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Tracking the Response of *Burkholderia cepacia* G4 5223-PR1 in Aquifer Microcosms

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The introduction of bacteria into the environment for bioremediation purposes (bioaugmentation) requires analysis and monitoring of microbial population dynamics to define persistence and activity from both efficacy and risk assessment perspectives. *Burkholderia cepacia* G4 5223-PR1 is a Tn*5* insertion mutant which constitutively expresses a toluene *ortho*-monooxygenase that degrades trichloroethylene (TCE). This ability of G4 5223-PR1 to degrade TCE without aromatic induction may be useful for bioremediation of TCE-containing aquifers and groundwater. Thus, a simulated aquifer sediment system and groundwater microcosms were used to monitor the survival of G4 5223-PR1. The fate of G4 5223-PR1 in sediment was monitored by indirect immunofluorescence microscopy, a colony blot assay, and growth on selective medium. G4 5223-PR1 was detected immunologically by using a highly specific monoclonal antibody which reacted against the O-specific polysaccharide chain of the lipopolysaccharides of this organism. G4 5223-PR1 survived well in sterilized groundwater, although in nonsterile groundwater microcosms rapid decreases in the G4 5223-PR1 cell population were observed. Ten days after inoculation no G4 5223-PR1 cells could be detected by selective plating or immunofluorescence. G4 5223-PR1 survival was greater in a nonsterile aquifer sediment microcosm, although after 22 days of elution the number of G4 5223-PR1 cells was low. Our results demonstrate the utility of monoclonal antibody tracking methods and the importance of biotic interactions in determining the persistence of introduced microorganisms.

The use of chloroethenes, including trichloroethylene (TCE), as degreasing agents has led to extensive contamination of groundwater in the United States (27). Strategies for remediation of TCE-contaminated groundwater aquifers often rely on techniques which are expensive and ineffective (1, 36). The use of nonnative or recombinant bacteria for environmental in situ bioremediation (bioaugmentation) could be a promising alternative method (16, 18), although survival of cultured bacteria in the environment at densities sufficient for effective bioremediation has been problematic. *Burkholderia cepacia* G4 5223-PR1 (formerly *Pseudomonas cepacia* [15]) is a Tn*5* insertion mutant which constitutively expresses the toluene *ortho*-monooxygenase responsible for TCE degradation (32); this organism is a potential candidate for use in remediation of TCE-polluted aquifers. To remediate aquifers, an introduced organism must survive and maintain its activity. The functional integration of nonnative bacteria into natural microbial communities provides a challenge for both microbial ecologists and applied microbiologists. Analyses of the population and trophic interactions in such situations will be critical for successful bioaugmentation.

Risk assessment associated with uncontained biotechnological processes also requires careful monitoring of the survival and dispersal of released microorganisms. Although releases of nonnative or recombinant bacteria have not been reported to result in adverse environmental effects to date, workers have a responsibility to ensure that released microorganisms will be constrained by the selective pressures of the target environment. Monitoring released populations of microorganisms is thus critical to both success and safety. Microorganisms may be monitored by plating on selective media or by using specific genetic elements. In addition, monoclonal antibodies (MAbs) are potentially very useful for detecting and quantifying targeted bacterial cells (14, 22, 29).

In this paper we discuss the following two aspects of bioaugmentation: (i) the development and testing of an MAb with species or even strain specificity for a surface antigen of *B. cepacia* G4 5223-PR1 for tracking purposes, and (ii) preliminary laboratory testing of the response of G4 5223-PR1 to abiotic and biotic factors that may affect persistence and activity at a planned release site. Our results highlight the importance of biotic interactions and particle association in the survival of an introduced organism.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The strain used in this study was *B. cepacia* G4 5223-PR1 (32) harboring aromatic compound-degrading plasmid pTOM (31a). Minimal salt medium (33) containing 10 mM phthalate and 50 μg of kanamycin sulfate per ml (Ph/Km medium) was used for routine cultivation and for all plating experiments. Solid media contained 1.5% purified agar. To generate the inoculum used for the groundwater microcosm experiment, cells in the late logarithmic growth phase were harvested by centrifugation at 9,000 × *g*. The resulting cell pellet was washed twice in 0.01 M phosphate-buffered saline (PBS) (pH 7.0). The cells were resuspended in 1 ml of autoclaved groundwater and injected through silicon membranes into the groundwater microcosms. We used a basal salts medium for the continuous cultures of G4 5223-PR1 used to provide cells for sediment columns. This medium contained (per liter of distilled water) 0.57 g of Na_2HPO_4, 0.187 g of KH_2PO_4, 0.04 g of NH_4Cl, 10 mg of $MgSO_4 \cdot 7H_2O$, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.03 mg of $MnSO_4 \cdot H_2O$, 0.01 mg of $CuSO_4 \cdot 5H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $Na_2SiO_3 \cdot 9H_2O$ and 2 mg of NaCl; 50 μM phthalate was added as a carbon and energy source. Aquifer material was obtained from the Canadian Armed Forces Base in Borden, Ontario, Canada. Groundwater was collected from a sampling well at a depth of 6.5 m, and sediment was obtained from a core sample near the top of the saturated zone.

