Methanotrophic Bacteria and Facilitated Transport of Pollutants in Aquifer Material

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In situ stimulation of methanotrophic bacteria has been considered as a methodology for aquifer remediation. Chlorinated aliphatic hydrocarbons such as trichloroethylene are fortuitously oxidized by the methane monoxygenase produced by methanotrophic bacteria. Experimental results are presented that indicate that both colloidal suspensions containing methanotrophic cells and the soluble extracellular polymers produced by methanotrophic cells have the potential to enhance the transport and removal of other environmental contaminants such as polynuclear aromatic hydrocarbons and transition metals in aquifer material. Three well-characterized methanotrophic bacteria were used in the experiments: Methylobomonas albus BG8 (a type I methanotroph), Methylosinus trichosporium OB3b (a type II methanotroph), and Methylocystis parvus OBBP (a type II methanotroph). Isotherms were obtained for sorption of two radiolabeled pollutants, [14C]phenanthrene and 106Cd, onto an aquifer sand in the presence and absence of washed cells and their extracellular polymer. Column transport experiments were performed with the washed methanotrophic cells and phenanthrene. The distribution coefficients for Cd with extracellular polymers were of the same order as that obtained with the aquifer sand, indicating that polymers from the methanotrophic bacteria could act to increase the transport of Cd in a porous medium. Polymer from BG8 significantly reduced the apparent distribution coefficient for Cd with an aquifer sand. [14C]phenanthrene also sorbed to extracellular polymer and to washed, suspended methanotrophic cells. The exopolymer of BG8 and OBBP significantly reduced the apparent distribution coefficient ($K_d$) for phenanthrene with aquifer sand. The distribution coefficients for phenanthrene with the methanotrophic cells were an order of magnitude greater than those previously reported for other heterotrophic bacteria. Cells of the methanotrophs also significantly reduced the apparent $K_d$ for phenanthrene with an aquifer sand. The three strains of methanotrophs tested displayed mobility in a column of packed sand, and strain OBBP reduced the retardation coefficient of phenanthrene with an aquifer sand by 27%. These data indicate that both extracellular polymer and mobile cells of methanotrophic bacteria display a capacity to facilitate the mobility of pollutant metals and polynuclear aromatic hydrocarbons in aquifer material.

The focus of the research reported here was on the potential for mobile methanotrophic cells and their extracellular polymers to facilitate the transport of hydrophobic organic compounds and heavy metals through porous material. Obligate methane-oxidizing bacteria, the methanotrophs, are a diverse group of bacteria and are relatively ubiquitous. They have been divided into three types, I, II, and X, based on intracytoplasmic membrane ultrastructure, enzymatic characteristics, metabolic pathways, percent G+C values, and 16S rRNA sequences (15). Methane monoxygenase (MMO), the enzyme complex that oxidizes methane to methanol, has a broad substrate range and can metabolize a variety of hydrocarbons (8). Wilson and Wilson (37) reported that trichloroethylene was dechlorinated in soils supplied with natural gas, suggesting the potential of methanotrophs for in situ bioremediation of soil and groundwater environments contaminated with chlorinated aliphatic compounds. Semprini et al. (31) reported biodegradation of trichloroethylene in a confined aquifer after supplying it with methane and oxygen and stimulating an indigenous population of methane-oxidizing bacteria. Pfiffner et al. (27) verified in situ stimulation of methanotrophs after addition of an exogenous supply of methane and air to an aquifer environment contaminated with trichloroethylene. These reports support the feasibility of in situ bioremediation of chlorinated solvents with indigenous populations of methanotrophs. Stimulation of methanotrophs with methane selectively increases the population density of these organisms in soil (19) and groundwater (27) environments and may also result in the production and release of extracellular polymer by the methanotrophic populations.

Rates of contaminant transport that are greater than those calculated on the basis of hydrodynamic flow of groundwater and sorption of the contaminant to the solid phase of the porous medium are indicative of facilitated transport (25). Because of the strong sorption of polynuclear aromatic hydrocarbons (PAHs) and transition metals such as Cd and Pb to the stationary porous media, their transport and removal are severely retarded. Nevertheless, researchers have observed facilitated transport of these types of contaminants (20, 34). Therefore, processes that increase the mobility and transport of these contaminants are of considerable interest both in the design of a treatment process to enhance their removal and in risk assessment.

Previous work has demonstrated that extracellular polymers produced by heterotrophic bacteria from subsurface and soil environments can facilitate transport of phenanthrene, a model PAH (9, 10), and transition metals such as Cd and Pb (5). Biocolloids, including mobile bacterial cells, can also facilitate transport of PAHs in aquifer material (18). There have been reports of methane-oxidizing bacteria producing extracellular polymer with the potential for industrial applications (6, 16, 17). Pfiffner et al. (27) have reported significant increases in the population densities of methanotrophs in...
groundwater samples from >0.1 methanotrophs ml⁻¹ before to >10³ methanotrophs ml⁻¹ after exogenous methane and oxygen were introduced into the aquifer. The presence of methanotrophic cells as biopolymers in the groundwater samples strongly indicates that components of the methanotrophic population were mobile in the pore water of the aquifer.

