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Fluorescence In Situ Hybridization-Flow Cytometry-Cell Sorting-Based Method for Separation and Enrichment of Type I and Type II Methanotroph Populations

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A fluorescence in situ hybridization-flow cytometry (FISH/FC)-based method was optimized using artificial mixtures of pure cultures of methanotrophic bacteria. Traditional oligonucleotide probes targeting 16S rRNAs of type I (MG84/705 probe) and type II (MA450 probe) methanotrophs were labeled with fluorescein or Alexa fluor and used for FISH, followed by fluorescence-activated FC analysis and cell sorting (FACS). The method resulted in efficient separation of target cells (type I or type II methanotrophs) from the artificial mixtures. The method was then applied for detection and enrichment of type I and type II methanotroph populations from a natural sample, Lake Washington sediment. Cells were extracted from the sediment, fixed, and subjected to FISH/FC/FACS. The resulting subpopulations were analyzed by reverse transcriptase PCR surveys of 16S rRNA, *pmoA* (encoding a subunit of particulate methane monooxygenase), and *fae* (encoding formaldehyde-activating enzyme) genes. The functional gene analysis indicated specific separation of the type I and type II methanotroph populations. 16S rRNA gene analysis revealed that type I methanotrophs comprised 59% of the subpopulation separated using the type I-specific probe and that type II methanotrophs comprised 47.5% of the subpopulation separated using the type II-specific probe. Our data indicate that the FISH/FC/FACS protocol described can provide significant enrichment of microbial populations of interest from complex natural communities and that these can be used for genetic tests. We further tested the possibility of direct whole-genome amplification (WGA) from limited numbers of sorted cells, using artificial mixtures of microbes whose genome sequences are known. We demonstrated that efficient WGA can be achieved using 10^4 or more cells separated by 16S rRNA-specific FISH/FC/FACS, while fewer cells resulted in less specific WGA.

Methanotrophs are a physiologically specialized group of bacteria capable of utilizing methane as a sole source of carbon and energy, and they have been recognized as major players in local and global elemental cycling in aerobic environments (20). Methanotrophs have been detected in a variety of environments, and in some they represent significant fractions of total microbial communities (9, 40). Estimates of methanotroph abundance in natural samples are based on a number of complementary techniques, such as determination of methane oxidation rates (3, 9, 29, 40), determination of the fatty acid composition of the total microbial population (9, 29, 40), PCR-based (2, 14, 15, 19, 25, 26, 28), quantitative PCR-based (5, 27, 29), or reverse transcriptase PCR (RT-PCR)-based (24, 32) surveys, and direct counting using microscopy combined with fluorescence in situ hybridization (FISH) (6, 8, 10). The most widely used PCR primers for detecting type I and type II methanotrophic bacteria target rRNA genes (15, 19) or functional genes encoding particulate methane monooxygenase (*pmoA*) (14), methanol dehydrogenase (*mxaF*) (14), formaldehyde-activating enzyme (*fae*) (25), or the D subunit of the formyltransferase/hydrolase complex (*fhcD*) (25), and large databases of these genes have been compiled from a variety of environments (5, 11, 14, 16, 19, 24, 26, 28, 31, 33, 39, 40). The most widely used FISH probes target rRNA (15, 19); however, several protocols have been developed recently for mRNA-targeted (*pmoA* or *mxaF*) FISH detection (12, 34).

In recent years, applying fluorescence-activated flow cytometry (FC) to the goals of environmental microbiology has received much attention (1, 38, 42), based on the promise of fast and accurate detection of small particles, potentially translated into qualitative and quantitative detection of microbial populations in natural environments. So far, FC has been applied successfully to studies of bacterial community structure, composition, and activity in aquatic ecosystems (7, 18). The approach has also been tested for analyzing soil and sediment microbial populations as well as for viral detection (13). However, the recovery and separation of microbial cells from soil or sediment particles still remain challenging tasks (13, 35). One of the most intriguing potential applications of FC is the possibility of direct extraction of specific subpopulations from environmental samples, omitting the cultivation step, followed by genetic or even genomic characterization. The aim of the present study was to establish a protocol for FISH-FC analysis and separation of methanotroph populations, allowing for subsequent genetic and genomic analyses. We first tested the feasibility of this approach and optimized the protocol by separating specific subpopulations from artificial mixes of cultured methanotrophs. We then applied the protocol to separate

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methanotroph populations from a complex microbial community inhabiting the top layer of Lake Washington sediment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Methylobacterium extorquens* AM1, *Methylobacillus flagellatus* KT, *Methylosinus trichosporium* OB3b, *Methylosarcina lacus* LW14, *Methylomonas* sp. strain LW13, *Methylosinus* sp. strain LW2, and *Methylococcus capsulatus* Bath were employed in the optimization experiments. *M. extorquens* and *M. flagellatus* were grown in a previously described minimal medium (21) supplemented with 100 mM methanol. Methanotroph cultures were grown in NMS medium (44). For optimization of *mmoX*-targeted FISH, *Methylomonas* LW13 cells were grown with or without a Cu supplement, as previously described (2).

Cell extraction. Lake Washington sediment samples were collected as described previously (26). Cells were extracted from 2 to 5 ml of the sediment. The following three methods for cell extraction were tested: (i) shaking for 20 min at 200 rpm at room temperature, (ii) vortexing using an MBB-8 apparatus (Biospec Products) for 2 min with 0.1-mm zirconia-silica beads (Biospec Products) at 5°C, and (iii) homogenization using a PRO200 homogenizer 115V (PROScientific) for 5 min at position 3 on ice. Samples were then diluted 20 to 50 times with filter-sterilized Lake Washington water (LWW) supplemented with the following: 0.1 M NaCl, 1% sucrose, and 0.1% of either Triton X or Tween 80. To eliminate sediment particles, the blended material was either filtered through 5-μm NY20 filters (Millipore) or centrifuged at 750 × *g* for 3 min. Cells from the resulting filtrates or supernatants were pelleted by centrifugation at 8,000 × *g* for 15 min and resuspended in either LWW or phosphate-buffered saline (PBS; 0.1 M Na_2HPO_4, 20 mM KH_2PO_4, 137 mM NaCl, 27 mM KCl).

