methane- and ammonia-oxidizing bacteria from free-living and attached communities in the Eastern Snake River Plain aquifer. The following three methane monooxygenase (MMO) PCR primer sets were used: A189-A682, which amplifies an internal region of both the *pmoA* gene of the MMO particulate form and the *amoA* gene of ammonia monooxygenase; A189-mb661, which specifically targets the *pmoA* gene; and mmoXA-mmoXB, which amplifies the *mmoX* gene of the MMO soluble form (sMMO). Whole-genome amplification (WGA) was used to amplify metagenomic DNA from each community to assess its applicability for generating unbiased metagenomic template DNA. The majority of sequences in each archive were related to oxygenases of type II-like methanotrophs of the genus *Methylocystis*. A small subset of type I sequences found only in free-living communities possessed oxygenase genes that grouped nearest to *Methylobacter* and *Methylomonas* spp. Sequences similar to that of the *amoA* gene associated with ammonia-oxidizing bacteria (AOB) most closely matched a sequence from the uncultured bacterium BS870 but showed no substantial alignment to known cultured AOB. Based on these functional gene analyses, bacteria related to the type II methanotroph *Methylocystis* sp. were found to dominate both free-living and attached communities. Metagenomic DNA amplified by WGA showed characteristics similar to those of unamplified samples. Overall, numerous sMMO-like gene sequences that have been previously associated with high rates of trichloroethylene cometabolism were observed in both free-living and attached communities in this basaltic aquifer.

Trichloroethylene (TCE) is a common groundwater contaminant of toxic and carcinogenic concern (2, 32, 37, 48). Its extensive use as an industrial solvent, combined with inadequate disposal methods, has led to groundwater contamination of many sites worldwide. TCE itself is a suspected carcinogen, and its biotransformation product vinyl chloride, which can be produced in anaerobic aquifers, is a known carcinogen for animals (33, 35). Due to the established toxicity of this product, the Environmental Protection Agency has a nonenforceable maximum contaminant level goal of 0 μg liter^{-1} for TCE in drinking water supplies. Presently, the enforced maximum contaminant level for TCE is 5 μg liter^{-1} (16).

In 1989, Test Area North (TAN), located on the Idaho National Environmental and Engineering Laboratory site in southeastern Idaho, was added to the National Priorities List as one of the sites requiring cleanup (15). The TAN site contains a contamination plume within the Eastern Snake River Plain Aquifer (ESRPA) containing as much as 300 mg of TCE liter^{-1} and extending over an area of 25,000 m^2 (46). Wells have been monitored throughout the TAN site in order to reveal the boundaries of this TCE plume. Though the extent of this plume is quite large, it does not appear to be migrating farther from the initial point of contamination. A previous study ruled out abiotic loss as the only reason for this observed attenuation (47). In addition, direct evidence that TCE cometabolism is occurring at the TAN site within the plume of TCE contamination has been reported (M. H. Howard-Jones, W. K. Keener, R. A. Wymore, F. S. Colwell, K. S. Sorenson, and M. E. Watwood, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. Q-005, p. 514, 2003).

Previously, methanotrophs, propanotrophs, nitrifiers, and phenol oxidizers have been isolated from groundwater within the TAN site (7). Expression of a wide array of nonspecific oxygenase enzymes would allow these bacteria to use a variety of primary substrates that support cometabolic TCE dechlorination. Of these organisms, the methanotrophs in particular have been well studied for their ability to cometabolize groundwater pollutants (1, 9, 24, 31, 42, 49, 50).

Methanotrophs use methane monooxygenase (MMO) to catalyze the oxidation of their primary growth substrate methane to methanol. The enzyme can be found either in the cytoplasm in a soluble form (sMMO) or in the membrane-bound particulate form (pMMO) (17). The pMMO enzyme is found in all known methanotrophs, with the potential exception of an acidophilic methanotroph isolated by Dedysh et al. (13). Of the organisms also capable of sMMO production, the majority have been found to be of the type II and type X methanotrophs affiliated with the α subclass of *Proteobacteria* (17). Though rare, strains of type I methanotrophs (affiliated with γ-*Proteobacteria*) have also been found to produce sMMO (5, 39). The *mmoX* gene encodes the conserved α subunit of the hydroxylase component of the sMMO (6) and has been used previously as a marker for sMMO (36, 45).

Both the soluble and particulate forms of MMO have established potentials for the aerobic dechlorination of pollutants

* Corresponding author. Mailing address: University of Idaho, Environmental Biotechnology Institute, Food Research Center 204, Moscow, ID 83844-1052. Phone: (208) 885-6580. Fax: (208) 885-5741. E-mail: crawford@uidaho.edu.

such as TCE, though at different rates (3, 4). The sMMO enzyme is of particular interest to bioremediation studies, as it has been shown to exhibit a broader substrate range than the particulate form. In addition, the sMMO exhibits a higher level of activity than pMMO, which leads to a significantly higher rate of TCE degradation than that of pMMO (4, 10).

Ammonia monooxygenase (AMO) is similar to pMMO in both structure and function. AMO is used to obtain energy for carbon dioxide fixation through the oxidation of ammonia and is found in ammonia-oxidizing bacteria (AOB), such as *Nitrosomonas* and *Nitrosospira* spp., that group within the β subclass of *Proteobacteria* (17, 22). The genes encoding AMO and those encoding pMMO share high sequence identity and are believed to be evolutionarily related. This concept is supported through predicted amino acid sequence alignments showing that both primary and secondary structures are well conserved between subunits of both enzymes (19). For example, *Nitrosomonas europaea* can oxidize TCE through the action of AMO. The affinity of TCE for AMO is very close to its natural substrate, ammonia (22).

The objective of this study was to use molecular techniques to examine the presence and diversity of oxygenase genes from methane-oxidizing bacteria (MOB) and ammonia-oxidizing bacteria within a pristine region of the TAN site of the Idaho National Environmental and Engineering Laboratory, focusing on MMO and AMO genes of the total microbial community (free-living and attached). This information will assist us in understanding the responses of the natural microflora of the pristine aquifer to the appearance of contaminants such as TCE. Beyond simply confirming the presence of genes that enable TCE cometabolism, our aim was to assay the diversity of these genes as a first step in understanding how different versions of the same class of degradative enzymes might collectively contribute to cometabolism.