Since an exogenous supply of methane and oxygen to aquifer environments can stimulate the growth of indigenous methanotrophic bacteria (27, 31), populations of methanotrophs may be considered a manipulable source of biopolymers and colloids, both of which may facilitate the transport of hydrophobic organic pollutants, and transition metals in porous media. These biopolymers and colloids naturally occur in soils, unlike alternative remediation agents, such as exogenously supplied xenobiotic surfactants or complexing agents that, at least in some cases, may be viewed as additional forms of subsurface contamination. The objective of this study was to establish by laboratory experiments if extracellular polymers and cells of pure type I and II methanotrophic cultures can act as carriers of PAHs and toxic metals and facilitate their transport in aquifer material.

MATERIALS AND METHODS

Growth and preparation of methanotrophic bacteria and their extracellular polymers. The following three strains of methanotrophic bacteria were used in our experiments and were provided by William C. Ghirose (Section of Microbiology, Cornell University): *Methylomonas albus* BG8 (American Type Culture Collection), a type I methanotroph; *Methylomonas trichosporium* OB3b(LB), a type II methanotroph; and *Methylcystis parvus* OBBP(LB), a type II methanotroph. These strains were transferred to plates of minimal nitrate salts medium (NSM; described by Graham et al. [14]). In our preparation of NSM, the phosphate salts were autoclaved separately and added after cooling to 50°C. Cu-free NSM was also prepared for obtaining production of the soluble form of MMO (sMMO). All of the glassware used to prepare sMMO was acid washed. Nobel agar (1.5%) from Difco was added to NSM when plates were poured.

Inoculated plates were put into a vented Gas-Pak (BBL Gas-Pak System) and exposed to a 1:2 mixture of methane and oxygen with a trace of carbon dioxide. The sealed Gas-Pak was placed in a 25°C incubator. Fresh plates were used to inoculate 25 ml of NSM in sterile serum bottles that were crimp sealed with sterile Teflon-faced rubber septa (Supelco). The air in the bottles was replaced, under aseptic conditions, with the same mixture of methane, oxygen, and carbon dioxide. A fresh gas mixture was administered to the culture bottles every 24 to 36 h until an optical density (at 600 nm) between 0.9 and 1.1 was obtained. Cells were harvested by centrifugation. The supernatant was saved and was dialyzed against distilled-deionized water in Spectra/Por Membrane tubing with a molecular weight cutoff of 6,000 to 8,000 to obtain extracellular polymer. The dialysate was freeze-dried, and the recovered polymer was stored at 4°C.

Cell suspensions were examined with a Zeiss universal transmitted-light microscope to measure cell dimensions and observe any cyst or spore formation. Cell concentrations were determined with a Coulter Multisizer II and by total organic carbon (TOC) analysis with an O.I. Corp. 700 TOC analyzer.

Test for sMMO activity of OB3b and its ability to hydroxylate phenanthrene. sMMO was reported to oxidize naphthalene to naphthol (4); however, the ability of the enzyme to oxidize other PAHs has not been established. Transformation of a PAH to the corresponding alcohol would give a product with increased aqueous solubility and greater mobility in porous material. Strain OB3b, known to produce sMMO in the absence of Cu (4), was grown in Cu-free NSM. After sufficient growth was visible, 1 ml of this suspension was used to inoculate a fresh serum bottle of Cu-free NSM. The methane, oxygen, and carbon dioxide mixture was replenished daily until a cell density approximating an *A₅₀₀* of 1.0 was obtained. By using the method of Brusseau et al. (4), the suspension of OB3b was diluted with fresh NSM containing no additional Cu to an *A₅₀₀* of 0.2. Crystals of naphthalene and phenanthrene were placed in sterile Hungate tubes, each in triplicate. Three milliliters of the diluted cell suspension was pipetted into the tubes, which were sealed with septa and crimp caps and incubated at 30°C on a shaker at 200 rpm for 1 h. The following controls were used: (i) cells without PAH and (ii) naphthalene and phenanthrene in Cu-free NSM. The reaction mixtures were transferred to screw-cap tubes, and 100 μl of 4.21 mM o-dianisidine was added to detect naphthol and phenanthrol; the tubes were capped, mixed with a vortex mixer, and read immediately in a Bausch & Lomb Spectronic 20 at 525 nm.

Test for cell surface hydrophobicity. The cell surface hydrophobicity of the methanotrophs was quantified by the microbial adhesion to hydrocarbons test as previously described (18, 29, 30).