The efficiency of recovery was tested by CFU counts, microscopic observations, and estimations of DNA yields. To ensure optimal CFU counts, cells were plated onto the following media prepared with LWW: 0.1× nutrient agar (BD Diagnostics), 0.1× Luria-Bertani (LB) agar (BD Diagnostics), 0.1× TGY (0.5% tryptone, 0.1% yeast extract, 0.1% glucose) agar, or MMB agar (http://www.dsmz.de/media/med628.htm). To specifically address the methanotroph extraction efficiency, cells were plated on LWW agar or on 0.1× NMS agar, and plates were incubated either under a methane-air (50:50) atmosphere or under an air atmosphere to subtract colonies growing on agar alone. Plates were incubated aerobically at 16°C, room temperature (approximately 24°C), or 30°C for up to 1 month. The maximal CFU counts were obtained on 0.1× LB agar. DNAs were extracted using an UltraClean Soil DNA kit (MO BIO Laboratories, Inc.), and their concentrations were measured spectrophotometrically (37).

Probes and conditions for FISH. The following probes were used in this study: 16S rRNA-targeted probes MA450, MG84, and MG705 (15) and a polynucleotide *mmoX*-targeted probe. The polynucleotide probe was PCR amplified using the chromosome of *Methylomonas* sp. strain LW13 as a template and PCR primers described earlier (2) and either labeled with Alexa fluor 488-dUTP included in the PCR mix or labeled using a Fluorescein-High Prime kit (Roche). The PCR fragments were purified by ethanol precipitation and digested by HincII, HinfI, HpaII, and AluI, resulting in 30- to 60-bp fragments.

Cells of pure cultures (10^8 to 10^9) or cells extracted from the sediment (10^8 CFU) were harvested by centrifugation, resuspended in 1 ml of PBS, and fixed with 4% paraformaldehyde (1:3 [vol/vol]) for 8 to 12 h on ice. The fixed cells were washed with 1 ml of PBS twice and resuspended in 200 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.5, 20% formamide, 0.1% sodium dodecyl sulfate). Each sample was divided amongst three tubes, and the tubes were prewarmed at 50 to 58°C. One of the tubes was used as a control, the second was mixed with a specific fluorescent probe (5 ng/μl of oligonucleotide probe or 50 ng/μl of polynucleotide probe), and the third was mixed with a nonspecific probe. Cell suspensions were incubated at an appropriate hybridization temperature (50°C for the MA450 probe, 58°C for the MG84/705 pair, and 56°C for the polynucleotide probe) for 8 to 12 h. After hybridization, cells were pelleted by centrifugation (8,000 × *g* for 3 min), separated from the supernatant, and incubated in 500 μl of hybridization buffer for an additional 20 min, followed by incubation in 500 μl of wash buffer (20 mM Tris-HCl, pH 8.0, 0.9 mM NaCl, 0.1% sodium dodecyl sulfate) for 20 min. Cells were collected by centrifugation, resuspended in 1 to 3 ml of cold PBS, homogenized for 15 to 30 s, and used for microscopic observations, FC analysis, and cell sorting. Cells were observed using an epifluorescence microscope (PASCAL LSM 5) and a ×100 oil immersion objective, and data were analyzed using Zeiss LSM Image software.

Flow cytometry and cell sorting. A BD LSR benchtop flow cytometer (Becton Dickinson) was used to measure the forward angle light scattering, right angle light scattering, and fluorescence of microbial cells. These parameters were acquired as pulse height signals for 10,000 events at a rate of 600 to 3,000 events per second. Subsequent analysis and cell sorting were performed using a BD FACS Vantage SE instrument. The instrument tubing was sterilized using, sequentially, 10% bleach, 3% hydrogen peroxide, 70% ethanol, and sterile PBS. Data analysis and graphics were acquired using the WinMDI 2.1 software package (http://facs.scripps.edu/software.html). Cells were collected at 1,000 events per 0.25-ml PCR tube, with a total of eight tubes per experiment.

Diagnostic RT-PCR. RT-PCR amplifications were carried out directly with sorted cells, using a one step RT-PCR kit (QIAGEN). *pmoA* was amplified using the primer set A189 (5′-GGNGACTGGGACTTCTGG-3′)/A682 (5′-GAASG CNGAGAAGAASGC-3′) (23). *mmoX* was amplified using the primer set mmoXA (5′-ACCAAGGARCARTTCAAG-3′)/mmoXD (5′-CCGATCCAGA TDCCRCCCCA-3′) (3). *fae* was amplified in two steps, as described previously (25), using the primer sets fae1f (5′-GTCGGCGACGGCAAYGARGTCG-3′)/ fae1r (5′-GTAGTTGWANTYCTGGATCTT-3′) and fae2f (5′-GCACACATC GACCTSATCATSGG-3′)/fae2r (5′-CCAGTGRATGAAVACGCCRAC-3′). Eubacterial 16S rRNA genes were amplified using the EUB27f (5′-AGAGTTT GATCMTGGCTCAG-3′) and EUB1492r (5′-TACGGYTACCTTGTTACGA CTT-3′) primers. The resulting PCR fragments were cloned into the pCR2.1 vector using a TOPO TA kit (Invitrogen). Plasmids were purified using a QIAprep spin miniprep kit (QIAGEN). Sequencing reactions were performed using a BlueDye3.1 kit. Reaction analyses were performed by the Department of Biochemistry sequencing facility at the University of Washington, using an ABI 3700 high-throughput capillary DNA analyzer.