MATERIALS AND METHODS

Sampling. The microbial communities present in the ESRPA were sampled in the fall of 2003. Two wells, ANP-9 and ANP-10, located in a pristine region of the aquifer proximal to the TCE-contaminated plume, were used for this study. A detailed site description has recently been reported by Newby et al. (40). The close proximity of the wells to each other (ca. 200 m) suggests that their respective water chemistries and microbial community structures should be similar. Previous analysis indicates comparable water chemistry characteristics between the two wells and values that are typical of the ESRPA. For example, selected water chemistry values for ANP-9 and this region of the aquifer in general include temperature of 14.1 to 14.5°C, a pH of 7.2 to 7.9, a dissolved oxygen level of 7.5 to 8.4 mg liter^{-1}, a dissolved organic carbon level of 1.5 to 2.6 mg liter^{-1}, and a methane concentration of 72 nmol liter^{-1} (40). Well ANP-9 was used in the collection of free-living cells, while well ANP-10 was used for attached community sampling.

The free-living community in this study was defined as all cells freely dispersed within the aquifer groundwater or only loosely associated with the basalt. The attached community consisted of all cells that colonized the surfaces of basalt substrates that were incubated in well ANP-10. Methods were designed to facilitate the sampling of each community by using existing wells (18, 27-29).

A KrosFlo Pilot hollow-fiber, tangential flow filtration system with a DynaFibre microporous membrane (0.2 μm pore size and 3.9 m^2 in surface area; Spectrum Labs, Rancho Dominguez, Calif.) was employed for the collection of free-living organisms from ANP-9. Prior to the initiation of filtration, the well was flushed with 3 well volumes (1 volume equal to the standing water in the well) to remove built-up sediments and debris from the casing. Approximately 13,300 liters of aquifer water was then passed through the hollow-fiber apparatus and concentrated to approximately 3 liters. The concentrate was collected by reversing the pump motor and directing the retentate into sterile 1-liter containers. This concentrate was then stored at −80°C and thawed at room temperature prior to being used.

TABLE 1. Primer sequences used for the molecular characterization of microbial communities derived from the Eastern Snake River Plain Aquifer

Primer	Sequence (5′ to 3′)	Target	Reference
A189	GGNGACTGGGACTTCTGG	*amoA*/*pmoA*	18
A682	GAASGCNGAGAAGAASGC	*amoA*/*pmoA*	18
mb661	CCGGMGCAACGTCYTTACC	*pmoA*	10
mmoXA	ACCAAGGARCARTTCAAG	*mmoX*	6
mmoXB	TGGCACTCRTARCGCTC	*mmoX*	6

The attached communities were sampled through the use of substrate columns. These columns consisted of crushed basalt chips (4 to 7 mm sieve size) housed in a 76-cm-long nylon mesh tubing (inside diameter, 2 cm; mesh size, 0.25 cm; InterNet, Inc., Minneapolis, Minn.). Prior to use, the columns were rinsed with deionized water to remove fine particles and then autoclaved (60 min) on three successive days. The sterile columns were then suspended in the well to a depth of 80 m for 6 months. At the end of this period, the columns were removed and the attached microbial mass was harvested.

Metagenomic DNA isolation. The UltraClean Mega Prep soil DNA kit (MoBio Laboratories, Inc., Solana Beach, Calif.) was used to harvest the attached community metagenomic DNA from the basalt chips. Four 50-ml polypropylene tubes were filled with 40 g of the substrates and secured horizontally in a rotating incubator (Innova 4300; New Brunswick Scientific Co., Inc., Edison, N.J.) at 200 rpm at 60°C for 30 min to remove bacterial biomass from the basalt substrates. Metagenomic DNA was recovered as per the manufacturer's protocol. After recovery, the extractions were pooled and concentrated by using a Microcon YM-30 ultrafiltration centrifugal device (Millipore, Billerica, Mass.).

Free-living cells were recovered by filtering 50 ml of the concentrated material through a 25-mm (0.20-μm)-pore-size Nuclepore polycarbonate membrane filter (Whatman, Inc., Clifton, N.J.). DNA was then extracted from the filtered material by use of the UltraClean soil DNA isolation kit from MoBio. Briefly, the filter was placed into the bead solution and vortexed on high for 10 s to disperse the filtered material. The tubes were then placed in a 65°C water bath for 30 min. The purification of the lysed material was performed as per the manufacturer's protocol. This method was performed on three additional aliquots so that a total concentrate volume of 200 ml was processed. The resulting DNA from each extraction was pooled and stored at −20°C. DNA recovered from the substrate-attached bacteria by the modified MoBio Mega Prep method resulted in 140 μl of DNA at a concentration of 24 ng μl^{-1} (A_{260}/A_{280} = 1.5).

Whole-genome amplification. Purified DNA from both communities was amplified by using the Repli-*g* whole-genome amplification (WGA) kit (Molecular Staging, Inc., New Haven, Conn.) to produce two additional community archives, W9 and W10. Briefly, the kit utilizes ϕ29 polymerase in conjunction with exonuclease-resistant random hexamer primers to isothermally amplify DNA 10^4- to 10^6-fold with minimal bias (26). Based on multiple-displacement amplification technology, exponential amplification occurs through a hyperbranching mechanism (12, 25). Long products, averaging 12 kb in length, are obtained due to the high processivity rate of >70,000 nucleotides incorporated for each primer binding event (8). ϕ29 also exhibits the highest fidelity rate of any known polymerase, with an error rate of only 1 in 10^6 to 10^7 nucleotides incorporated (14).

For each sample, 10 ng of genomic DNA was chemically denatured by being mixed with 2.5 μl of denaturation solution (40 mM KOH, 1 mM EDTA [pH 8.0]) and incubated at room temperature for 3 min. The reaction mixture was neutralized by the addition of 5 μl of neutralization solution (a 1:10 dilution of supplied solution B in distilled water). Amplification reactions were performed in 50-μl volumes containing 12.5 μl of 4× WGA PCR mix (as supplied by the manufacturer), 0.5 μl of DNA polymerase mix, 27 μl of sterile H_2O, and 10 μl of denatured sample. The reaction mixtures were then incubated at 30°C for 16 h. Amplification of the free-living DNA was only successful after two washes with distilled water followed by concentration with a Microcon filter device. Amplified products were visualized by using 1% agarose gel electrophoresis stained with ethidium bromide. The amplified DNA was quantified by densitometry using Kodak 1D software (Kodak, Rochester, N.Y.).

Functional gene PCR amplification. Three sets of PCR primers were used in this study to elucidate the oxygenase genes associated with methane- and ammonia-oxidizing bacteria from each community (attached and free-living) and WGA material (W9 and W10) (Table 1). To date, no single primer set has been

demonstrated to amplify *mmoX* genes from all sMMO-producing bacteria.