Design of the groundwater experiment. Two 2-liter aspirator bottles were filled with 1 liter of groundwater from the Borden aquifer to examine the survival of

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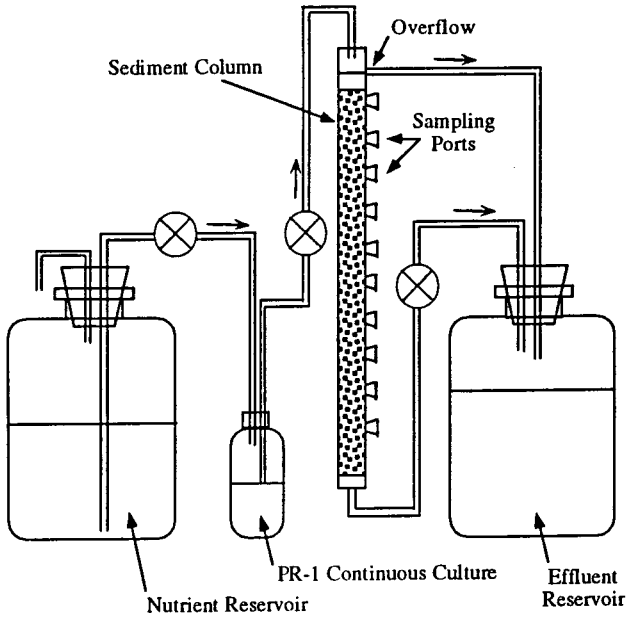

FIG. 1. Schematic diagram of the flow system which we used (not to scale). PR-1, G4 5223-PR1.

G4 5223-PR1. Each bottle was equipped with a silicon septum at the bottom for sampling. One of the bottles was sterilized at 120°C after it was filled with groundwater. The experiment was started by injecting 0.5 ml of washed G4 5223-PR1 cells through the silicon septum of each bottle. Immediately after injection, the cell density in both microcosms was 5×10^6 cells ml^{-1}. Both microcosms were kept in the dark at 18°C. Samples were removed with a sterile syringe after agitation.

Design of the continuous flow system. Sediment column systems with a continuous flow of either G4 5223-PR1 cells or basal salts medium were constructed as illustrated in Fig. 1. Plexiglas tubes that were 57 cm long and had an inside diameter of 2.9 cm were used for the sediment columns. Ten sampling ports that were 0.9 cm in diameter and 5 cm apart were closed with silicon stoppers. Samples of porewater from different depths in the columns were obtained with sterile syringes inserted through the silicon stoppers; the size of each sample was 100 to 200 μl. One column was filled with sediment sterilized by autoclaving, and another was filled with nonsterile sediment. We did not anticipate that the column containing the autoclaved sediment would be completely sterile because of the limitations of sterilizing the acrylic column tubes. The flow rate through each column was controlled by a peristaltic pump at the outflow end; the flow rate was set at 1.1 ml h^{-1} (11 cm day^{-1}), which approximated the flow rate found in the Borden aquifer (35). The void volume of each column was approximately 310 ml, and it took 4.7 days to replace 1 void volume. Inflow at the top of the columns from the continuous cultures in excess of the flow through the column was shunted off to waste receptacles. The columns were covered with aluminum foil and kept at 18°C. For the first 7 days, both columns received outflow from a continuous G4 5223-PR1 culture with a cell density of 3×10^7 cells ml^{-1} and a generation time of approximately 100 h. After the columns were saturated with G4 5223-PR1, no more cells were added, and we attempted to elute the columns by changing the inflow preparation to sterile basal salts medium without a carbon source. After 22 days of elution, column stoppers were removed from sampling ports and sterile glass tubing (diameter, 0.75 mm) was used to obtain horizontal core samples of the column sediment at each depth. These sediment samples were partitioned into fixative vials and diluted with 10 ml of 0.1% sodium PP_i (pH 7.0) in preweighed, screw-cap, disposable tubes. The diluted PP_i sediment samples were mixed by vortexing them at a high speed for 30 s, and the supernatant fluids were used for plate counting. The tubes were then placed in a drying oven to obtain the dry weights of the sediments used for these analyses. The remainder of the column material was frozen at −70°C for future use.

Determination of the growth rate of a protozoan on G4 5223-PR1. An unidentified kinetoplastid flagellate was isolated with a drawn micropipette and was used to establish a clonal culture. This flagellate was maintained on washed G4 5223-PR1 cells. To demonstrate the ability of the flagellate to utilize G4 5223-PR1 as prey, the bacterium was grown on phthalate-containing medium, pelleted by centrifugation, and washed so that it was free of culture fluid, and the optical density at 480 nm of the preparation was adjusted to 0.1. Flagellates inoculated into this cell suspension were counted in periodically fixed aliquots with a hemacytometer. The growth rate of the flagellate was calculated as the slope of natural log-transformed data versus time.

Isolation and characterization of LPS. G4 5223-PR1 was grown overnight in Luria-Bertani medium and harvested by centrifugation. Lipopolysaccharide (LPS) was isolated by using an ethanol extraction procedure, as previously described (6). The LPS was separated by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20), using a 13% polyacrylamide stacking gel. The gels were stained with silver to visualize the LPS (37).

MAb production. An overnight culture of G4 5223-PR1 grown in Luria-Bertani medium was fixed in 4% formaldehyde–PBS for 1 h on ice. The fixed cells were pelleted by centrifugation and washed twice in PBS. About 10^7 cells in 0.25 ml of PBS were mixed with 0.25 ml of Freund's incomplete adjuvant and injected intraperitoneally into BALB/c mice. Two booster injections were given at 2-week intervals. At 3 days before fusion, the last injection was made without Freund's adjuvant. Fusion was performed by using the method of Peters et al. (25) and 50% (wt/vol) polyethylene glycol. The fusion products were plated on Optimem 1 (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum, 2 mM glutamine, and azarine-hypoxanthine (Gibco). Hybridoma supernatants were assayed with an enzyme-linked immunosorbent assay (ELISA) by using purified LPS or whole cells as the antigen. Hybridoma cells that produced specific antibodies were subcloned and cultured in roller flasks. After 2 weeks of incubation at 37°C, the supernatants were harvested by centrifugation at 16,000 × *g* for 20 min and then supplemented with NaCl (final concentration, 3.3 M) and boric acid (final concentration, 0.1 M). The pH was adjusted to pH 8.9, and the antibodies were isolated and concentrated by affinity chromatography by using a protein A-Sepharose CL 4B column (30).