Whole-genome amplification. Cells of *M. capsulatus* were labeled with the MG84/705 probe set and sorted from a mixed culture as described above. Pools containing desired numbers of cells were collected, and cells were pelleted by centrifugation at 10,000 × *g* for 10 min. DNA was amplified using a GenomePhi whole-genome amplification kit (GE Healthcare) according to the manufacturer's instructions. The amplified DNA was digested with BamHI (NEB) and randomly cloned into the pUC19 vector (NEB). Clone libraries were generated using *Escherichia coli* JM109 as a host, up to 50 clones from each library were verified by the presence of inserts, and these were sequenced as described above.

Phylogenetic analysis. Sequences were aligned using the Clustal W program (41). The Phylip program package (17) was used for phylogenetic analysis.

Nucleotide sequence accession numbers. The sequences obtained in this work were deposited with GenBank under the following accession numbers: DQ367733 to DQ367735 (16S rRNA genes), DQ367737 to DQ367742 (*pmoA* genes), and DQ367743 to DQ367746 (*fae* genes).

RESULTS AND DISCUSSION

FISH-based cell sorting with artificial mixtures of methylotrophic bacteria. Previously designed probes for 16S rRNA-targeted methanotroph detection and standard FISH protocols (15) were first tested with laboratory strains of the methanotrophs *M. capsulatus*, *M. lacus*, *Methylomonas* sp. strain LW13 (type I methanotrophs), *M. trichosporium*, and *Methylosinus* sp. strain LW2 (type II methanotrophs). Microscopic observations and FC data demonstrated that cells of the first three strains were successfully labeled with the MG84/MG705 probe pair specific for type I methanotrophs (Fig. 1a to c) and that cells of the last two strains were successfully labeled with the MA450 probe specific for the *Methylosinus*/*Methylocystis* group of type II methanotrophs (data not shown). In addition, we tested a polynucleotide probe for detection of a functional gene mRNA translated from the *mmoX* gene previously detected in *Methylomonas* sp. strain LW13. This probe only resulted in a good signal with cells grown in the absence of copper, in accordance with the known expression pattern for the soluble methane monooxygenase (30). Thus, we demonstrated that not only 16S rRNA but also mRNA can be detected by FISH and FC (Fig. 1d to f).

We then tested the applicability of these probes to the specific separation of cells of interest from mixed bacterial populations. We produced artificial mixtures of fixed cells of four cultures, *M. capsulatus*, *M. trichosporium*, *M. extorquens*, and *M. flagellatus*, in which target cells were present in different