Primers that targeted the *mmoX* gene and the *pmoA* gene specifically were used, as was a set that allowed the corecovery of *amoA* gene fragments along with *pmoA*. All PCR products were verified on 1% agarose gels stained with ethidium bromide and visualized by using UV light. Images were captured and recorded by using Kodak 1D software. Reagents and enzymes were purchased from Fisher Scientific (Hampton, N.H.) unless otherwise specified. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa) with standard desalting.

pMMO diversity within the communities was assessed through the use of primers designed by Holmes et al. (19) and Costello and Lidstrom (11) (Table 1). Both groups utilized the same forward primer, A189, in conjunction with one of two reverse primers. The first reverse primer, A682, was designed to target a highly conserved region in both the *pmoA* and *amoA* genes. These genes are believed to encode the active site containing the 27-kDa polypeptide subunit of both the MMO and the AMO. The A189-A682 primer set has been shown previously to amplify a 525-bp internal section of both *pmoA* and *amoA* (19). The second reverse primer, mb661, has been shown to preferentially amplify only an internal section of approximately 470 bp from the *pmoA* gene (11). PCR amplifications with both primer sets were performed in 50-μl volumes containing a 200 μM concentration of each deoxynucleoside triphosphate, 1× PCR buffer, 10 μg of bovine serum albumin, 1.2 mM $MgCl_2$, 10 pmol of each forward and reverse primer, 2.5 U of *Taq* polymerase, and 20 ng of DNA template. Reaction mixtures were subjected to initial denaturation for 5 min at 94°C followed by 30 cycles of 57°C for 1 min, 72°C for 1 min, and 94°C for 1 min. The final step consisted of a 57°C annealing for 1 min, followed by a 10-min elongation at 72°C.

The primer set mmoXA and mmoXB was used to amplify an approximately 1,230-bp fragment from the *mmoX* gene as described by Auman et al. (6). This primer set is designed to target the region of *mmoX* that encodes the conserved fragment of the α subunit of the hydroxylase component of sMMO. PCR amplifications were performed in 50-μl volumes containing 200 μM concentrations of each deoxynucleoside triphosphate, 1× PCR buffer, 10 μg of bovine serum albumin, 1.2 mM $MgCl_2$, 10 pmol of each forward and reverse primer, 2.5 U *Taq* polymerase, and 20 ng of DNA template. Reaction mixtures were subjected to an initial denaturation of 5 min at 94°C followed by 30 cycles of 60°C for 1 min, 72°C for 1 min, and 94°C for 1 min. The final step consisted of a 60°C annealing for 1 min, followed by 10 min of elongation at 72°C.

A PCR on a metagenomic DNA template isolated from an undefined groundwater methanotroph enrichment which had been grown on methane was used as a positive control for each primer set.

Following amplification, all PCR products were purified by a Wizard spin column PCR cleanup (Promega, Madison, Wis.). Some products required additional purification by agarose gel electrophoresis in order for clones with the proper insert size to be obtained. This procedure was done by loading approximately 1 μg of PCR product into 1% SeaPlaque GTG LMP agarose, followed by extraction via Qiaex II gel extraction (QIAGEN, Valencia, Calif.).

Archives were coded based on the well numbers used in the sampling (9 and 10 for free-living and attached communities, respectively) and the primer sets used in the PCR amplification. For instance, the archive produced from free-living cells isolated from well ANP-9 and amplified with the PCR set A189-A682 was coded as 9-682. Archives produced from whole-genome-amplified DNA were designated with a "W" preceding the well identifier.

Functional gene fragment library production. PCR amplicons for each sample were cloned by using the TOPO TA cloning kit for sequencing (version K) (Invitrogen Corporation, Carlsbad, Calif.). Briefly, 4 μl of each fresh PCR product was incubated with 1 μl of salt solution (1.2 mM NaCl, 0.06 M $MgCl_2$) and 1 μl of pCR4-TOPO vector. Electrocompetent Top10 *Escherichia coli* cells were electroporated at 2.4 kV in 0.2-cm cuvettes with 2 μl of the cloning reaction mixture. Positive transformants were selected by spread plating onto Luria-Bertani agar plates with either kanamycin (50 μg ml^{-1}) or ampicillin (60 μg ml^{-1}) used as the selective agent and incubated overnight at 37°C. Kanamycin was used when direct PCR amplicons were cloned, whereas ampicillin was used when the amplicon was purified by gel electrophoresis.

Ninety-six colonies were isolated for each library archive and inoculated into 96-well plates containing 200 μl of Luria-Bertani broth and 50 μg of kanamycin ml^{-1} amended with 1× Haugness buffer (4% glycerol, 3.6 mM K_2HPO_4, 1.3 mM KH_2PO_4, 2 mM trisodium citrate, 1 mM $MgSO_4$, pH 7.5) as a cryoprotectant. These plates were then incubated in a GeneMachines HiGro microtiter-plate orbital shaker (Genomic Solutions, Ann Arbor, Mich.) for 18 h at 420 rpm and 37°C with an airflow rate of 4 standard liters per min per chamber. Ten wells were randomly sampled for insert verification by PCR using vector-specific flanking primers (M13f and M13r supplied with the kit). Amplicons were visualized on 1% agarose gels prestained with ethidium bromide. After verification, plates were sealed with aluminum tape and stored at −80°C for future use.

Restriction fragment length polymorphism (RFLP) analysis. Plasmid DNA was isolated from the 96-well cultures by using the Montage Plasmid $Miniprep_{96}$ kit (Millipore). Cultures were prepared by inoculating one round-bottom, 96-well cell culture block (supplied with the kit) containing 1 ml of Terrific broth with 50 μg of kanamycin ml^{-1} per well for each archive. Blocks were incubated in the HiGro incubator for 24 h under the conditions described above. Cells were harvested by centrifugation at 1,500 × *g* for 5 min with subsequent removal of supernatant by decanting. The blocks were sealed and pellets were frozen at −20°C prior to processing.

Plasmid DNA was isolated from the cell pellets as per the manufacturer's full lysate protocol. Five wells were selected from each plasmid preparation and DNA quantified by measuring the absorbance at 260 nm.

RFLP patterns were produced for each clone by the digestion of 1 μg of plasmid DNA with 5 U each of MspI and HinPII (New England Biolabs, Beverly, Mass.) in a final reaction mixture volume of 50 μl. Reaction mixtures were incubated at 37°C for 2 h, followed by enzyme inactivation at 65°C for 10 min. The manual grouping of fragmentation patterns was performed by analyzing 10 μl of each digest on 4% NuSieve 3:1 agarose (Cambrex Bio Science, Baltimore, Md.). Gels were stained with ethidium bromide and rinsed with deionized H_2O prior to imaging. One representative from each RFLP group was chosen for further analysis.