Characterization of MAbs. Antibody classes were determined by an ELISA in which isotype-specific antisera (Dianova, Hamburg, Germany) were used. The O-antigen specificity of MAbs was determined by semidry Western blotting (immunoblotting) (17), using a current of 5 mA/cm^2 and a blotting time of 1.5 h. ELISA in which purified LPS or whole cells were used were performed to verify the specificity of the MAbs. For whole-cell testing overnight cultures were preserved in 2% formaldehyde and washed in PBS. Approximately 10^7 cells or 1.5 μg of purified LPS in 50 μl of PBS was added to each well of a microtiter plate (type F96 Maxisorp Immuno Plate; Nunc, Roskilde, Denmark). After overnight incubation at 4°C, the wells were blocked with 200 μl of 10% fetal calf serum in PBS for 1 h at 30°C. Then, 50 μl of undiluted supernatant was added, and the preparation was incubated for 1 h at 30°C. The plate was washed three times with 250 μl of PBS and then incubated with 50 μl of a 10^3-fold dilution of peroxidase-conjugated goat anti-mouse immunoglobulins (Dianova). After incubation for 1 h at 30°C, the plate was washed as described above. Staining was performed with *o*-phenylenediamine.

Fluorescent-antibody and DAPI staining of sediment and groundwater samples. All bacterial cell samples were fixed with formaldehyde (final concentration, 2%) prior to immunofluorescence staining. The fixed cell preparations were filtered with black Nuclepore filters (diameter, 25 mm; pore size, 0.2 μm; Nuclepore, Pleasanton, Calif.) on filtration towers (diameter, 13 mm). The filters were washed with PBS and blocked for 1 h with 0.5 ml of 2% bovine serum albumin (BSA) in PBS to prevent nonspecific binding. The filters were incubated with 0.5 ml of a 1:200 dilution of purified MAb in PBS containing 1% BSA and incubated for 1 h at room temperature (RT). After the filters were washed three times with 1 ml of PBS, 0.5-ml portions of 1:200 dilutions of fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins were added. The secondary antibody was incubated for 1 h at RT, and the filters were washed as described above. After fluorescent-antibody staining, the bacteria were stained with 4′,6-diamidino-2-phenylindole (DAPI) as described by Porter and Feig (26). Total direct counts were determined with DAPI alone. DAPI preparations were also used to estimate the numbers of bacteriovorous protists. The stained filters were examined with a Nikon Optiphot microscope equipped with a mercury light source and filters for FITC and DAPI fluorescence. A computer-assisted image analysis of the fluorescent video images was performed by using a Nikon model SA fluorescence microscope equipped with an MTI-DAGE CCD-75 camera and a GEN II photo intensifier and MicroComp particle analysis software.

Colony blot assay. Nitrocellulose filters (pore size, 0.45 μm; diameter, 82 mm; Schleicher & Schuell, Dassel, Germany) were placed on the surfaces of agar plates until they were saturated. The filters were removed and covered on both sides with a single sheet of Whatman filter paper. The sandwich was exposed for 20 min to dry heat at 65°C. After each reverse side was wetted, the filters were carefully removed from the Whatman paper and blocked overnight at 4°C by using 5% (wt/vol) skim milk in PBS. The filter disks were washed three times in PBS and then incubated for 2 h at RT in a 10^3-fold dilution of the purified MAb in PBS containing 0.1% (wt/vol) BSA. All incubation and washing steps were carried out on a low-speed shaker. After the filter disks were washed three times in PBS, they were incubated for 1 h at RT in a 1:1,000 dilution of peroxidase-conjugated, secondary, goat anti-mouse antibodies (Bio-Rad, Munich, Germany). The filter disks were washed three times in PBS and then developed by using 4-chloro-1-naphthol as the substrate.

Postembedding labeling. Exponentially growing cells were fixed in 0.2% glutaraldehyde–0.5% formaldehyde for 1 h on ice. The fixed cells were sedimented by centrifugation at 10,000 × *g* for 15 min, washed in PBS containing 10 mM glycine, and encased in 2% Noble agar. The cores were then dehydrated by using a series of solutions containing increasing concentrations of ethanol (30 to 100%) and embedded in LR-gold, a new low-temperature acrylic resin (23). Ultrathin sections were mounted on Formvar-coated Ni grids and incubated for 2 min on