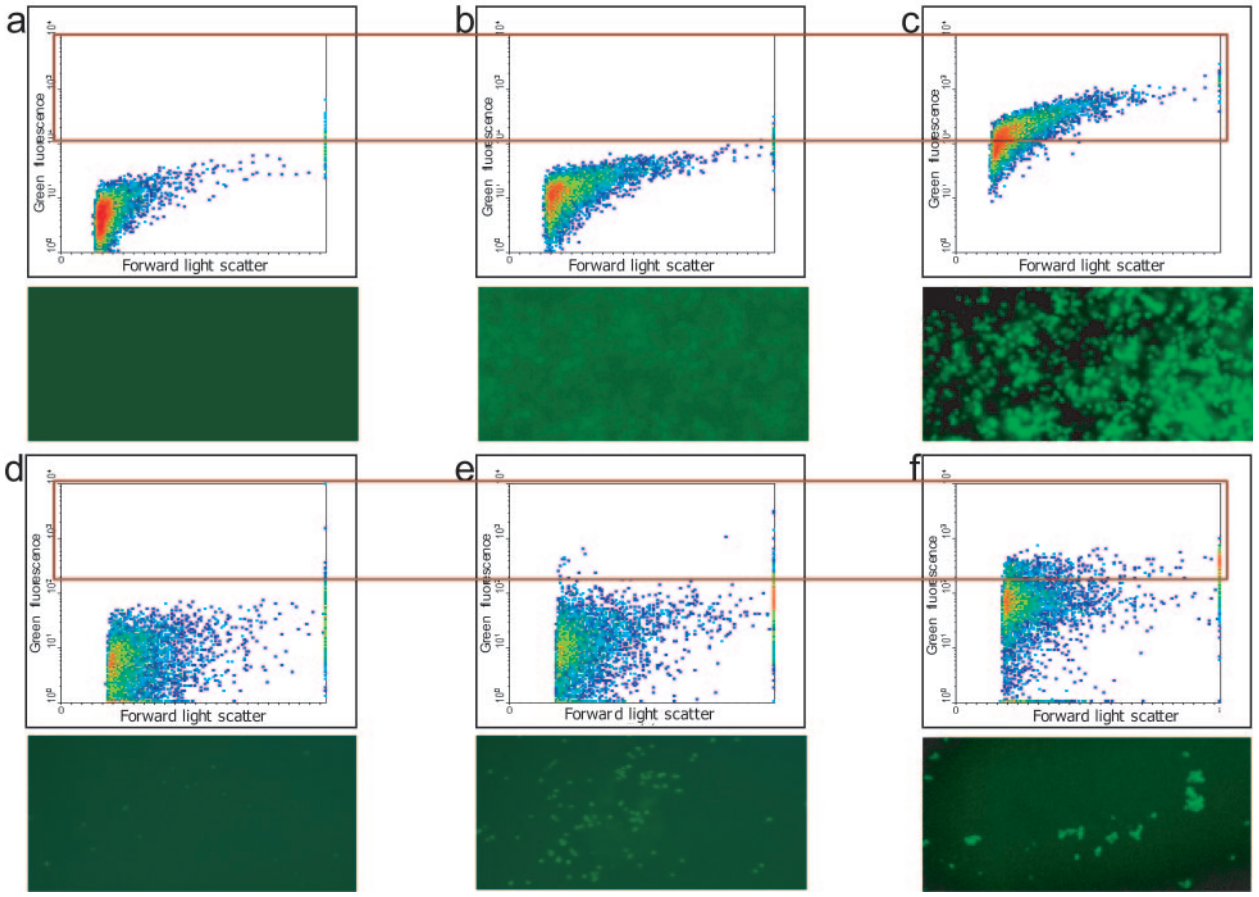

FIG. 1. FC (top) and fluorescence microscopy (bottom) analyses of fixed cells of *M. capsulatus* (a to c) and *Methylomonas* sp. strain LW13 (d to f). (a and d) No probe; (b) nonspecific probe (NON-EUB338); (c) type I methanotroph-specific oligonucleotide probe (MG84/705); (e and f) *Methylomonas* sp. strain LW13 *mmoX*-specific polynucleotide probe; (e) cells grown in the presence of Cu; (f) cells grown without Cu. Each plot contains 10,000 events. The boxed part of the plot indicates the events gated for sorting.

proportions (5 to 25% of total cells). These were hybridized with either the MG84/705 probe set (targeting *M. capsulatus*) or the MA450 probe (targeting *M. trichosporium*), followed by FC and cell sorting. Cells displaying high fluorescence were collected and analyzed by RT-PCR amplification of 16S rRNA and *pmoA* genes, followed by cloning and sequencing. A total of 25 randomly chosen clones from each library were analyzed. All clones in both 16S rRNA gene and *pmoA* libraries originating from the MG84/705-based sort belonged to the target organism, *M. capsulatus*. Similarly, all clones originating from the MA450-based sort belonged to the target organism, *M. trichosporium*. We performed similar experiments with a bacterial mixture including *Methylomonas* sp. strain LW13, *M. extorquens*, and *M. flagellatus*, using with the *mmoX*-targeted polynucleotide probe, and obtained similar results: DNAs amplified from the sorted cells belonged to *Methylomonas* sp. strain LW13. These results demonstrated that FISH-FC-based separation of cells from simple bacterial mixtures allowed discrimination with high efficiency between target and nontarget cells, and thus this protocol could be applied to enriching cells of interest from more complex natural populations.

Recovery of bacterial cells from Lake Washington sediment. Effective extraction of bacterial cells from sediment is the first essential step for correct assessment of natural populations, and it remains a challenging task. We tested several types of extraction procedures for their efficiency of recovering bacterial cells from Lake Washington sediment. The shaking protocol produced the lowest cell counts ($6.2 \times 10^4 \pm 1.8 \times 10^4$ CFU ml^{-1} plated onto 0.1× LB agar). The MBB-8 vortexing protocol resulted in higher cell counts ($4.5 \times 10^6 \pm 1.2 \times 10^6$ CFU ml^{-1}), but the best results were achieved by applying homogenization using a PRO200 homogenizer ($2.8 \times 10^8 \pm 0.2 \times 10^8$ CFU ml^{-1}). The addition of 0.1 M NaCl and dilution of treated samples (1:20 or 1:50) in LWW supplemented with 1% sucrose and 0.1% Triton X-100 further increased the extraction efficiency by 10 to 15%. The number of CFU in a methane atmosphere was 6×10^5 colonies after this extraction protocol, compared to 8×10^2 CFU when sediment dilutions were plated without extraction. Therefore, a protocol involving the homogenization of lake samples followed by dilution was applied for all cell sample preparations and resulted in extraction of approximately 3×10^8 cells from 1 ml of lake sediment, on average. Since filtration resulted in a significant decrease in cell number (data not shown), it was replaced by two steps of centrifugation, first at $750 \times g$ for 3 min and then at $5{,}000 \times g$ for 15 min. The efficiencies of these steps were followed by

