Tracking Genetically Engineered Bacteria: Monoclonal Antibodies against Surface Determinants of the Soil Bacterium Pseudomonas putida 2440

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Assessment of potential risks involved in the release of genetically engineered microorganisms is facilitated by the availability of monoclonal antibodies (MAbs), a tool potentially able to monitor specific organisms. We raised a bank of MAbs against the soil bacterium Pseudomonas putida 2440, which is a host for modified TOL plasmids and other recombinant plasmids. Three MAbs, 7.3B, 7.4D, and 7.5D, were highly specific and recognized only P. putida bacteria. Furthermore, we developed a semiquantitative dot blot assay that allowed us to detect as few as 100 cells per spot. A 40-kDa cell surface protein was the target for MAbs 7.4D and 7.5D. Detection of the cell antigen depended on the bacterial growth phase and culture medium. The O antigen of lipopolysaccharide seems to be the target for MAB 7.3B, and its in vivo detection was independent of the bacterial growth phase and culture medium. MAb 7.3B was used successfully to track P. putida (pWW0) released in unsterile lake mesocosms.

Recent achievements in molecular biology have provided efficient ways to manipulate prokaryotic and eukaryotic organisms, and a number of possible applications for these “new” organisms are envisaged in the food, health, and agricultural industries. Bioremediation is an area of increasing interest for decontamination of polluted areas; such treatments involve, among other strategies, release of large numbers of wild-type or modified microorganisms to decontaminate polluted soils, water, and sediments.

Among soil bacteria, the pseudomonad group exhibits an enormous range of metabolic activities against biogenic and xenobiotic chemicals (31). Furthermore, the catabolic pathways of pseudomonads have been used as model systems to expand the range of recalcitrant chemicals that microorganisms can mineralize (34).

We have centered our attention on Pseudomonas putida 2440 (12), a restriction-negative strain which was derived from P. putida mt-2, the natural host for archetypal TOL plasmid pWW0 (49). P. putida 2440 has been used as the host for recombinant TOL plasmids able to eliminate aromatic hydrocarbons that cannot be degraded by non-pWW0-modified pathways (1, 35). Furthermore, P. putida 2440 has been shown to be an excellent recipient for recombinant plasmids through conjugation and transformation (4), and it has been used in several biotransformation processes, e.g., production of benzyl alcohol (9).

Concerns about the potential risks associated with deliberate or accidental release of large numbers of genetically engineered microorganisms have been raised because of the unpredictability of their immediate and long-term behavior in the environment. The assessment of potential risks will be facilitated by the availability of monoclonal antibodies (MAbs), which make it possible for an organism to be specifically monitored and efficiently quantitated.

We raised highly specific MAbs against surface determinants of P. putida 2440 (lipopolysaccharide [LPS] and proteins) and developed a solid semiquantitative assay that allowed us to detect, within hours, as few as 100 cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this work were P. putida 2440 (49), P. putida 2440(pWW0) (12), P. putida 2440(pWW0-EB62) (1), PaW340 and PaW94 (S. Harayama), and 29 and 43 (D. Springael); Pseudomonas sp. strain B13 (10); P. aeruginosa (this laboratory); P. fluorescens (17); P. syringae (17); P. oleovorans (M. Wubbolts); P. putida (45); Rhizobium melloti 4GRB13 (5); R. leguminosarum 3855 (19); Erwinia chrysanthemi (47); Acinetobacter calcoaceticus (29); Azotobacter vinelandii (20); Agrobacterium tumefaciens 281 (14); Alcaligenes eutrophus (32); Escherichia coli JM101 (40); Klebsiella pneumoniae (J. Casadesús); K. aerogenes (30); Salmonella typhi-murium (J. Casadesus); a Corynebacterium sp. (this laboratory); Bacillus subtilis (32); Flavobacterium marinospticum (Spanish Culture Collection 578) (44); and Synchocystis sp. strain PCC 6803 (A. Muro).

Bacterial strains were grown at 30°C in LB medium (40), except for R. mellioti GRB13, R. leguminosarum 3855, and A. tumefaciens 281, which were grown at 28°C on Ty medium (5). Cyanobacteria were grown on BG11 medium (37), and P. marinotpticum was grown on nutrient medium II (40).

P. putida strains were also grown on M9 minimal medium (1) with glucose (0.5% [wt/vol]), 10 mM succinate, and 5 mM m-methyl- and p-ethylbenzoate as the sole C source.

Reagents and solutions. Phosphate buffer solution (PBS) was 20 mM sodium phosphate and 15 mM sodium chloride,
pH 7.4. This solution was supplemented with 0.05% (vol/vol) Tween 80 (PBST), albumin at the concentrations indicated (PBSA), or 0.05% (vol/vol) Tween 80 and albumin (PBSTA) at the concentrations indicated. PBST-azide was PBS supplemented with 0.1% (wt/vol) sodium azide. Poly-l-lysine solution was 1 mg of poly-l-lysine in 100 ml of PBS. Tris buffer solution (TB) was 50 mM Tris-HCl, pH 7.5.