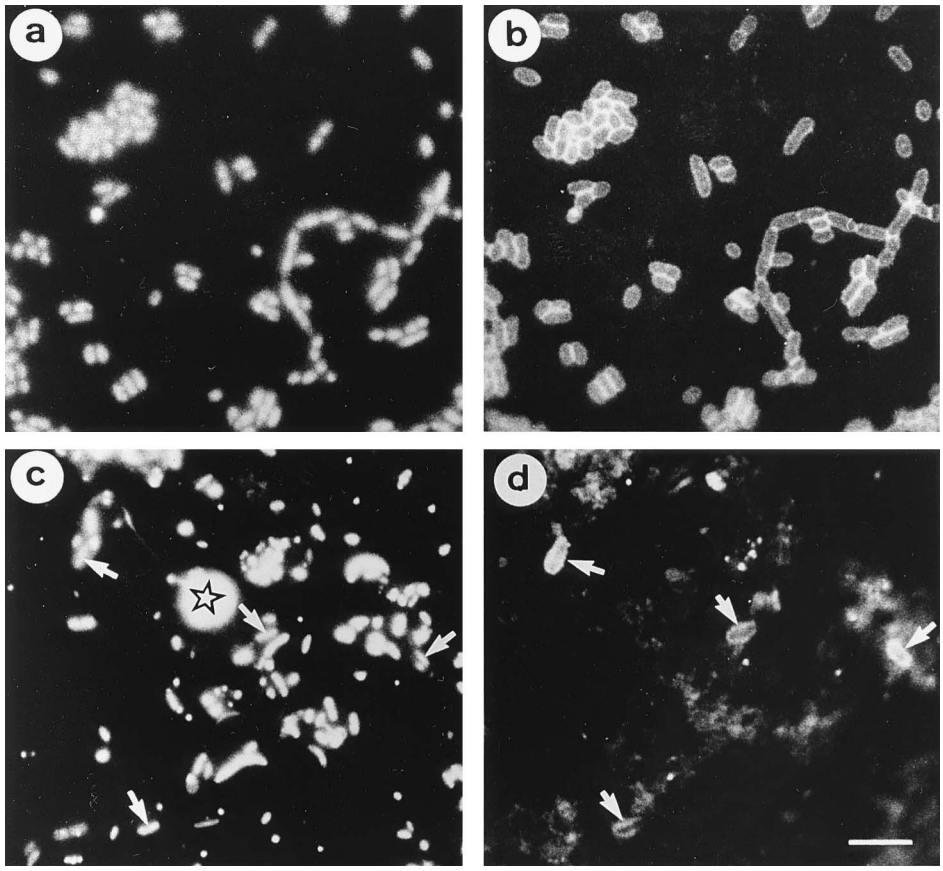

FIG. 2. DAPI- and FITC-stained *B. cepacia* cells. (a and b) DAPI-stained (a) and FITC-stained (b) G4 5223-PR1 cells from a pure culture growing in 10 mM phthalate–minimal salt medium. The cells grew as chains, clusters, and single cells. (c and d) DAPI-stained (c) and FITC-stained (d) G4 5223-PR1 cells released in nonsterile groundwater microcosm. Samples were obtained 4 weeks after G4 5223-PR1 was introduced into the microcosm. The star indicates a DAPI-stained eucaryotic cell. The arrows indicate G4 5223-PR1 cells. Bar = 5 μm.

drops of 1% ovalbumin. After 3 h of incubation on drops of a 1:200 dilution of MAb at RT, the grids were rinsed in PBS and incubated for 2 h at RT on drops containing protein A-coated, 15-nm gold particles (8). The sections were rinsed in PBS and distilled water. The sections were stained with 4% uranyl acetate in distilled H_2O for 20 min at RT; this was followed by washing and a final incubation in lead citrate for 20 min (38). Ultrathin sections were washed and allowed to air dry before observation. Electron micrographs were taken with a Zeiss model EM 10 B transmission electron microscope operating at an acceleration voltage of 80 kV.

RESULTS

Isolation of LPS-specific hybridomas. A pellet of fused hybridoma cells was distributed into 360 tissue culture wells, and hybridoma supernatants were screened against purified LPS. This reduced the number of positive reactions of supernatants compared with the results obtained when whole bacteria were screened, which gave positive reactions for 40 to 80% of the preparations (unpublished data) and did not have the desired specifications. In a second screening, 36 supernatants which had reacted positively in the first ELISA were screened against G4 5223-PR1 cells fixed in formalin and also against a mixture of *Pseudomonas putida* KT 2440 and *Pseudomonas fluorescens* 55. Eight hybridoma supernatants did not react with the other pseudomonads, but did react with both purified G4 5223-PR1 LPS and G4 5223-PR1 cells. These supernatants were used for immunofluorescence staining of G4 5223-PR1 in which FITC-labeled, goat anti-mouse antibodies were used. Three of the eight supernatants yielded strong positive reactions (i.e., brightly shining circles around the cells) (Fig. 2a and b). Hybridomas of these three supernatants were cloned by using a limiting dilution technique. One clone, which secreted immunoglobulins belonging to the immunoglobulin G3 subclass, was chosen because of its strong immunofluorescence signal and production of high levels of MAb; this clone was designated NL1.

Characterization of the MAb. PAGE of purified LPS from G4 5223-PR1 resulted in separation of the LPS into a lipid A-core region and ladder-like bands typical of the O-antigen of the smooth form of LPS (Fig. 3, lane a). An immunoblot of the fractionated LPS revealed the specific reactivity of the NL1 antibody toward the O-antigen bands (Fig. 3, lane b). Figure 4 shows the peripheral immunocytochemical labeling of the O-antigen in an ultrathin section of G4 5223-PR1 cells by NL1 antibody and protein A-gold. The distribution of the gold particles on the bacterial cell periphery was random; there was no area where the density was distinctively higher than other areas. The specificity of the MAb from NL1 was assessed by performing ELISA with formalin-fixed cells of bacteria which may be found in soil or water (Table 1). We found that the MAb from NL1 reacted exclusively with G4 5223-PR1. Culturable isolates, as well as indigenous bacteria washed out of aquifer sediment obtained from the target release site, exhibited no reactivity when we used the MAb either for the colony blot assay or for immunofluorescence microscopy.