Sequencing and phylogenetic analysis. Sequencing of insert DNA was performed on an ABI Prism model 3730 sequencer (Applied Biosystems, Inc., Foster City, Calif.) by using BigDye version 3 chemistry. Contiguous sequences were produced by using ContigExpress software of the Vector NTI suite, version 9. Nucleotide-nucleotide BLAST (blastn) was used to search GenBank for nearest relative sequences. BLAST results and representatives for each archive were aligned by using AlignX software from the Vector NTI suite, version 9.

Edited alignments were evaluated with the maximum likelihood method by using the PAUP package (version 4.0b10; Sinauer Associates, Inc., Sunderland, Mass.). Evolutionary distance calculations were generated by using the model best describing the data for each tree as determined using the DT-ModSel program (38). Confidence estimates for the nodes within phylogenetic trees were performed by bootstrap analysis (100 replicates). Trees were visualized with TreeView software, version 1.6.6 (43).

RESULTS

Sampling. Free-living cell collection produced 3 liters of concentrate at 4.2×10^6 cells ml^{-1} (1.26×10^{10} cells in total), as determined by direct microscopic counts of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells. After taking into account a 1:4,433 dilution factor, these results suggest a concentration of approximately 10^3 cells ml^{-1} in the original ground water. This number agrees with previous cell density estimates from this same well (40). Biomass from the substrate columns was not quantified.

Metagenomic DNA isolated from sampling of free-living cells by the heat-lysis method used in conjunction with the MoBio kit produced a total of 160 μl of DNA at 12.5 ng μl^{-1} (A_{260}/A_{280} = 1.61). The DNA was ~25 kb in size.

Whole-genome amplification. In an effort to assess the applicability of using whole-genome amplification as a means to generate additional metagenomic template DNA for analyses, archives generated from amplified and unamplified templates were compared. WGA samples resulted in quantities of 550 ng μl^{-1} and 800 ng μl^{-1} (quantified by densitometry) for the free-living and attached communities, respectively. These concentrations represent nearly 10^4-fold amplifications. Both amplified samples migrated between 20 and 25 kb when analyzed by agarose gel electrophoresis.

PCR amplification. DNA extracted from the free-living and attached communities resulted in PCR amplicons of the expected size for a given primer pair. The only exception was the

absence of a PCR product from DNA isolated from the attached community with the *mmoX*-specific primers. These primers were also unsuccessful at amplifying the targeted gene fragment from WGA attached community DNA. In some instances, the products did not resolve as single, isolated bands even after PCR optimization.

Gene fragment library production. It was found initially that high transformation rates could be obtained, although a large percentage (30 to 50%) of those clones contained nonspecific inserts. In order to increase the percentage of clones containing the proper insert size, it was necessary to gel purify the PCR product prior to cloning. This was the case even in the absence of other visible contaminating DNA, such as multiple bands or primer dimers (data not shown). After gel purification, the percentage of clones not containing inserts of the proper size dropped well below 10%.

In general, DNA from all communities transformed at a very low efficiency compared to positive controls supplied with the kit, though some efficiencies were far lower than others. The reason for these differences remains unclear, though we speculate that it may be due to the nature of the oligonucleotide primers used in the PCR. In most cases, multiple cloning and transformation reactions were necessary to produce 96 colonies for the archives.

RFLP and phylogenetic analysis. A total of 96 clones were screened for each of the 10 functional gene archives. Initially, many RFLP patterns were observed in all archives, and a majority of those groups contained only a single representative. Subsequent sequencing revealed that many of these patterns were a result of false-positive clones that did not contain the proper insert. These RFLP groups were discarded from the archives. Figure 1 shows a representative gel containing multiple RFLP patterns from 9-661, 9-682, W9-661, W9-682, and W10-682.

Archives produced with primer set A189-A682 resulted in a combined total of 327 clones. From these clones, only type II methanotroph and AOB sequences that corresponded to α-*Proteobacteria* and β-*Proteobacteria*, respectively, were found (Fig. 2). Archive compositions of whole-genome amplified and unamplified communities were similar except that group I uncultured (*pmoA*) sequences were obtained with less frequency in the WGA free-living community archive than in the unamplified archive, while *Nitrosospira* spp. (*amoA*) were represented to a much greater degree in the WGA-derived archive than in the unamplified archive. Interestingly, this difference was not observed for the attached community. A comparison of the free-living and attached communities showed differences between the two communities. Group II uncultured (*pmoA*) sequences were more prevalent in the attached community than in the free-living community. Furthermore, representative sequences from group I and III uncultured (*pmoA*) bacteria and *Nitrosospira* were not found in the attached community archive (Table 2).

Archive 9-682 produced a total of 83 clones from which 13 unique RFLP patterns were obtained (Fig. 2). Just over half (59%) of the clones were found to contain oxygenase genes that group within α-*Proteobacteria*, while the remainder (41%) were from subclass β-*Proteobacteria*. BLAST results for groups 1, 3 to 7, 11, 13, 16, and 19 revealed sequences closely (95 to 98%) related to those of previously identified *pmoA*/*amoA*-containing bacteria. However, groups 2, 9, and 17 showed only 88% sequence identity to the oxygenase genes of uncultured bacterium M84-P36. These three groups branched separately from M84-P36 and formed a distinctive cluster with W9-682 7 (Fig. 2).

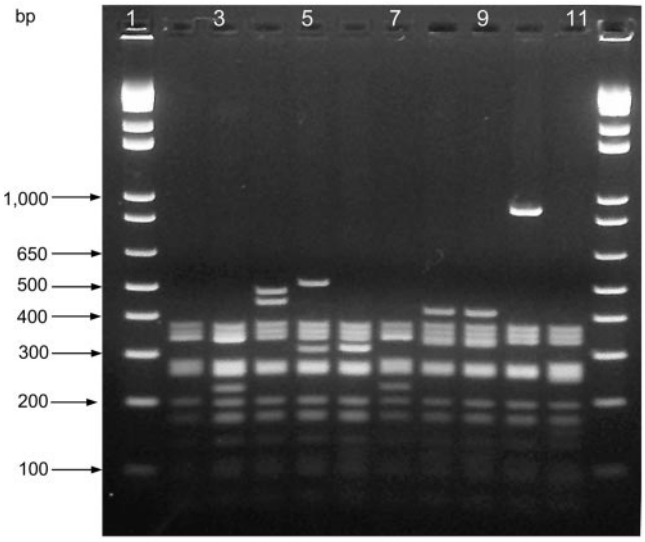

FIG. 1. Representative RFLP analysis. Plasmid DNA isolated from clones produced in the functional gene archives was digested with HinPII. Two hundred fifty nanograms of each digest was separated by using 2% LMP GTG agarose run at 5 V cm^{-1} for 1.75 h in 1% Tris-acetate-EDTA. Lanes 1 and 12 contain 500 ng of 1-kb Plus DNA molecular weight marker. Lanes 2 and 3, 9-661; lanes 4 and 5, 9-682; lanes 6 and 7, W9-661; lanes 8 and 9, W9-682; lanes 10 and 11, W10-682. The gel was stained with ethidium bromide. Images were captured and recorded by using Kodak 1D software and UV transillumination.