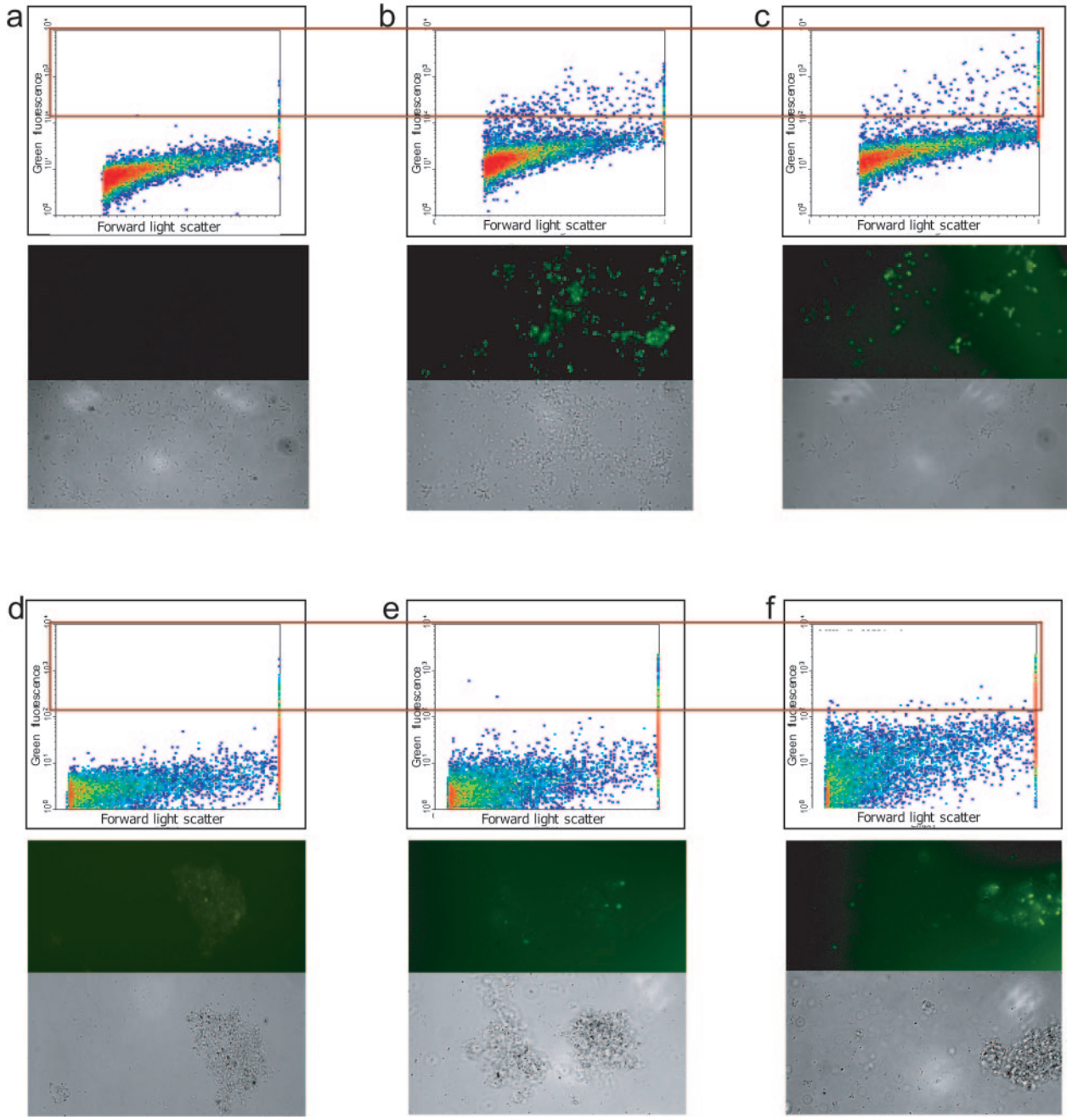

FIG. 2. FC (top), fluorescence microscopy (middle), and phase-contrast microscopy (bottom) analyses of fixed cells of an artificial mixture of *M. capsulatus*, *M. trichosporium*, *M. extorquens*, and *M. flagellatus* (a to c) and of cells extracted from Lake Washington sediment (d to f). (a and d) No probe; (b, c, and f) type I methanotroph-specific oligonucleotide probe (MG84/705); (e) type II methanotroph-specific probe (MA450). For panels b and c, 10% and 5% of target cells (*M. capsulatus*), respectively, were included in the mixture. Each plot contains 10,000 events. The boxed part of the plot indicates the events gated for sorting.

CFU counts and estimations of DNA recovery. The first centrifugation step resulted in the collection of sediment debris and eukaryotic organisms (algae, protists, etc.), with a small number of bacterial cells and DNA (6×10^3 CFU and 30 μg DNA per g of sample), while the second centrifugation step resulted in relatively pure bacterial cell samples (2.8×10^8 CFU and 200 μg DNA per g of sample). Flow cytometric analysis of the samples showed that 98% of the detected events stained with the DNA stain 4′,6′-diamidino-2-phenylindole (DAPI).

Separation and enrichment of type I and type II methanotrophs from Lake Washington sediment. Cells extracted from the lake sediment as described above were fixed and hybridized with the group-specific probe set MG84/705 (type I methanotrophs) or MA450 (type II methanotrophs), as done for pure culture mixtures. Hybridization with the MG84/705 probe