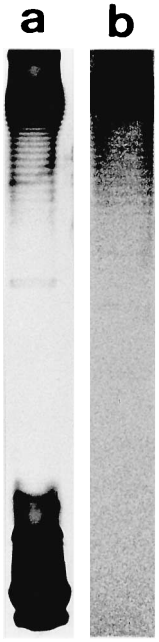

FIG. 3. Silver staining pattern of *B. cepacia* LPS and immunological recognition of the LPS by MAb NL1. Lane a, silver staining pattern of G4 5223-PR1 LPS (3 μg); lane b, immunoblot obtained with purified G4 5223-PR1 LPS and NL1 antibodies, revealing the reactivity toward the O-antigen bands.

Survival of G4 5223-PR1 in groundwater. In sterilized groundwater the G4 5223-PR1 population was stable for 30 days, as determined by total DAPI counting, immunofluorescence counting, and selective plate counting (Fig. 5a). The immunofluorescence values accounted for 100% of the total DAPI counts throughout the incubation period, indicating that there was no loss or change of epitopes due to starvation. The culturable counts declined within 5 days and leveled off at a value (7×10^5 cells ml^{-1}) which was approximately 1 order of magnitude lower than the microscope count value. In nonsterile groundwater G4 5223-PR1 cells could be clearly identified by using FITC-labeled NL1 antibodies (Fig. 2c and d). The culturable counts and the immunofluorescence counts were indistinguishable and declined to levels below the limit of de-

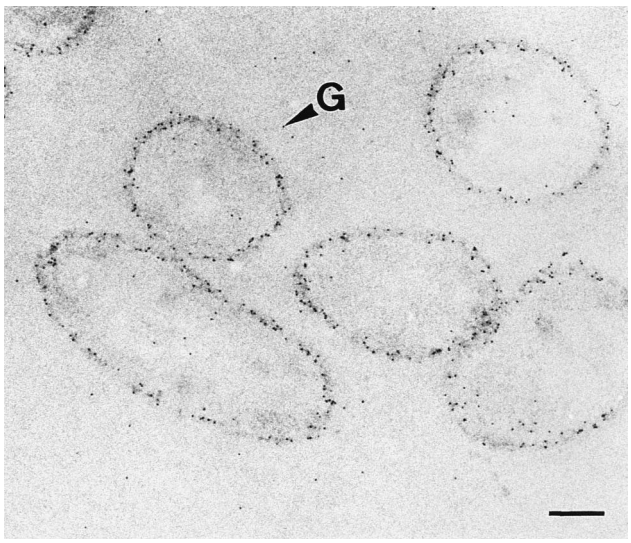

FIG. 4. Immunoelectron microscopic labeling of the O-specific side chains of G4 5223-PR1: thin section of G4 5223-PR1 cells incubated with NL1 antibody and then with protein A-gold particles (15 nm). G, gold particle. Bar = 1 μm.

TABLE 1. Strains used in serological tests

Strain	Source or reference[a]	ELISA results[b]
Burkholderia cepacia G4 5223-PR1	32	+
Burkholderia cepacia LMG 1222[Tc]	LMG	−
Burkholderia cepacia LMG 6889	LMG	−
Pseudomonas putida KT2440	28	−
Pseudomonas fluorescens 55	13	−
Comomonas acidovorans LMG 1226[T]	LMG	−
Comomonas testosteroni LMG 1800[T]	LMG	−
Rhizobium sp. strain DSM 30138	DSM	−
Sphingobacterium spiritivorum LMG 8347[T]	LMG	−
Sphingomonas sp. strain RW1 (= DSM 6014)	DSM	−
Cytophaga johnsonae LMG 1341[T]	LMG	−
Rhodococcus rhodochrous DSM 43241	DSM	−

[a] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Ghent, Belgium.
[b] The strains were tested for reactivity by using an ELISA with NL1 as the primary antibody. +, positive reaction; −, negative reaction.
[c] T, type strain.

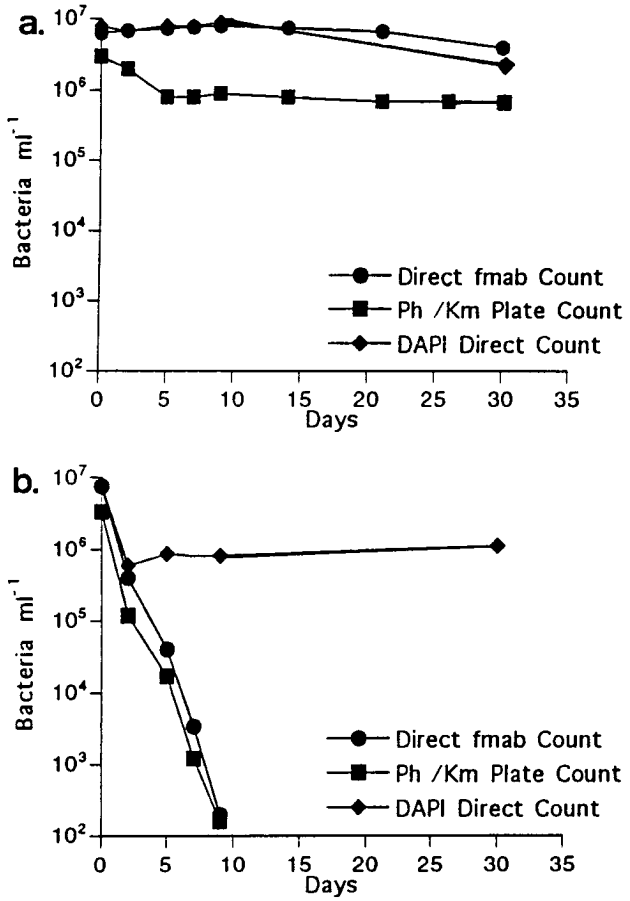

FIG. 5. Survival of G4 5223-PR1 after introduction into groundwater microcosms. (a) Sterilized groundwater. (b) Nonsterile groundwater. Survival was monitored by plate counting on Ph/Km medium, DAPI counting, and direct immunofluorescence counting (fmab).