W9-682 produced a total of 91 clones that resulted in 10 unique RFLP patterns, of which 14% were α-*Proteobacteria* and 86% were β-*Proteobacteria* (Fig. 2). All groups, with the exception of group 7, yielded high sequence identity (94 to 99%) to previously identified *pmoA*/*amoA*-containing bacteria similar to those in 9-682. Group 7, like groups 2, 9, and 17 of 9-682, had a highest identity value of 88% for the uncultured bacterium M84-P36. Group 11 formed a distinct branch within the tree.

Archives 10-682 and W10-682 were both very similar in composition and showed less oxygenase diversity than the free-living community archives, as only five RFLP patterns were seen for 10-682 and six patterns were seen for W10-682 (Fig. 2). Both archives contained a large majority (89 and 97%, respectively) of β-*Proteobacteria*, of which nearly all showed high levels of similarity to oxygenase genes of the uncultured bacterium gp22 and are distantly related to the AOB *Nitrosospira* (43 of 45 nucleotides were identical). Two of the groups of α-*Proteobacteria*, 10-682 4 and W10-682 10, clustered near the MOB *Methylocystis* sp. 42/22, while the third group of W10-682 15 clustered near other uncultured α-*Proteobacteria*.

Archives produced with primer set A189-mb661 resulted in a combined total of 317 clones. An overall high percentage of α-*Proteobacteria* was found in each of these archives. Archive 9-661 and W9-661 contained 97 and 77% *Methylocystis*, respec-

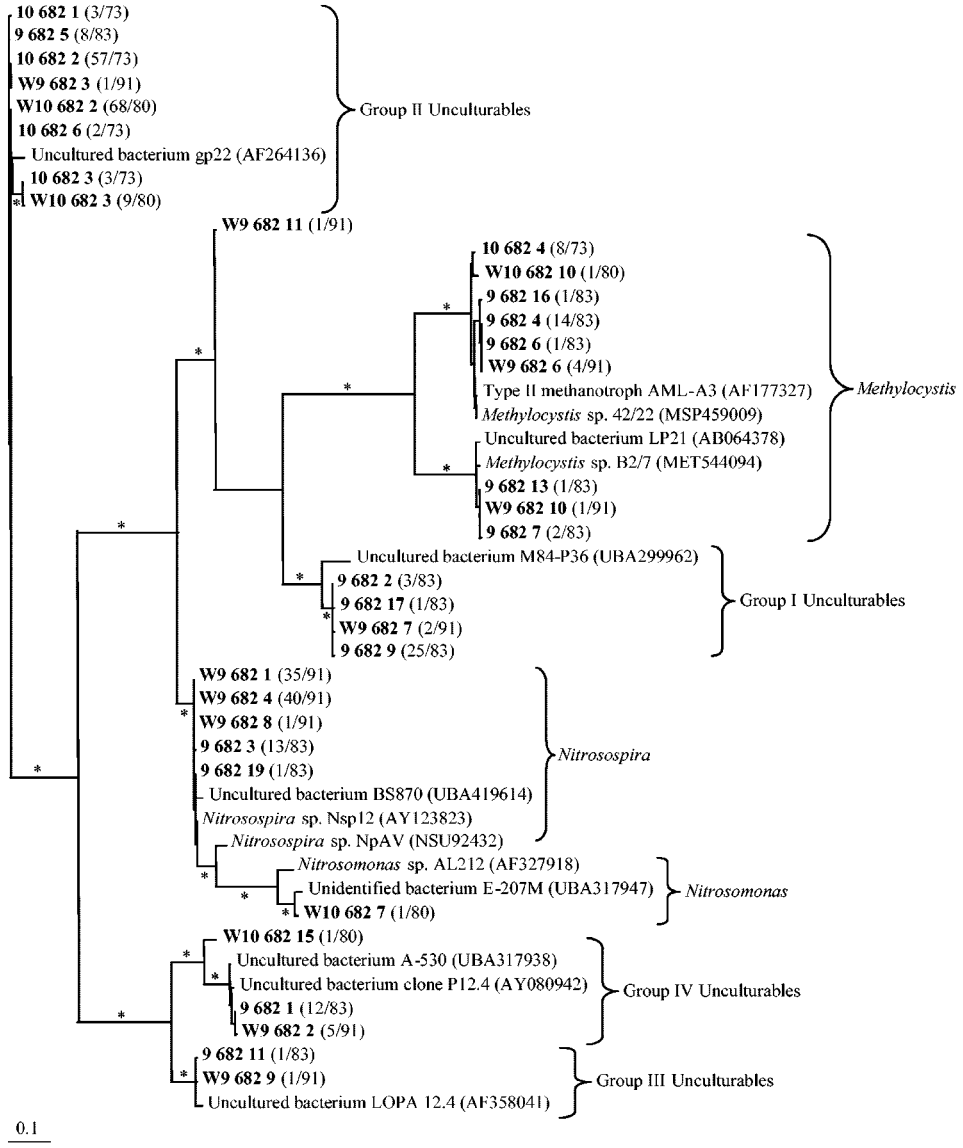

FIG. 2. Phylogenetic relationships among partial *amoA*/*pmoA* gene sequences produced from archives of unamplified and WGA free-living and attached communities. Gene fragments were obtained by using the primer set A189-A682 (540 base positions were considered). The bar represents 10% sequence divergence as determined by the length of the horizontal lines connecting any two species. The phylogenetic tree was constructed by using PAUP based on maximum likelihood analysis using the K80+G correction model. Bootstrap values greater than 75% are represented by asterisks. Unique clones obtained from the archives are indicated by boldface type. Each clone is named in the following manner: the first number (9 or 10 in bold) refers to the well number, the second number (e.g., 682 in bold) refers to the reverse primer that was used to obtain this sequence, the third number (in bold) is the RFLP group number, and the numbers in parentheses refer to the fraction of clones represented by this phylotype.

tively, and both 10-661 and W10-661 were made up of 100% *Methylocystis*-like sequences (Table 3).