Characteristics of sorbents used in isotherm and column experiments. Sands from two different aquifer sources were used. They were obtained from a local quarry in Newfield, N.Y. The oxic status of the sands was not characterized; however, contact with atmospheric oxygen was freely permitted during storage and handling. One sand was used in all experiments with phenanthrene, and the other was used in experiments with Cd. Selected physical and chemical characteristics of the aquifer sands are presented in Table 1. More detailed characterization of these porous materials has been reported else-
where (5, 9). The sands were air dried and tumbled to obtain homogeneity before use in experiments.

**Determination of binding constant for phenanthrene with extracellular polymers.** Fluorescence quenching, as described by Gauthier et al. (13), was used to determine the distribution coefficients for phenanthrene with the extracellular polymers (\(K_{d,\text{polymer}}\)) from the methanotrophs tested. Freeze-dried polymer was dissolved in 5 mM CaSO\(_4\)-0.02% Na\(_2\)SO\(_4\) and 50-\(\mu\)l increments were added to a 3-ml sample of a phenanthrene solution (phenanthrene was dissolved in the same electrolyte as the polymer). A photon-counting spectrophotometer (SLM 8000tm), excitation monochromator (MCN640), and emission monochromator (FP-100) (SLM Instruments, Inc.) were used to measure fluorescence at excitation and emission wavelengths of 234 and 367 nm for each increment of polymer added. Results were interpreted by using the Stern-Volmer equation (13),

\[
F_0/F = 1 + K_{\text{polymer}} \tag{1}
\]

where \(F_0\) is the fluorescence of the phenanthrene solution in the absence of polymer, \(F\) is the fluorescence with polymer, \(K_{\text{polymer}}\) is the distribution coefficient for the added polymer, and \(K\) is \(K_{d,\text{polymer}}\). Dilution effects were accounted for in the calculations. Duplicate or triplicate analyses were performed for each polymer tested.

**Determination of the distribution coefficient for the polymers with the aquifer sand (\(K_{d,\text{polymer}}\)).** Three concentrations of polymer ranging from 10 to 100 mg liter\(^{-1}\) were combined in acid-washed centrifuge tubes with 300 mg of an aquifer sand and 30 ml of 5 mM CaSO\(_4\)-0.02% Na\(_2\)SO\(_4\). Each treatment was duplicated. The tubes were tumbled slowly for 24 h at 25°C. The tubes were then centrifuged at 870 × g for 20 min. The supernatant was removed and analyzed for TOC with an O.I. Corp. 700 TOC analyzer. Controls without polymer were used to make corrections for the organic matter associated with the sand. The distribution coefficient for the polymer with the sand was determined by using the equation

\[
C_i = K_iC_i \tag{2}
\]

where \(K_i\) is the distribution coefficient \(K_{d,\text{polymer}}\), \(C_i\) is the concentration (in grams per milliliter) of polymer in the supernatant, and \(C_i\) is the concentration (in grams per gram) of polymer sorbed to the sand. \(C_i = (C_i - C_i)\)\(\frac{V_i}{M}\), where \(C_i\) is the initial concentration of polymer, \(V_i\) is the volume of solution containing the sorbent, and \(M\) is the sorbent mass.

**Determination of the distribution coefficient for Cd sorption to polymer.** At constant pH and ionic strength, the sorption of a trace metal to a macromolecule can be quantified in terms of a conditional stability constant (\(K_{d,\text{polymer}}\)) as follows:

\[
K_{d,\text{polymer}} = \frac{[ML]}{[M^{2+}][L]} \tag{3}
\]

where \([M^{2+}]\) is the concentration of the free metal ion, \([L]\) is the concentration of the binding molecule, and \([ML]\) is the concentration of the metal-molecule complex. To quantify the Cd sorbed to a polymer, the method described by Kellemes and Lion (21) was used. A 94-48 solid-state Cd ion-selective electrode (Orion) in conjunction with a 90-02 double-junction reference electrode (Orion) was used to measure free Cd(II) in solution.

**Sorption isotherm experiments with whole bacterial cells.** The method of Jenkins and Lion (18) was used to determine distribution coefficients for phenanthrene with cells and sand in the presence of cells. Briefly, azide-poisoned solutions of phenanthrene were added to cell suspensions with and without aquifer sand in funnel top ampoules. After addition of \(1^\text{4}C\)phenanthrene (13.1 mCi of \(9^\text{4}C\)phenanthrene [Sigma Chemical Co.] per mmol), the ampoules were flame sealed and equilibrated for 24 h at 25°C on a slowly rotating tumbler. After equilibration, cells were removed by centrifugation at 330 × g for 30 min. In experiments with sand, the sand was separated from the aqueous phase and suspended cells by sedimentation for 24 h. The aqueous solutions were assayed for \(1^\text{4}C\) activity by liquid scintillation counting.