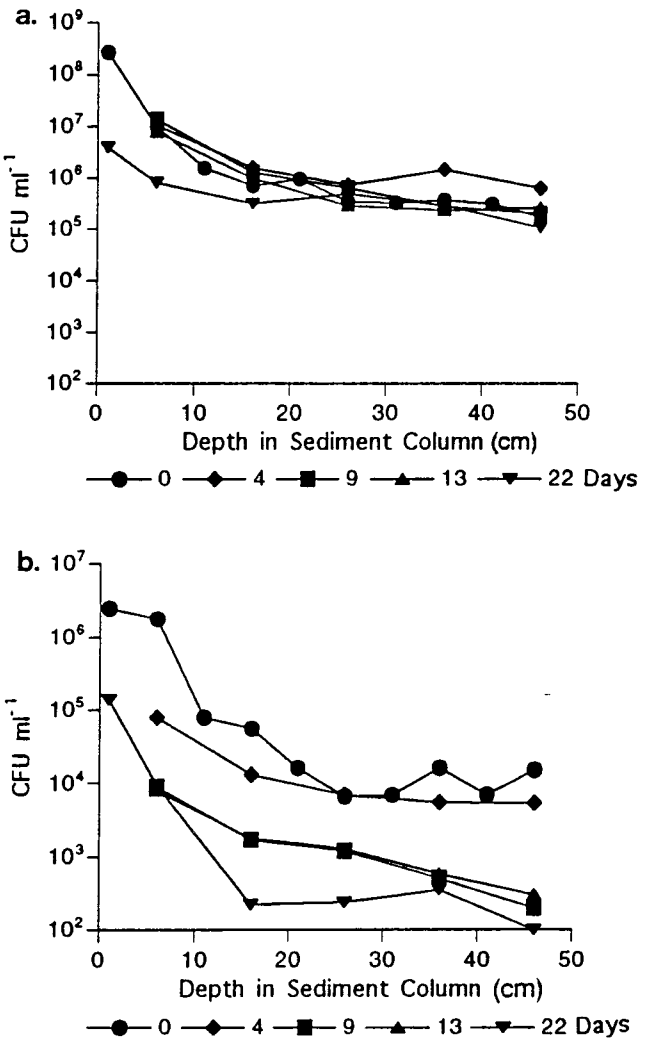

FIG. 6. Survival of *B. cepacia* after introduction into sediment columns: CFU on Ph/Km medium. (a) Sterilized sediment column. (b) Nonsterile sediment column. The plate counts indicated that greater losses of G4 5223-PR1 occurred in the nonsterile sediment column and that relatively stable populations of cells persisted after 9 days of elution.

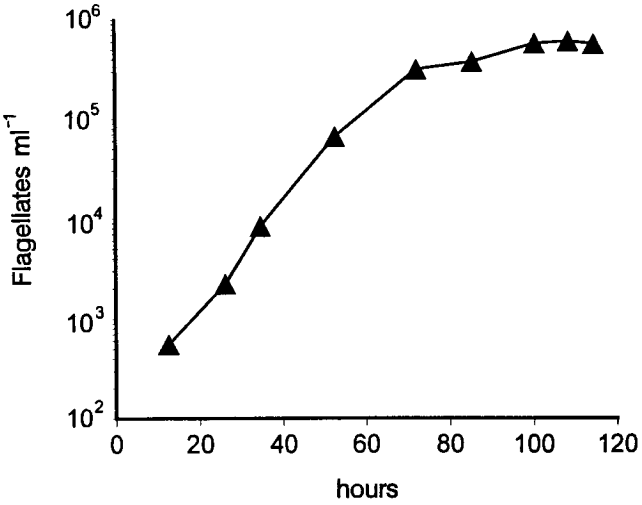

FIG. 7. Growth of an unidentified kinetoplastid flagellate from Borden aquifer material on a suspension of washed G4 5223-PR1 cells. This organism was a dominant predator in the aquifer microcosms.

tection (20 cells ml^{-1}) within 10 days, while the total number of cells remained stable after 5 days at levels of 8×10^5 to 1×10^6 cells ml^{-1} (Fig. 5b).

Addition of G4 5223-PR1 to aquifer sediment columns. A continuous culture of G4 5223-PR1 grown with 50 μM phthalate as the sole carbon source was pumped to the top of both sediment columns (Fig. 1). The total direct count for bacteria in the column inflow was 3×10^7 cells ml^{-1}. G4 5223-PR1 was transported through sterilized and nonsterile sediment columns within 4.5 days. Since the water content was 0.39 ml per ml of sediment and the flow rate through the columns was 11 cm day^{-1}, the transport of G4 5223-PR1 occurred during the time that it took for approximately 1 void volume to be replaced.