Archives 9-661 and W9-661 were very similar in composition, with a high percentage of each corresponding to *Methylocystis* sp. (Fig. 3). Seven RFLP patterns were seen in archive 9-661, whereas W9-661 resulted in 13 patterns. Four of the groups, 9-661 9, W9-661 5, W9-661 11, and W9-661 13, branched with the type I methanotroph *Methylomonas* sp., though they formed a divergent cluster. Another type I methanotroph *Methylobacter* sp. was distantly related (81% sequence identity) to the groups 9-661 3 and W9-661 12. The nearest identity of these two groups is to the uncultured bacterium LP20 (89%), though the sequences were divergent enough to branch separately.

Archives 10-661 and W10-661 each produced six unique RFLP patterns and were similar in composition (Fig. 3). All of the clones screened in each archive were highly related to oxygenase genes of *Methylocystis*, with >98% sequence identities in most cases and >95% sequence identities in all cases. These clones clustered very closely to the *Methylocystis* sp. used as the reference in the phylogenetic tree. No type I methanotrophs or AOB were found.

TABLE 2. Diversity of *pmoA* and *amoA* functional genes obtained in archives generated by primer set A189-A682 for free-living (9-682 and W9-682) and attached (10-682 and W10-682) microbial communities within the ESRPA

Group or species (relevant gene)	% Archive composition			
	Free-living		Attached	
	9-682	W9-682	10-682	W10-682
Group I uncultured (*pmoA*)	35	2	0	0
Group II uncultured (*amoA*)	10	1	89	96
Group III uncultured (*pmoA*)	1	1	0	0
Group IV uncultured (*amoA*)	14	6	0	1
Nitrosospira sp. (*amoA*)	17	84	0	0
Methylocystis sp. (*pmoA*)	23	6	11	1
Nitrosomonas sp. (*amoA*)	0	0	0	1
Total	100	100	100	99

Archives produced from primer set mmoXA-mmoXB resulted in a total of 143 clones. Of these clones, 12 RFLP patterns were produced from 9-mmoX, and 7 patterns were produced from W9-mmoX. Overall, both archives resulted in similar compositions, with *Methylocystis*-like oxygenase sequences resulting in 95 and 100% of the total clones for 9-mmoX and W9-mmoX, respectively (Table 4).

Phylogenetic analysis of the two archives revealed three distinct clusters, two closely related to *Methylocystis* sp. and the other to *Methylomonas* sp. Type I methanotrophs found only in 9-mmoX (groups 5, 8, and 11) were related to *Methylomonas* sp. LW15 (>91% sequence identity). These three groups collectively account for 5% of the overall archive. Type II methanotrophs represented in 9-mmoX include 87% *Methylocystis* sp. IMET 10486 (groups 1, 2, 4, and 14), 7% *Methylocystis* sp. 51 (groups 3, 13, 15, and 16), and 1% *Methylocystis* sp. SE12 (group 12). Type II methanotrophs represented in W9-mmoX include 68% *Methylocystis* sp. SE12 (groups 1, 5 and 7), 30% *Methylocystis* sp. IMET 10486 (groups 6, 10, and 12), and 2% *Methylocystis* sp. 51 (group 14) (Fig. 4).

DISCUSSION

Sampling. It has been reported that most bacteria in an aquifer are attached to the aquifer solids, and only a small fraction exist as free-living cells (18, 29). The sampling of attached biomass from aquifer boreholes is usually accomplished through the collection of core samples. This method can be expensive, as well as challenging, depending on the aquifer to be sampled. In this study, we chose to use porous columns filled with material simulating the existing fractured basalt of the aquifer and incubated in a borehole in the aquifer.

TABLE 3. Diversity of the *pmoA* functional gene obtained in archives generated by primer set A189-mb661 for free-living (9-661 and W9-661) and attached (10-661 and W10-661) microbial communities within the ESRPA

Species	% Archive composition			
	Free-living		Attached	
	9-661	W9-661	10-661	W10-661
Methylocystis sp.	97	77	100	100
Methylomonas sp.	1	17	0	0
Methylobacter sp.	2	6	0	0
Total	100	100	100	100

The use of artificial substrata incubated in wells to sample attached communities close to the study site described herein has been described recently by Lehman et al. (29). The study by Lehman et al. found that the use of dialysis chambers containing basalt (similar to the substrate columns used here) resulted in the recovery of a greater range of microbial diversity compared to that from authentic core samples. Therefore, boreholes may not be ideal for collecting representative samples of microbes colonizing aquifer solids. Free-living communities sampled by filtration of groundwater are also subject to biases. Due to the large volume of ground water used in this study (13,300 liters), it is likely that many different microbial niches were sampled, not all necessarily corresponding to the free-living community. Sediment particles were present in the filtrate, and bacteria colonizing these sediment layers would have been extracted along with free-living cells. It has been observed that bacteria display a dynamic equilibrium between attached and free-living phases (51). These bacteria form biofilms as part of their normal life cycle that culminates with the release of free-living cells capable of colonizing new habitats. Thus, it is difficult to draw a distinct line between attached and free-living communities. Though the communities may differ in overall composition, it is not surprising that we found no distinct disparity between the attached and free-living communities sampled. It is likely that free-living cells present in the free-living samples are also represented, to some extent, in the attached samples.

Whole-genome amplification. WGA technology is based on the unbiased amplification of limited DNA samples (21, 26). This technology was able to amplify our starting DNA to produce a product equal in size to the starting template when visualized by agarose gel electrophoresis (data not shown). PCR of WGA products also resulted in amplicons equal in size to unamplified samples. The differences noted between archives were most likely due not to the WGA process but rather to bias introduced by the PCR. Since multiple PCRs were not pooled, bias generated through a single PCR amplification may have skewed the resulting archives.

Functional gene library production and phylogenetic analysis. The primer sets A189-A682, A189-mb661, and mmoXA-mmoXB were used successfully as functional gene probes in the assessment of MOB and AOB in most of the communities tested. It is unclear why no amplification was seen from the attached and WGA attached DNA with the mmoXA-mmoXB primer set. Sequences were amplified from both attached and WGA attached communities by using the primer sets A189-A682 and A189/mb661, and these were closely related to sequences of the sMMO-producing MOB *Methylocystis*.