Sorption of phenanthrene to cells and to the aquifer sand in the presence and absence of suspended cells was evaluated by using the following linearized form of the mass-balance equation (24):

\[
\frac{(C_iV_i)}{(C_fV_f)} = 1 + K_{d}(M/V) \tag{4}
\]

where \(C_o\) is the initial concentration (in disintegrations per minute) of \(1^\text{4}C\)phenanthrene, \(V_o\) is the volume of the solution, \(C_i\) is the concentration of sorbate in equilibrium with sorbent, \(V_i\) is the volume of solution containing the sorbent, \(K_{d}\) is the apparent linear distribution coefficient (a function of the cell concentration), and \(M\) is the mass of sorbent. Plotting \(M/V\) on the \(x\) axis and \((C_iV_i)/(C_fV_f)\) on the \(y\) axis yields a linear slope of the value which equals the apparent distribution coefficient (\(K_{d}\)). In experiments without sand, \(M\) is the mass of cells (as quantified by TOC) and \(K_{d}\) is \(K_{d,\text{cell}}\), the phenanthrene distribution coefficient to cells.

**Batch sorption isotherm with polymer, aquifer sand, and Cd.** Three hundred milligrams of a low-carbon aquifer sand, variable quantities of \(1^\text{10}Cd\) (to give a specific activity of approximately 1 \(\mu\)Ci of \(Cd\) g\(^{-1}\)), variable quantities of \(CdCl_2\) (to give \(CdCl_2\)) were combined with silanized glass centrifuge tubes at a final volume of 30 ml. The suspensions were equilibrated for 24 h at 25°C while being tumbled slowly. After the Cd was allowed to equilibrate, polymer was added to yield a concentration of 10.6 mg liter\(^{-1}\). The suspensions were then allowed to equilibrate for another 24 h, after which the tubes were centrifuged at 1,000 × g for 20 min. Controls contained no polymer, and treatments were duplicated. The concentration of \(1^\text{10}Cd\) in the supernatant was determined (±2%) by crystal scintillation counting of the \(1^\text{10}Cd\) 88 keV \(\gamma\) decay (Packard Auto-Gamma 5650). The quantity of Cd sorbed was determined by subtracting the amount of Cd in solution from the amount of Cd added. The Cd distribution coefficient was determined by using equation 2.

**Bacterial cell mobility in porous media.** Cell mobility was determined by using columns packed with aquifer sand and the methods described by Jenkins and Lion (18). A chloride breakthrough curve (BTC) was obtained before the BTC for the cells. The BTC for cells was obtained by injecting the cell suspension into the column as a 4- to 6-h pulse followed by a continuous flow of sterile 5 mM CaSO\(_4\). Cell concentration was determined by using the methods described above. On the basis of the Cl\(^-\) BTC and use of CXTFIT, a model developed by Parker and van Genuchten (26), the dispersion coefficient...
and pore water velocity were estimated. The fitted pore water velocity was in good agreement with that calculated from the pore volume and flow rate.

The retardation coefficient ($R$) for the methanotrophic bacteria was estimated by using the first temporal moment of the BTC (24) as follows:

$$\begin{align*}
R &= \left\{ \Sigma (C/C_0) \theta d\theta \right\} / \left\{ \Sigma (C/C_0) d\theta \right\} - \theta_p/2 
\end{align*}$$

(5)

where $C$ is the effluent concentration of cells, $C_0$ is the influent concentration of cells, $\theta$ is pore volumes, and $\theta_p$ is the duration (in pore volumes) of the input pulse of cells. By definition, $R$ is the ratio of the velocity of the pore water to the velocity of the bacterial cells.

The distribution coefficient for the bacterial cells with the sand ($K_{cell}^s$) was calculated from $R$ by using the following equation:

$$\begin{align*}
R &= 1 + K_{cell}^s \rho/\eta 
\end{align*}$$

(6)

where $\rho$ is the bulk density of the sand and $\eta$ is porosity.

**Column experiments with phenanthrene plus cells.** A stainless steel chromatography column (12.5 by 1 cm [inside diameter]) with all-steel fittings and 2-µm stainless steel frits (Alltech) was used for the packed-column experiments for developing BTCs for phenanthrene alone and phenanthrene plus cells. The column was dry packed, under conditions of constant tapping, with aquifer sand. The bulk density of the packed sand was determined gravimetrically to be between 1.6 and 1.7 g cm$^{-3}$. As described by Jenkins and Lion (18), the column was connected to a continuous-flow syringe pump (Pharmacia LKB P-500) and a fraction collector (Gilson 222); the column was housed in a constant-temperature chamber that was kept at 25°C. Before initiation of a BTC determination, the column was saturated with a solution of 5 mM CaSO$_4$-0.02% Na$_2$SO$_4$. A chloride BTC determination preceded the phenanthrene BTC determinations. On the basis of the excellent agreement of previously replicated BTCs for phenanthrene with and without the presence of bacterial cells (18, 24), development of one BTC for phenanthrene and for phenanthrene in the presence of OBBP cells was considered sufficient to demonstrate a significant difference in the retardation coefficients for each respective BTC.