Elution of G4 5223-PR1 from aquifer sediment columns. After 7 days the continuous input of the G4 5223-PR1 suspension was stopped and replaced by sterile inorganic salts. Figure 6 shows the distribution of CFU at different depths in both columns over time. Colonies that grew on Ph/Km medium were still detected in porewater samples obtained from throughout both columns after 22 days of elution (the time that it took for about 5 void volumes to pass through a column). The decrease in the nonsterilized sediment column was much more rapid than the decrease in the sterilized sediment column. The plateable bacteria were reduced to low numbers (10^2 to 10^3 cells ml^{-1}), but the bacteria were not completely eliminated, in contrast to the result obtained with the nonsterile groundwater microcosm. The loss of G4 5223-PR1 cells was presumably due to predation by bacteriovorous protists. High numbers (10^4 to 10^5 cells ml^{-1}) of two species of flagellate protists were observed in the nonsterile column. The ability of a dominant unidentified kinetoplastid flagellate (data not shown) obtained from the Borden aquifer to grow on a monospecific suspension of G4 5223-PR1 is shown in Fig. 7. The maximum growth rate of the flagellate in this batch culture was 0.109 h^{-1}, corresponding to a generation time of 6.38 h. This growth rate is among the highest growth rates achieved with bacterial species with this flagellate in the laboratory (data not shown) and indicates that G4 5223-PR1 is readily utilized by this protist. During the entire experiment the numbers of CFU in the sterilized sediment were between 1 and 3 orders of magnitude higher than the numbers of CFU at equivalent depths in the nonsterilized sediment (Fig. 6), and the numbers of CFU in the sterilized sediment appeared to be relatively stable over time.

Sediment extraction. After 22 days of elution with sterile medium, the column flow was stopped, and sediment samples were removed from the columns at each depth and extracted by using 0.1% sodium PP_i buffer. The number of CFU obtained after PP_i extraction of the sterilized column samples decreased 1 order of magnitude with depth, whereas the number of CFU in the nonsterile column samples decreased 3 orders of magnitude with depth (Fig. 8). On the basis of the porewater volume in sediment samples and dilution by PP_i buffer, the numbers of CFU (as determined on Ph/Km medium) per milliliter of porewater from sediment extraction samples were, on average, 10-fold higher than the numbers of CFU in porewater samples obtained with syringes from the sterilized sediment column. This difference was greater (160-fold) in the nonsterile sediment column, indicating that a significantly greater loss of porewater-free cells occurred.

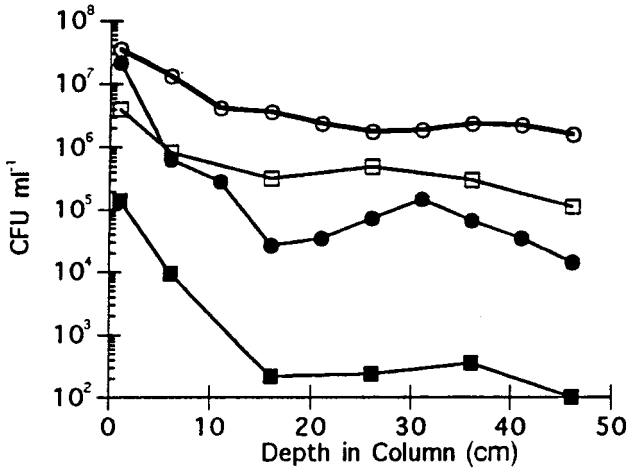

FIG. 8. Numbers of particle-associated and porewater-free *B. cepacia* cells (after 22 days of elution), expressed as the number of CFU per milliliter of porewater or sodium PP_i extract. Symbols: ○, sterilized sediment, PP_i extract; □, sterilized sediment, porewater; ●, nonsterile sediment, PP_i extract; ■, nonsterile sediment, porewater. The numbers of CFU in PP_i extracts were normalized to the calculated porewater volume of the amount of sediment extracted to determine the discrepancies in numbers due to particle-associated cells.

Immunodetection of G4 5223-PR1 in sediment column samples. Quantitative immunofluorescence direct counting of G4 5223-PR1 in aquifer material was impeded by high levels of background fluorescence. The fluorescence of the cells from the columns was also weaker than the fluorescence of the inflow cells. However, immunolabeled G4 5223-PR1 cells could be clearly identified in column outflow samples. The MAb was most useful for distinguishing G4 5223-PR1 colonies from colonies of other bacteria that grew on Ph/Km medium plates. After 22 days of elution, the NL1 antibody in a colony blot immunoassay unambiguously identified all G4 5223-PR1 colonies (Fig. 9).

Plasmid transfer from G4 5223-PR1 into indigenous bacteria. Selective plating of column sediment samples obtained from different depths resulted in growth of colonies which could be clearly identified as non-G4 5223-PR1 colonies (Fig. 9). Subsequent spraying of the plates with 0.5 M catechol produced yellow-stained colonies as a result of catechol 2,3-dioxygenase activity and conversion to α-hydroxymuconic ϵ-semialdehyde. All colonies that were negative when they were examined by colony blotting with the NL1 antibody failed to turn yellow after catechol treatment. Thus, when plasmid-encoded catechol 2,3-dioxygenase of G4 5223-PR1 was used as the reporter enzyme, no evidence that pTOM was transferred from G4 5223-PR1 into indigenous bacteria was found.

DISCUSSION

There is growing interest in the release of cultured bacteria for bioremediation of contaminated aquifers (18). Reliable and sensitive detection and enumeration of these nonnative bacteria in environmental samples are fundamental requirements for such projects. MAbs against the O-specific side chains of gram-negative bacteria provide a very specific tracking mechanism. Antibodies against more conserved, peripheral cell structures, such as outer membrane proteins, often cross-react with different serotypes (10–12). Moreover, LPS is a constituent of the cell wall and thus is expressed under a wide variety of nutritional conditions.