An explanation for our observations may lie in the distinct difference in the species of *Methylocystis* found in free-living versus attached communities. None of the sequences amplified from the free-living samples with A189-A682 and A189-mb661 was found in the mmoXA-mmoXB archives and vice versa. It appears that the three primer sets used exhibit specificity towards different *Methylocystis* spp. This fact supports the conclusion that the methanotroph species targeted by mmoXA-

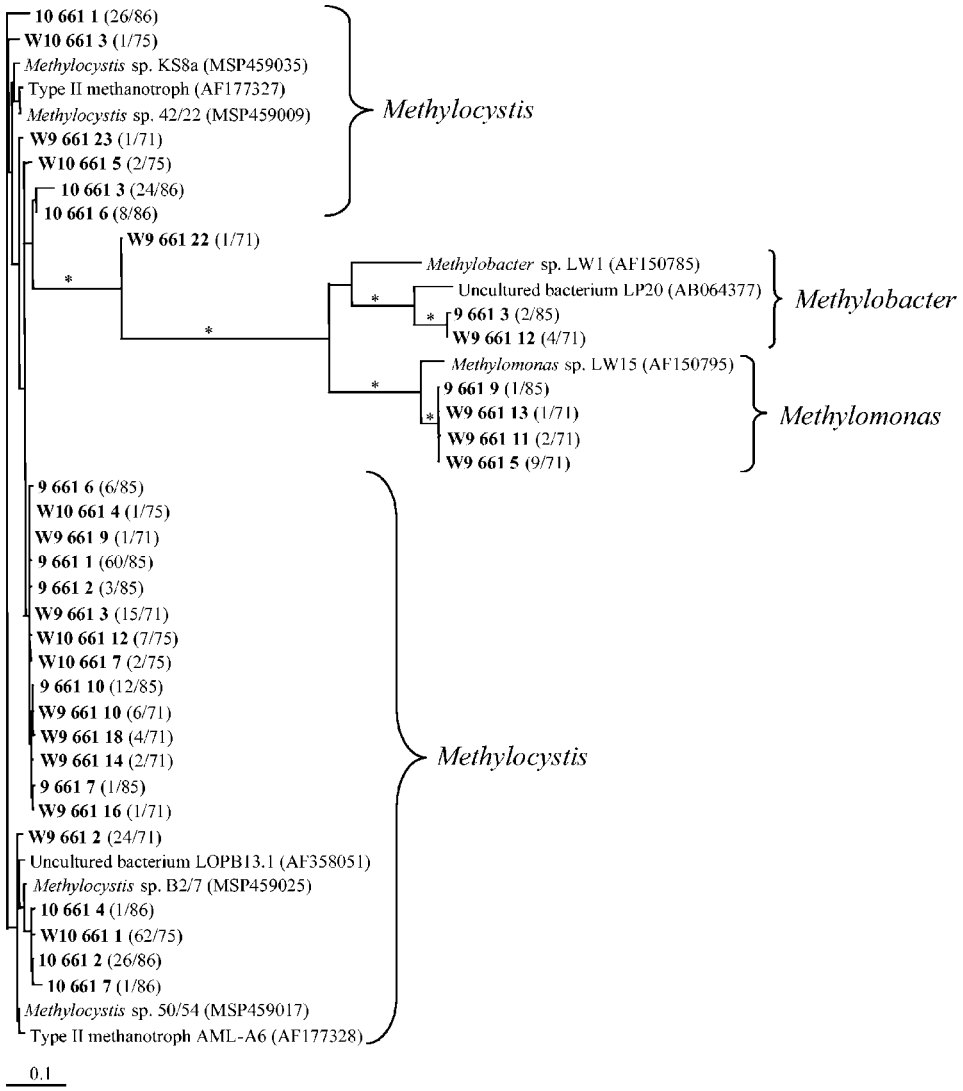

FIG. 3. Phylogenetic relationships among partial *pmoA* gene sequences produced from archives of unamplified and WGA free-living and attached communities. Gene fragments were obtained by using the primer set A189-mb661 (550 base positions were considered). The bar represents 10% sequence divergence as determined by the length of the horizontal lines connecting any two species. The phylogenetic tree was constructed by using PAUP based on maximum likelihood analysis using the HKY+G correction model. Bootstrap values greater than 75% are represented by asterisks. Unique clones obtained from the archives are indicated by boldface type. Clones are named according to the manner described in the legend to Fig. 2.

mmoXB were not present in the attached samples in sufficient numbers to be detected.

The number of available *mmoX* sequences on which to base primer design is small. The primer set *mmoX* f882-*mmoX* r1403, widely used for *mmoX* amplification, is based on sMMO gene clusters from only two methanotrophs (34). The mmoXA-mmoXB primer set, which was designed 5 years after the introduction of *mmoX* f882-*mmoX* r1403, is based on only six known full *mmoX* sequences (6). Deduced amino acid alignments show a high percentage of conservation from sequences amplified by Auman et al. (6) with these primers. This result could be due to the fact that the primer set is specific for only highly unique genes. Another study done by Auman and Lidstrom failed to amplify any type II *mmoX* genes from their samples, even though type II-specific 16S rRNA PCR showed these bacteria to be present (5). Since we observed a similar result in a different environment, it may be that genes found to be conserved in cultivable organisms may not be sufficiently similar to genes in the environment, as noted by Hanson and

TABLE 4. Diversity of the *mmoX* functional gene obtained in archives generated for free-living communities within the ESRPA

Species	% Archive composition	
	9-mmoX	W9-mmoX
Methylocystis sp.	95	100
Methylomonas sp.	5	0
Total	100	100