For the phenanthrene BTC determination, a pulse of 0.56 µM [14C]phenanthrene (~16,400 dpm ml$^{-1}$) was applied to the column for 6 h, followed by elution with 5 mM CaSO$_4$-0.02% Na$_2$SO$_4$. When a methanotrophic culture was incorporated into the column experiments, washed cells of a known concentration were mixed with the [14C]phenanthrene used in the pulse; the pulse was then followed by a suspension of cells of the same cell concentration. One-milliliter fractions of the eluate were collected directly into scintillation cocktail. The concentration of the radiolabel was determined with a Beckman LS 9800 liquid scintillation counter. CXTFIT (26) was used to determine the pore water velocity and the dispersion coefficient on the basis of data from the chloride BTC. The retardation coefficient of phenanthrene was determined by use of equation 6 for the first temporal moment. BTCs for hydrophobic organic compounds such as phenanthrene under saturated conditions are generally asymmetric. This asymmetry is seen in peak tailing (28). Because peak tailing affects the temporal first moment of the BTC, all values of $R$ are based on experiments that yielded 100% recovery of added labeled phenanthrene.

**Model predictions.** The mathematical model developed by Magee et al. (24) for predicting the effect of an organic carrier on the transport of PAHs in a porous medium was adapted to predict the effect of bacterial cells on PAH transport by Jenkins and Lion (18). The model has the following form:

$$\begin{align*}
R &= \frac{1 + K_{cell}^s \rho}{1 + \{K_{cell}^s/\eta\}} 
\end{align*}$$

(7)

where $K_{cell}^s$ is the distribution coefficient for sorption of phenanthrene to cells (in milliliters per gram of cell carbon), $\rho$ is the bulk density of the sand, and $\eta$ is the porosity of the sand. $R$ values calculated from the model were converted to apparent $K_d$ values by using equation 6 for comparison to observations in batch sorption experiments with phenanthrene in the presence of cells and the aquifer sand.

**RESULTS**

**Hydrolylation of phenanthrene.** Preliminary tests verified that the reagent o-dianisidine may be used to detect both phenanthrol and naphtol. As expected, type II strain OB3b expressed sMMO, as indicated by its ability to hydroxylate naphthalene. Under the conditions of the assay, OB3b did not hydroxylate phenanthrene. It was possible that the low aqueous concentration of phenanthrene may have been lower than the minimum substrate concentration of the enzyme. Therefore, methanol, at a concentration of 1%, was added to the phenanthrene solution as a cosolvent to increase its concentration. However, the sMMO of OB3b also failed to hydroxylate phenanthrene with added methanol in solution.

The ability of OB3b to hydroxylate naphthalene did not give it the ability to hydroxylate phenanthrene, an activity which would have increased the bioavailability of this PAH. Since sMMO activity has been reported for an environmental strain of type I methanotroph (22) and type II methanotrophs distinct from OB3b that express sMMO exist (3), there may be other methanotrophic strains that express sSMOs that can hydroxylate phenanthrene.

**Cell surface hydrophobicity.** Results of the microbial adhesion to hydrocarbons test (29, 30) indicated that the three methanotrophic strains, grown under the conditions described, displayed a hydrophilic character, as evidenced by their negligible association with the immiscible hexadecane phase.

**Effect of extracellular polymer of the methanotrophs on the mobilization of phenanthrene and Cd.** Results of the experiments to determine the distribution coefficients for phenanthrene with the extracellular polymers from the methanotrophs and for the polymers with the sand and the effect of the
polymers' presence on the distribution coefficient for phenanthrene with the sand (Table 2) were generally similar to those previously reported for polymers from aerobic heterotrophic bacteria (9). Given the low values for the extracellular polymer distribution coefficients with the sand, the polymers would be anticipated to be less retarded than phenanthrene. With the exception of the polymer from strain OB3b, the presence of extracellular polymer significantly reduced the apparent \( K_d \) for phenanthrene with the sand. Similarly, results of the Cd titrations and the batch isotherm experiment with the extracellular polymer from strain BG8 (Table 3) were comparable to previous results obtained with aerobic heterotrophic bacteria (5). All extracellular polymers complexed Cd(II), and the polymer from BG8 reduced the distribution coefficient for Cd with the sand significantly (89%).

**Effect of mobile cells of type I and II methanotrophs on mobilization of phenanthrene in aquifer sand.** Results of experiments to determine the distribution coefficients for phenanthrene with cells of the three methanotrophic strains and for the methanotrophic cells with the sand (Table 4) were similar to values previously determined for other aerobic heterotrophic bacteria, although the \( K_d^{cell} \) values for the methanotrophs were an order of magnitude greater than for most other bacteria tested (18). Moderate cell concentrations (0.4 \( \times \) 10^8 to 5 \( \times \) 10^9 cells ml\(^{-1}\)) decreased the \( K_d \) for phenanthrene with the sand by \( \approx 10\% \) (Table 4).