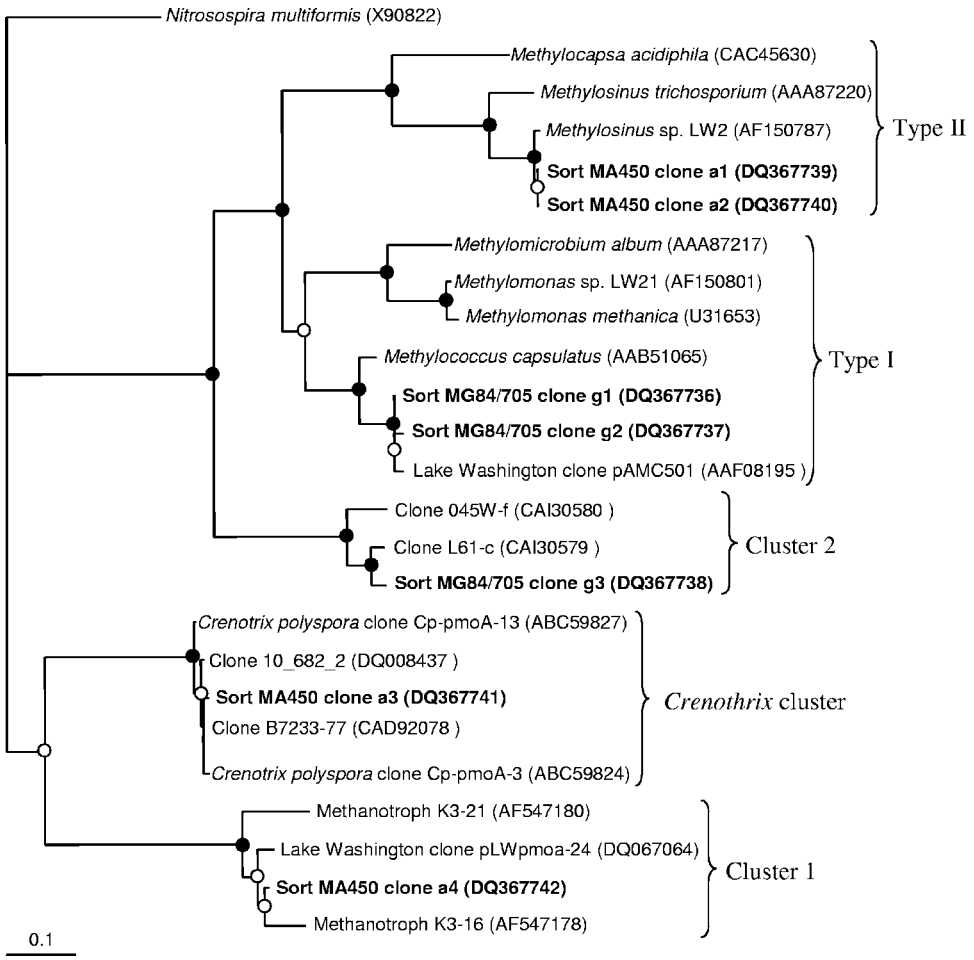

FIG. 3. Phylogenetic tree showing relationships of the translated PmoA sequences (160 positions) uncovered in this work to the sequences from known type I and type II methanotrophs and to the sequences of uncultivated organisms. The *Nitrosospira multiformis* sequence was used as an outgroup. A distance algorithm (Protdist) was employed, with 1,000 bootstrap analyses. Closed circles indicate bootstrap support of over 90%, and open circles indicate bootstrap support of over 55%.

pair resulted in a larger number of positive (bright) cells than the number of cells hybridizing with the MA450 probe (Fig. 2d to f). Similarly, cell counts deduced from FC analysis correlated with previous observations on the dominant presence of type I methanotrophs (4.7% ± 1.3% of total events) at the site compared to type II methanotrophs (1.2% ± 0.4% of total events) (9). Cells were sorted as described above. To increase the efficiency of cell separation and to decrease the background, only cells displaying the highest fluorescence signals were gated (2% of cells hybridized with the MG84/705 probe and 0.2% of cells hybridized with the MA450 probe) and collected (Fig. 2). The enrichment of the target cells was tested via RT-PCR amplification of *pmoA*, *fae*, and 16S rRNA gene fragments. For each gene, a clone library was constructed, and 25 to 50 randomly chosen clones were sequenced and analyzed.

Three types of *pmoA* sequences were identified in the library obtained from sorted cells hybridized with the MG84/705 (type I) probes. Two of these (clones g1 and g2 [37 and 42% of total clones, respectively]) were closely related to each other and to the sequences from type I methanotrophic bacteria, while the third (g3 [21% of the total clones]) was closely related to a group of deeply branching sequences not represented so far by any cultivated organisms (Fig. 3). These sequences were recently ascribed to cluster 2 of *pmoA* genes (28, 36). Two types of *fae* sequences were amplified from the same cells and were equally represented in the library, and these were closely related to each other and to type I methanotroph sequences (Fig. 4). Eighty-six percent of 16S rRNA sequences amplified from the same cells were related to gammaproteobacterial sequences, with 59% being related to the sequences of type I methanotrophs (Fig. 5) and 27% being related to the sequences of *Pseudomonas* sp. The remaining 14% of sequences were related to the sequences of gram-positive bacteria.

Four types of *pmoA* sequences were identified in the library obtained from sorted cells hybridized with the MA450 (type II) probe. Clones a1 and a2 (11 and 9%, respectively, of all sequences) were closely related to each other and to *pmoA* genes of the *Methylosinus*/*Methylocystis* group. Clone a4 (50% of total clones) was related to the group of deeply diverging type II *pmoA* genes previously described for tundra soil isolates related to *Methylosinus*/*Methylocystis* (Fig. 3) (33) and recently ascribed to cluster 1 of *pmoA* genes (28, 36). Closely related