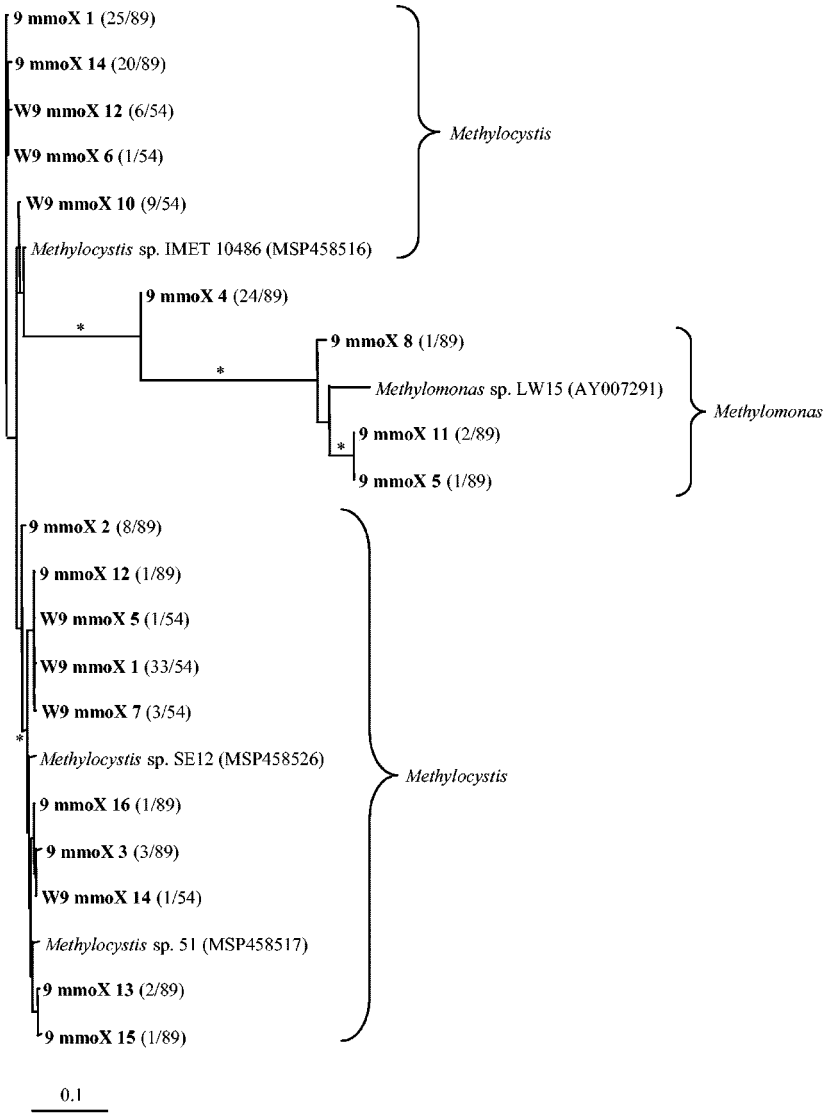

FIG. 4. Phylogenetic relationships among partial *mmoX* gene sequences produced from archives of unamplified and WGA free-living communities. Gene fragments were obtained by using the primer set mmoXA-mmoXB (1,271 base positions were considered). The bar represents 10% sequence divergence as determined by the length of the horizontal lines connecting any two species. The phylogenetic tree was constructed by using PAUP based on maximum likelihood analysis using the TrN+G correction model. Bootstrap values greater than 75% are represented by asterisks. Unique clones obtained from the archives are indicated by boldface type. Clones are named according to the manner described in the legend to Fig. 2.

Hanson (17), and therefore must be considered when interpreting data generated by such primer sets. To date, no single primer set has been demonstrated to amplify *mmoX* genes from all sMMO-producing bacteria.

As expected, the primer set A189-A682 amplified sequences corresponding to both *pmoA* and *amoA* from all four communities. The abundance of *amoA*-related sequences compared to *pmoA* found in all four archives except 9-682 suggests that nitrifying bacteria might dominate the samples. Of particular interest is the presence of the large subset of clones that cluster with the uncultured bacterium M84-P36. These clones make up a total of 35% of the archive and are only distantly related to other uncharacterized *pmoA* clones (20), suggesting the presence of a potentially significant novel group of *pmoA*-carrying methanotrophs. The proportion of sequences in clone libraries, however, cannot be used to accurately predict the relative abundance of the AOB or the novel *pmoA* group in the natural communities, due to biases that are inherent in PCR amplification and the number of sequences analyzed. Such proportions, however, are useful for comparisons of archive data within a given data set.

Characterization of the communities with *pmoA*-specific primers (A189-mb661) suggested that bacteria related to the type II methanotroph *Methylocystis* dominate free-living as well as attached methanotroph communities. Newby et al. obtained similar results in their study of the methanotroph communities from the ESRPA (40). However, their study sampled a much smaller volume of groundwater and characterized only free-

living cells. The free-living communities (amplified and unamplified) also showed the presence of type I methanotrophs related to *Methylomonas* sp. LW15, a known sMMO-containing bacterium (6). The small percentage of clones from archives 9-661 and W9-661 found to be related to the genus *Methylobacter*, which has not been shown to produce sMMO, suggests that a broad range of niches may have been sampled through the collection of free-living cells. Again, the Newby et al. study documented the presence of these type I methanotrophs by using a different molecular approach (40). A direct comparison of the gene fragments could not be made, due to differences in the targeted areas of the genes in question.

It has been proposed that growth of type II methanotrophs is favored over growth of type I methanotrophs under low-oxygen, high-methane conditions (17). The analysis of groundwater sampled from well ANP-9 showed a significant concentration of methane but also revealed an oxic environment (40). It is expected that an environment such as this should favor the growth of type I methanotrophs. The presence of copper, however, is another key factor responsible for regulating the growth of type I and type II methanotrophs. Copper is necessary for pMMO activity and plays a key role in the regulation of pMMO and sMMO expression (30). Under low-copper conditions, bacteria with the ability to produce sMMO are able to outcompete those able to produce only pMMO (17, 39). This mechanism is due to the fact that the active pMMO enzyme contains approximately 12 to 15 copper atoms mol^{-1} (41). While the exact nature of these Cu^{2+} ions in the function of pMMO is not clear, they are postulated to play a role in the active site of the protein (41, 44). Samples of groundwater from ANP-9 show that the copper levels are below the detection limit of 1.0 $\mu g\ ml^{-1}$ (23). Taking into account the conditions present in the wells sampled (the presence of dissolved methane and low copper concentrations), the community composition observed in this study is not surprising.

Our results, obtained through the molecular assessment of oxygenase genes, demonstrate that in both the attached and free-living communities, the majority of the methanotrophic populations present show a high degree of similarity to other type II methanotrophs. The natural attenuation of TCE observed at the TAN site may be due in part to the sMMO-producing communities described here. However, we did not attempt to enumerate methanotrophs from either free-living or attached communities. Because of this fact, the organisms represented in this study constitute an undetermined percentage of the overall bacterial population. Recently, Lehman et al. found that very few MOB or AOB could be recovered from authentic core or groundwater samples taken from within the TCE plume (29). The results from their study may reflect the fact that the enumerations were based on most-probable number assays performed with medium selective for type I methanotrophs and would not have detected the type II populations described here.

The presence of sMMO-producing bacteria alone does not account for the rate of TCE natural attenuation observed at the TAN site. It was our goal to provide evidence that the in situ bacterial populations within the pristine aquifer have the potential for TCE cometabolism. Future work to quantify type II methanotrophs specifically and monitor the production of sMMO is necessary in order to correlate the presence of these bacteria with the TCE attenuation rate observed at the TAN site within the contaminated plume.

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