The NL1 antibody was highly specific when it was tested with a range of different soil bacteria; it recognized only G4 5223-PR1. The epitope proved to be stable for 30 days of incubation in an oligotrophic groundwater microcosm. G4 5223-PR1 was easily detected in groundwater samples (Fig. 2c and d). In sterile groundwater we observed after 5 days quantitative discrepancies of about 1 order of magnitude between G4 5223-PR1 immunofluorescence direct counts and the numbers of CFU on selective plates. It has been reported previously that portions of bacterial populations may become unculturable but retain other characteristics of viable cells, including plasmids (3, 4, 14, 31); these previous findings suggest that we should be cautious in relying solely on plating techniques for tracking purposes. An alternate explanation for the difference between the direct count and plate count data is that cells aggregated and adhered to small particles during the first few days (3), although only minimal clumping was observed in direct microscopic examinations. After the initial decrease, the number of G4 5223-PR1 cells remained stable for at least 25 days, indicating that starvation was not a primary factor in decreases in the numbers of G4 5223-PR1 cells in the systems which we studied. Direct immunofluorescent counting of G4 5223-PR1 cells in samples extracted from sediment was limited by non-

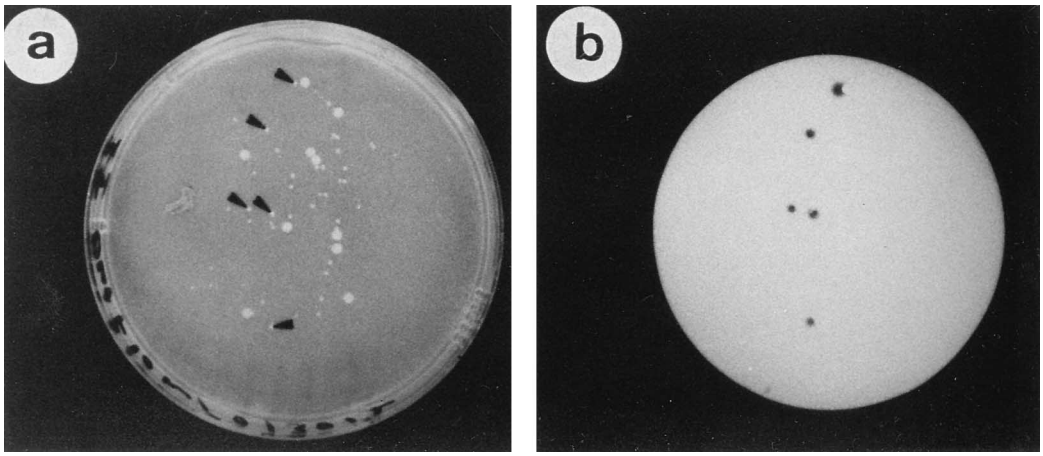

FIG. 9. Quantification of *B. cepacia* by a colony blot immunoassay. (a) Ph/Km agar plate inoculated with 50 μl of outflow material from the nonsterile column and incubated for 1 week at 18°C. The arrowheads indicate G4 5223-PR1 colonies, which were identified on the corresponding G4 5223-PR1-specific colony blot (b).

specific background fluorescence. This background fluorescence may have been due to a combination of nonspecific adsorption of the MAb, which could not be prevented by blocking agents, and autofluorescence of sediment material.

The colony immunoblot assay, in which antibodies specific for peripheral antigens are used, is a rapid method which has been used previously to identify pathogenic microorganisms (2, 34). This technique was used to detect G4 5223-PR1 in sediment samples, because it was not always possible to identify all G4 5223-PR1 colonies by the selective plating technique (Fig. 9). The colony blot assay provides a quantitative and definitive method for monitoring culturable cells in samples containing large numbers of particles. Semiquantitative immunological methods for detecting microorganisms, including ELISA (24) and dot blot microfiltration (29), either did not provide the desired sensitivity or are not practical for sediment samples because of limited filtration properties. Immunomagnetic beads that are coated with antibodies and used as capture probes (5, 7, 21) would be an appropriate immunological method for enrichment of target cells from the background population and would enable a further decrease in the limit of detection. In addition, the combination of MAb technology and screening for catechol 2,3-dioxygenase activity provides a method for monitoring both plasmid maintenance in G4 5223-PR1 and transfer of pTOM to indigenous bacteria under selective or even nonselective growth conditions.

The survival and activity of G4 5223-PR1 in batch systems containing sterile aquifer material have been described recently (19). The information presented in this paper for both static and flowthrough systems demonstrates that the number of G4 5223-PR1 cells in nonsterile systems appears to be subject to both transport and predation losses in a treatment zone. The constant removal of bacteria by protist grazing pressure should result in a reduction in the number of cells of nongrowing species as the bacterial assemblage turns over. Selective feeding by protists on larger bacterial cells has been observed previously (9) and could enhance rates of loss of larger cultured cells introduced into the system because of the population turnover effect. However, a significant refuge from predation and transport obviously is provided by sediment particles. Thus, monitoring groundwater for only free cells in native systems may miss most of the target bacteria. Brettar and Höfle (3) have demonstrated that survival of bacteria introduced into lake water was enhanced by association with particulate material. In our study, residual populations in the nonsterile column were relatively stable under our flow and predation pressure conditions during 22 days of elution. The high number of PP_i-extracted CFU in the nonsterile column suggests that this difference could be accounted for by survival of more particle-bound bacteria than bacteria living unbound within the porewater in the presence of predation pressure. The populations may be too small to effectively mineralize TCE (19), but they should be considered significant when the potential impact of introducing a nonnative bacterium into aquifer systems is studied.

The survival of G4 5223-PR1 in sterilized groundwater and sediment suggests that the abiotic conditions of the target environment will not limit survival of this organism. The rapid loss of G4 5223-PR1 in nonsterile groundwater demonstrated the importance of biotic interactions in limiting survival of cells in porewater. The persistence of G4 5223-PR1 in nonsterile sediment under flow conditions in the presence of high numbers of bacteriovorous protists suggests that particle surface-biofilm associations are important for the survival of nonindigenous microorganisms in aquifer environments.

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