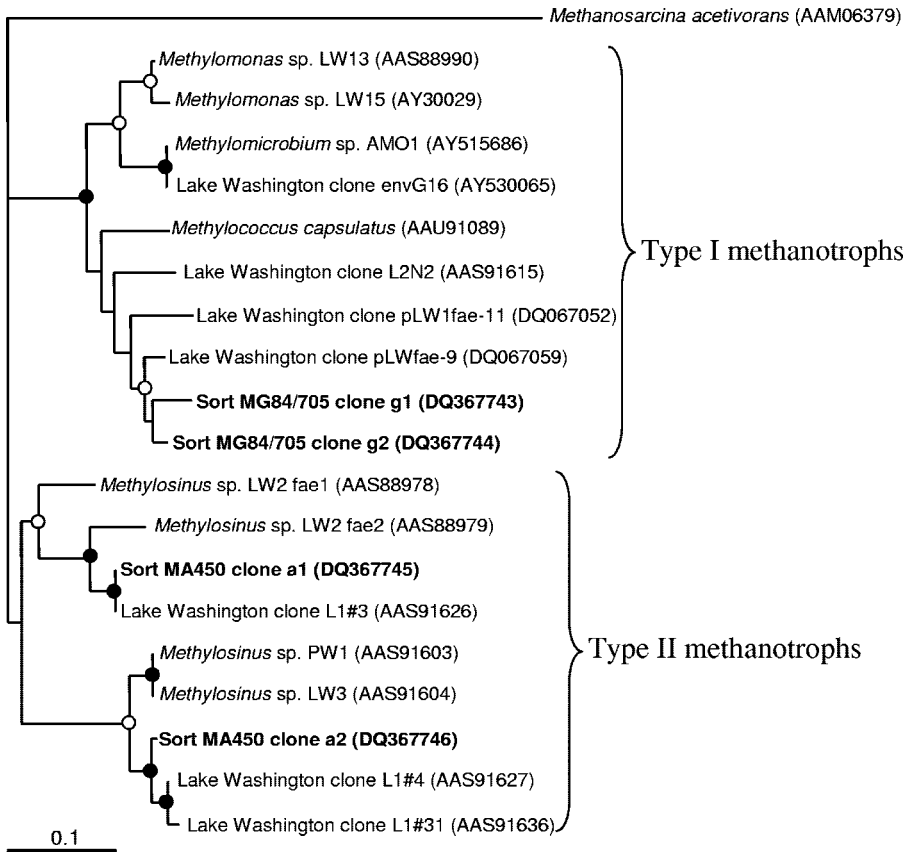

FIG. 4. Phylogenetic tree showing relationships of the translated Fae sequences (98 positions) uncovered in this work to the sequences from known type I and type II methanotrophs and to the sequences of uncultivated organisms. The *Methanosarcina acetivorans* sequence was used as an outgroup. A distance algorithm (Protdist) was employed, with 1,000 bootstrap analyses. Closed circles indicate bootstrap support of over 90%, and open circles indicate bootstrap support of over 50%.

sequences were also previously detected in the Lake Washington sediment via RT-PCR surveys (Fig. 3) (32). Clone a3 (30% of total clones) was closely related to *pmoA* genes of the recently described yet uncultivated filamentous methanotroph *Crenothrix polyspora* (39) and to a large group of sequences recovered from the Eastern Snake River Plain aquifer, designated group II unculturables (16). While *C. polyspora* is a gammaproteobacterium, its *pmoA* sequence has been noted to be more closely related to *amoA* sequences and to the divergent alphaproteobacterial sequences (cluster 1) (39) than to gammaproteobacterial sequences. Two types of *fae* sequences were identified in the library obtained from the same cells and were equally represented in the library, and both were closely related to *fae* genes described for type II methanotroph isolates from Lake Washington as well as to the sequences retrieved from Lake Washington via PCR surveys (Fig. 4) (26). In the 16S rRNA gene clone library obtained from the same cells, 62.5% of the sequences were related to alphaproteobacterial sequences, with 35% being most related to *Methylocystis* sp. sequences (approximately 98% similarity) and 12.5% being more distantly related to the sequences of alphaproteobacterial methanotrophs (Fig. 5), while 15% were related to the sequences of *Sphingomonas* sp. Of the 37.5% of nonalphaproteobacterial sequences, 5% were related to the sequences of *Pseudomonas* sp., and the rest were related to the sequences of gram-positive bacteria.

The presence of *Pseudomonas*-like sequences as well as sequences related to the sequences of gram-positive bacteria in both 16S rRNA clone libraries is likely due to nonspecific sorting based on natural bacterial fluorescence or due to cell adhesion. Similar problems with the specificities of FISH probes have been noted before (38). The majority of the sorted cells, however, seemed probe specific, as type I methanotroph-targeted sorting resulted in enrichment of sequences related to those of known type I methanotrophs, and type II methanotroph-targeted sorting resulted in enrichment of sequences related to those of known type II methanotrophs. The limited diversity of phylogenetic and functional genes in the analyzed PCR-based libraries likely reflects the dominant nature of the organisms in question in each subpopulation. While most of the sequences uncovered in this work were closely related to the sequences previously uncovered from the site by PCR or RT-PCR surveys, some of the sequences were novel and had not previously been detected in Lake Washington. The divergent 16S rRNA sequence distantly related to those of type II methanotrophs (clone a2) (Fig. 5) may represent a novel group of type II methanotrophs, the divergent *pmoA* clone (g3) may represent a divergent group of gammaproteobacterial *pmoA*