**Mobility of the two types of methanotrophs.** All columns were packed as similarly as possible; consequently, their physical characteristics fell within close tolerances. Bulk densities ranged from 1.55 to 1.65 g cm\(^{-3}\), porosity ranged from 0.32 to 0.35, and pore water velocities (determined from the Cl\(^{-} \) BTCs) ranged from 0.1 to 0.2 cm min\(^{-1}\). Replicate columns for strain OB3b displayed similar BTCs and, at 17 pore volumes, had an average percent mass recovery of 32.5\% \( \pm \) 0.5\% and an average \( R \) value of 2.5 \( \pm \) 0.5. These data indicated good reproducibility of column parameters and results.

**Results of the BT determination for the type I methanotroph BG8 (Fig. 1) indicated that this organism was mobile under the experimental conditions used. The \( R \) value for strain BG8 was 2.0 (it traveled through the packed sand column approximately half as fast as the chloride tracer) and was small compared with that of phenanthrene (\( R > 50 \)). Unfortunately, mass recovery of BG8 in the column effluent was low (10.8\%), indicating slow detachment kinetics or loss of cells by filtration. As a result, the calculated \( R \) value for BG8 cells should be considered to represent only the mobile fraction of the cell population applied to the column. The culture of OB3b that expressed sMMO [OB3b(sMMO)] displayed an apparent mobility greater than that of the chloride tracer (its \( R \) value was \( < 1 \)), although like that of BG8, its mass recovery was low (15\% over the duration of the experiment). Although the \( R \) values for the two type II methanotrophs that expressed particulate MMO were greater than that for BG8 (Fig. 1), their mass recovery over the duration of the experiment was significantly greater. The cell dimensions of strains BG8, OB3b, and OB3b were \( \approx 1.5 \) \( \mu \)m, a colloidal size that is relatively favorable for transport (38). The lack of complete mass recovery of the strains may be accounted for either by a reversible process of sorption-attachment to the solid matrix and slow desorption-detachment kinetics or by filtration. The apparent tailing in the BTCs of BG8 and OB3b, and, to a lesser extent, OB3B is indicative of slow desorption-detachment kinetics. The BTC for OB3b(sMMO) lacked evidence of tailing, and lack of complete mass recovery of this strain may be attributable to filtration, although its physical dimensions were similar to those of the other cultures. The three strains of methanotrophs investigated form resting cells (cysts or spores), the physiology of which may lend itself to sorption to or filtration by the porous solid matrix of the packed sand column.

**Phenanthrene BTCs determined with and without a bacterial carrier.** Transport of phenanthrene in the presence of the mobile type II methanotroph OB3B was significantly enhanced. The BTCs for phenanthrene with and without OB3B (Fig. 2) indicated that the presence of 27 \( \mu \)g of TOC cells of OB3B ml\(^{-1}\) reduced the retardation factor, \( R \), for phenanthrene with the aquifer sand by more than 25\%. These data

### Table 3. Distribution coefficients for cadmium with methanotrophic extracellular polymer (\( K_d^{polymer} \)) and reduction of the distribution coefficient for cadmium with aquifer sand in the presence of polymer

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>( K_d^{polymer} ) (mL/g)</th>
<th>% Reduction in ( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG8</td>
<td>2.64 ( \times ) 10^4</td>
<td>89%</td>
</tr>
<tr>
<td>OB3B</td>
<td>3.73 ( \times ) 10^4</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OB3b(sMMO)</td>
<td>4.66 ( \times ) 10^4</td>
<td>ND</td>
</tr>
<tr>
<td>OB3b(sMMO)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.74 ( \times ) 10^4</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(<a>\) Determined by using 10.6 mg of polymer liter\(^{-1}\).  
\(<b>\) OB3b was grown in low-[Cu] mineral salts medium and synthesized sMMO as measured by the ability to hydroxylate naphthalene (4).  
\(<c>\) Calculated from \( R \) values taken from BTCs by using equation 6.  
\(<d>\) ND, not determined.  

### Table 4. Distribution coefficients for phenanthrene with cells (\( K_d^{cell} \)) and for cells with sand (\( K_d^{sand} \)) and effect of cells on reduction of the apparent distribution coefficient for phenanthrene on aquifer sand

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>( K_d^{cell} ) (mL/g of cell C)</th>
<th>( K_d^{sand} ) (mL/g of cell C)</th>
<th>Cell density (( \mu )g of cell CmL(^{-1}))</th>
<th>% Reduction in ( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB3b</td>
<td>16.3 ( \times ) 10^3</td>
<td>0.50</td>
<td>52.7</td>
<td>20</td>
</tr>
<tr>
<td>OB3B</td>
<td>21.5 ( \times ) 10^3</td>
<td>0.34</td>
<td>55.5</td>
<td>39</td>
</tr>
<tr>
<td>BG8</td>
<td>11.2 ( \times ) 10^3</td>
<td>0.23</td>
<td>78.5</td>
<td>57</td>
</tr>
<tr>
<td>OB3b(sMMO)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>( \leq 0 )</td>
<td>83.3</td>
<td>23</td>
</tr>
</tbody>
</table>

\(<a>\) Calculated from \( R \) values taken from BTCs by using equation 6.  
\(<b>\) Cell concentration used to obtain the indicated percent reduction in \( K_d \).  
\(<c>\) ND, not determined.  
\(<d>\) Not determined.
were made. The deviation between the presence of strain OBBP. The inset details the tailing of the BTCs. The lower R value for phenanthrene in the presence of OBBP reflects the decrease in tailing of the BTC relative to that which occurred without OBBP (see inset).

indicate that the interaction between the ability of cells to sorb hydrophobic compounds, such as phenanthrene, and the mobility of methanotrophic cells in porous media can result in facilitated transport of hydrophobic pollutants. Comparable results have been reported for mobile heterotrophic bacteria (18).

**Model predictions.** Model calculations of the apparent Kd for phenanthrene with the aquifer sand in the presence of cells were made for strains BG8 and OBBP on the basis of equation 7 and measured values from the batch isotherm and column experiments. The calculated values were compared to observed reductions in Kd for phenanthrene with the aquifer sand (Fig. 3). Irreversible removal of cells is not considered by equation 7, and as anticipated, model predictions and observations diverged for cell strains that had low cell mass recovery in column experiments. The observed results for strain OBBP fell between the model predictions based on Kcell values of 0.34 (observed in column experiments) and 0 (unretarded cells). The deviation between observation and model calculations was greatest for strain BG8, which had the lowest cell mass recovery. Predicted values for BG8 based on Kcell values of 0 and 0.23 fell below the observed values.

**DISCUSSION**

**Extracellular polymers from methanotrophic bacteria as carriers of pollutants in aquifer material.** Bacterial extracellular polymers have been observed in groundwater (7). Dohse and Lion (10) reported that most (92%) of the heterotrophic bacterial isolates that they investigated produced extracellular polymers that facilitated the mobility of phenanthrene in an aquifer sand. In an analogous study, Chen et al. (5) reported that all of the extracellular polymers of the heterotrophic soil bacteria that were tested were capable of enhancing the desorption and mobilization of Pb and Cd from an aquifer sand. The results presented here demonstrate that the extracellular polymers of methanotrophic bacteria were only weakly sorbed by the aquifer material (as indicated by the values of their respective distribution coefficients with the aquifer sands [Kpolymer]). As a result, the polymers would be expected to be considerably more mobile than the PAH phenanthrene or the transition metal Cd. Fluorescence quenching measurement of

FIG. 3. Predicted and observed decreases in the apparent Kd for phenanthrene with the aquifer sand in the presence of methanotrophic strains BG8 and OBBP as determined by equation 7. Percent reduction was calculated as follows: % reduction = [(Kd' - Kd observed or predicted)/Kd'] × 100.

PAH binding to extracellular polymer and metal-polymer titration results showed that phenanthrene and Cd have a strong affinity for the polymers relative to the sand. These data suggest that the polymers produced by the methanotrophic bacteria have the potential for enhancing the mobility of both inorganic and organic environmental contaminants.

The batch isotherm experiments with phenanthrene, sand, and polymers demonstrated that the polymers from two of the methanotrophs had the capacity to reduce the apparent Kd of phenanthrene. The concentrations of polymer used in this study were an order of magnitude less than the polymer concentration used in a prior study with polymer produced by aerobic heterotrophs reported by Dohse and Lion (10). The ability to reduce the apparent Kd of phenanthrene with the sand in batch reactors was taken as strong evidence that these polymers would facilitate the transport of phenanthrene in an aquifer material, as Dohse and Lion (10) demonstrated for a selected bacterial polymer from an aerobic heterotroph.

The batch isotherm experiment with aquifer sand, Cd, and extracellular polymer of strain BG8 demonstrated the ability of a relatively dilute concentration of BG8 polymer to reduce the Kd for Cd with the aquifer sand. The reduction in the apparent distribution coefficient for Cd with the sand by polymer from BG8 was comparable to the reduction observed for a heterotrophic bacterium isolated from a subsurface environment (5).

The above data suggest that in situ stimulation of meth-
Methanotrophic cells as carriers of hydrophobic pollutants in aquifer material. Before a recent report of a type I methanoxidizing bacterium that expressed sMMO (22), it was generally believed that only type II and X methanotrophs are capable of expressing sMMO. In terrestrial ecosystems, all three types of methanotrophs have been shown to occur together in the same groundwater (3), soil (33), and sediment (35) environments. The three well-characterized methanotrophs used in the experiments reported here were chosen to represent typical components of an indigenous methanotrophic community. Type I strain BG8 expresses the particulate form of M MO only (4) and forms azotobacter-type cysts (36). Type II strain OB3B does not express sMMO (4) and forms lipid cysts (36). Strain OB3B expresses sMMO under conditions of low Cu concentration (4) and forms resting spores (36). The formation of cysts and spores has been observed to occur during nutrient deprivation, and the percentage of cyst and spore formation can vary between 5 and 95%, with a smaller percentage occurring after several transfers of the strains (36). Because nearly all of the experiments with cells reported here were performed on cells after their removal from the mineral salts medium and methane, their sole C source, the results in this report potentially reflect the presence of both vegetative cells and their cyst or spore forms. Microscopic observation indicated that at the beginning of experiments, negligible cyst or spore formation had occurred.