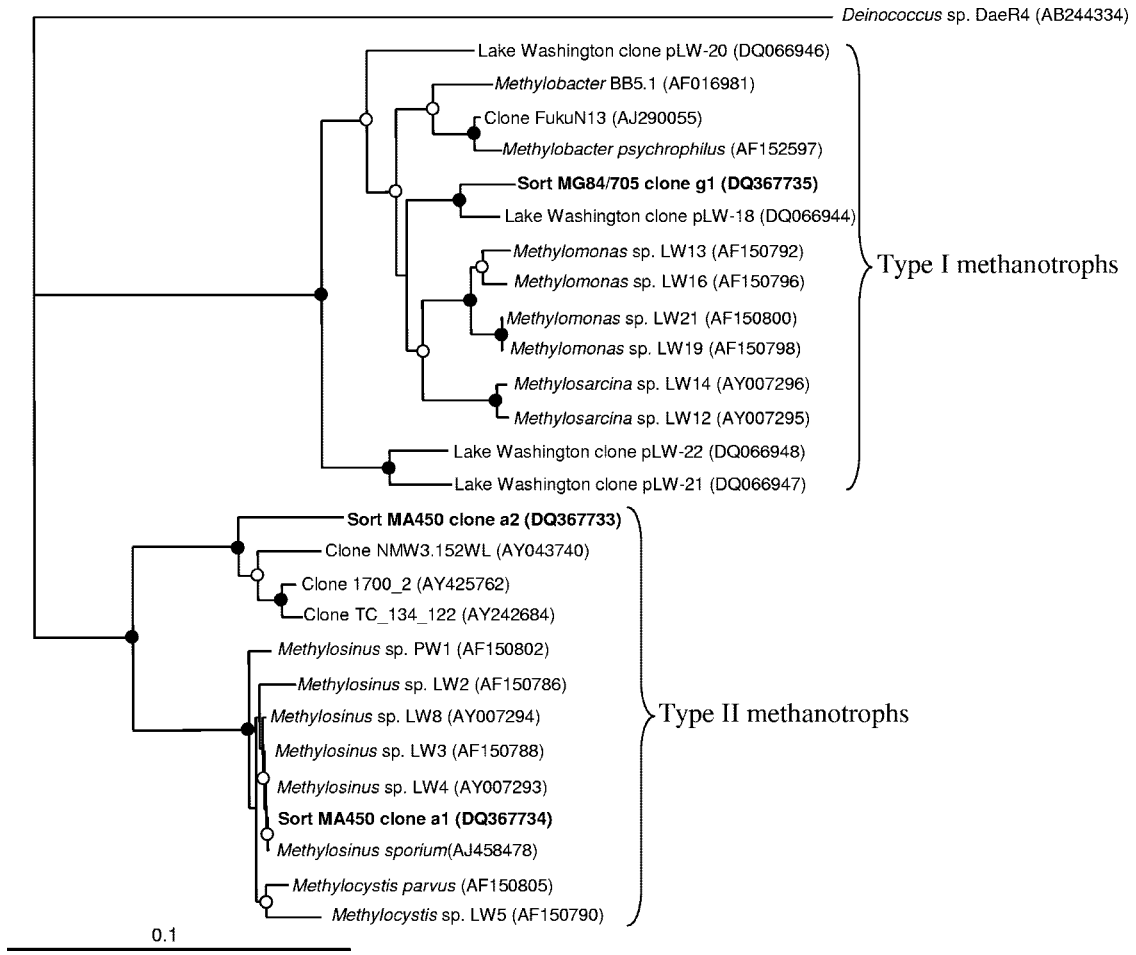

FIG. 5. Phylogenetic tree showing relationships of 16S rRNA gene sequences (543 to 605 positions) uncovered in this work to the sequences from known type I and type II methanotrophs and to the sequences of uncultivated organisms. A distance algorithm (DNAdist) was employed, with 1,000 bootstrap analyses. Closed circles indicate bootstrap support of over 90%, and open circles indicate bootstrap support of over 55%. The *Deinococcus* sequence was used as an outgroup.

genes (cluster 2) (Fig. 3), and another divergent *pmoA* clone (a3) may represent a group of *pmoA* genes shared by alpha- and gammaproteobacterial methanotrophs (*Crenothrix* cluster) (Fig. 3). While this last possibility is intriguing, additional experiments, such as double (rRNA and mRNA) FISH, are necessary to link this novel group of *pmoA* sequences to the phylogenetic identities of the organisms in question.

We were not able to perform FC analysis or fluorescence-activated cell sorting (FACS) using the *mmoX*-targeted polynucleotide probe (see above), as this probe produced a very weak fluorescent signal, consistent with the lack of or low expression of *mmoX* in the sediment community under ambient conditions (M. Kalyuzhnaya, unpublished observations).

Overall, the results described above suggest that the FISH/FC/FACS approach is effective in obtaining preparations of cells enriched in either type I or type II methanotrophs from lake sediment and is suitable for further genetic analysis of functional genes in this population.

WGA. One of the attractive applications of FISH-FC-based separation of specific populations of cells could be in the genomic characterization of uncultured microbes. While the amounts of DNA isolated from sorted cells would not be sufficient for traditional shotgun sequencing, DNAs could be amplified by using commercially available enzymes for whole-genome amplification (WGA). We tested the feasibility of such an approach and determined the number of cells required to produce sufficient amounts of DNA for specific amplification, using a model methanotroph, *M. capsulatus*, whose genome sequence is available (43). We used an artificial mix of *M. capsulatus* with *M. extorquens* and *M. flagellatus* in approximately equal proportions. The genomic sequences of the two control organisms are also available (http://www.integratedgenomics.com/genomereleases.html#6 and http://genome.jgi-psf.org/draft_microbes/metfl/metfl.home.html). Cells were labeled using the MG84/705 probe and subjected to FISH/FC/FACS as described above. Pools containing 10, 10^2, 10^3, 10^4, or 10^5 of the sorted cells were used for WGA. The resulting DNAs were used to construct shotgun libraries corresponding to each pool, and up to 50 clones from each library were sequenced. Our data indicated that a direct correlation existed between the number of cells used for WGA and the specificity of WGA. In the first three pools, 80%, 50%, and 5% of the tested clones contained nonspecific DNA. This DNA seemed to be noncoding, as judged by BLAST analyses with either a nonredundant nucleotide or nonredundant pro-

tein database, and likely was a product of nonspecific (background) amplification (11, 22). The remaining sequences in these libraries carried *M. capsulatus* DNA, as judged from BLAST analyses. One hundred percent of tested clones in libraries corresponding to pools with 10^4 and 10^5 cells carried *M. capsulatus* DNA, and the hits were distributed randomly along the chromosome, with only one hot spot, in the glutamine synthetase gene (data not shown). Likely, the cause of this hot spot was a cloning bias toward the respective BamHI fragment of 0.2 kb. Our results are in agreement with the results from other groups indicating that at least 10^3 microbial cells or at least 1 ng of DNA is necessary for high-fidelity WGA (4, 11, 22).

Conclusion. The FISH/FC/FACS approach for separation of microbial populations of interest from natural samples described here represents a promising tool for genetic and genomic characterization of as yet uncultivated or unculturable microbes. In this work, we demonstrated successful separation of type I and type II methanotrophs and enrichment of the desired type from a complex natural community, Lake Washington sediment, using traditional FISH probes. Future work will address the specificity of cell sorting using phylogenetic probes and will further explore the feasibility of probes targeting functional gene RNA transcripts for FISH/FC/FACS and subsequent WGA.

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