The hydrophilic character of the three methanotrophs further indicated either that the lipid cysts of strain OB3B, the azotobacter-like cysts of BG8, and the spores of OB3B occurred at a minimum or that the fraction of cysts and spores that may have formed during the experiment were as hydrophilic as the vegetative cells. As previously reported for heterotrophic bacteria associated with soil and subsurface environments (18), the hydrophilicity of the three methanotrophs examined did not influence their capacity to passively sorb phenanthrene. These data confirmed the observations of other researchers (18, 23) who have noted that cell surface hydrophobicity is not a salient factor in the ability of bacterial cells to passively sorb hydrophobic organic pollutants. The reductions in the apparent $K_s$ for phenanthrene with the aquifer sand in the presence of the three methanotrophic strains appeared not to be directly related to the $K_{s,max}$ for phenanthrene.

All of the strains of methanotrophs tested displayed partial mobility through the packed sand columns. Pfiffner et al. (27) enumerated methanotrophic cells from environmental groundwater samples. These results strongly suggest that components of indigenous methanotrophic communities were unattached or suspended and transported in the pore water environment of the aquifer and could thus be considered mobile. Because the $R$ value for strain OB3B(sMMO) was smaller than that for strains BG8, OB3B, and OB3B expressing the particulate form of M MO (Fig. 1), it might be considered the most mobile of the strains. Mass recovery of OB3B(sMMO), however, was low (13%) compared with the mass recoveries of OB3B expressing the particulate form of M MO and OB3B. The difference between the BTCs of OB3B and OB3B(sMMO) may be indicative of fundamental differences associated with the physiological conditions associated with the expression of sMMO as opposed to the expression of the particulate form of M MO. Previous studies on bacterial cell mobility (11, 12, 18) have indicated that there is no correlation between the mobility of a cell suspension and characteristics such as cell surface hydrophobicity, net surface charge, presence of capsular material, flagella, and cell dimensions. Both the $R$ values and mass recovery of OB3B and OB3B were comparable to those determined for other heterotrophic bacteria tested (18). The observation that the two type II methanotrophs, OB3B and OB3B, were more mobile than the type I methanotroph, BG8, is consistent with the observation that strain BG8 was bound to sand more readily than strains OB3B and OB3B (32) and that type II methanotrophs appeared to dominate groundwater samples (3) obtained from the same field site that Pfiffner et al. (27) sampled.

Type II strain OB3B showed the greatest capacity for binding phenanthrene. Because of its mobility, it moved through the packed sand column more quickly than phenanthrene and consequently facilitated the transport of phenanthrene that sorbed to the cells. The decrease in the $R$ value for phenanthrene in the presence of OB3B was comparable to that observed for a bacterium isolated from a subsurface environment contaminated with coal tar residues (18). The cell concentration of strain OB3B that facilitated the transport of phenanthrene through the packed column ($27 \mu g$ of cell C ml$^{-1}$ or $\sim 1 \times 10^8$ cells ml$^{-1}$) was less than half the cell concentration (on a C basis) of the bacterium that facilitated phenanthrene transport through a column of packed sand described by Jenkins and Lion (18) and is comparable to the high end of total cell counts for methanotrophic populations reported for soil environments unamended with exogenous methane and oxygen ($2.7 \times 10^7$ cells g of soil$^{-1}$) (33), for unamended sediment slurries ($5 \times 10^5$ cells ml of slurry$^{-1}$) (35), and for soils over methane-producing landfills ($2.6 \times 10^7$ g of soil$^{-1}$) (19). Because a fraction of methanotrophic cells either sorbed to or were filtered by the packed sand, they may also modify the sorptive properties of the porous medium and thus affect the mobility of phenanthrene (2).

For strain OB3B, the comparison between predicted and observed values for the decrease in the $K_s$ for phenanthrene with the aquifer sand (Fig. 3) indicated that the three-component model developed by Magee et al. (24) may be successfully applied to mobile methanotrophs as it has to other mobile cells (18). Because the calculated reduction in pollutant retardation is related to cell concentration (equation 7), an increase in pollutant mobility would be anticipated if the population density of mobile methanotrophs were increased by injection of methane and oxygen.

The facilitated transport of contaminants may or may not be desirable. It may lead to increased spread of a pollutant plume, or it may presumably be exploited to accelerate removal and biodegradation. Further study is needed to better understand the ecological and environmental ramifications of exogenous stimulation of indigenous methanotrophs and their interactions with organic and inorganic pollutants.

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