Peroxidase-conjugated and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins were from Dakopatts (Denmark). Nitrocellulose membranes were type HA (0.45-μm pore size; Millipore Corp.).

**MAb Production.** *P. putida* 2440 cells were grown overnight on LB medium. Cells were harvested by centrifugation and washed three times in PBS. About 10⁸ cells in 0.25 ml of PBS were intraperitoneally injected into BALB/c mice. The injection was repeated three times at 1-week intervals. In the fourth injection, 10⁹ cells were subepidermally injected in incomplete Freund adjuvant. Finally, 3 days prior to fusion, the mice received 10⁶ cells in PBS intravenously. About 1.2 × 10⁶ spleen lymphocytes from a previously immunized mouse were fused with 3 × 10⁶ cells of mouse myeloma cell line NS-1 using polyethylene glycol as described by Köhler and Milstein (21). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium and then amplified in hypoxanthine-thymidine medium. Production of antibodies against *P. putida* 2440 was assayed by indirect immunofluorescence. Hybridomas producing highly specific antibodies against *P. putida* 2440 were cloned by limiting dilution to less than 1 cell per well. Selected clones were then used to produce ascites tumors in pristane-primed mice. Cells were removed from the ascites fluids by centrifugation at 2,800 × g for 10 min, and aliquots of cleared ascites fluids were stored at −70°C.

**Isotype Analysis.** Antibody class was determined by Ouchterlony (double-diffusion) analysis using isotype-specific antisera.

**ELISAs.** Enzyme-linked immunosorbent assays (ELISAs) using whole cells were performed basically as described by Hermoso et al. (15). To improve the binding of whole cells to the polyvinyl chloride microtiter plates, 50 μl of poly-l-lysine solution was added per well and removed after 30 min at room temperature. About 10⁶ CFU in 50 μl of fixation buffer (100 mM sodium bicarbonate, pH 9.4) were then added and incubated overnight at 4°C. Cells were washed three times (5 min each time) with 200 μl of PBST. Subsequently, a first blocking step was carried out with 100 μl of PBSTA containing 0.1% albumin for 30 min. Fifty microliters of 10³-fold-diluted ascites fluid in PBSTA containing 0.1% albumin was immediately added and incubated for 2 h. Plates were washed as described above with PBST, and finally 75 μl of a 10³ dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins was added. Incubation conditions and washes were as described for the first antibody. As the substrate for peroxidase, 200 μl of 100 mM citrate–200 mM phosphate buffer (pH 5.0) containing 40 mg of o-phenylenediamine hydrochloride per 100 ml and 40 μl of hydrogen peroxide per 100 ml was added. The reaction was developed for 15 min and stopped by adding 100 μl of 4 M sulfuric acid. A₄₉₂ was determined with an ELISA plate reader.

**Immunofluorescence.** About 5 × 10⁶ *P. putida* 2440 cells were harvested by centrifugation and washed twice with 3 ml of PBS-azide. Washed cells were then incubated for 30 min with hybridoma supernatants or ascites fluids at appropriate dilutions. All steps were carried out at 4°C. The cells were then washed twice and incubated with 25 μl of FITC-conjugated rabbit anti-mouse immunoglobulin diluted to 1/20 in PBS-azide for 30 min. The cells were washed twice and resuspended in PBS-azide containing 10% (vol/vol) glycerol and 100 μg of o-phenylenediamine per ml to prevent fading (25). Immunofluorescence was evaluated with a Zeiss epifluorescence microscope (magnification, ×1,000).

**Polypeptide Analysis.** Cell lysates were prepared by mixing suspensions of cells with sample buffer (24). After the viscosity of the samples was reduced by sonication (35 W, 2 × 30 s), samples were boiled for 10 min and 100 μg of protein was electrophoresed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the Laemmli method (24). Running gels of 17.5% (wt/vol) acrylamide and stacking gels of 5% (wt/vol) acrylamide were poured. Proteins in Western blots (immunoblots) were stained with amido black (46). Protein concentrations were determined as described by Lowry et al. (26), with bovine serum albumin as the reference.

**Analysis of LPS Patterns.** For analysis of LPS, an overnight culture of *P. putida* 2440 was harvested by centrifugation, washed twice in PBS, and resuspended in this buffer to an optical density at 525 nm (OD₅₂₅) of 0.5 to 0.6. A 1.5-ml volume of the cells in PBS was centrifuged and suspended in 50 μl of SDS-PAGE lysing buffer (24). Pronase digestion was then carried out at 60°C for 60 min (16). LPS was selected separately electrophoretically under the conditions described above for SDS-PAGE, and LPS were stained by the Ag LPS- and protein-staining method of Hitchcock and Brown (16).

**Dot blotting method.** Dot blotting was performed with a Bio-Dot Microfiltration Apparatus (Bio-Rad). A nitrocellulose membrane was soaked in PBS for 30 min. One hundred microliters of PBSTA with 1% albumin was added per well to block the filter. Then, 50 μl of serial dilutions (from 10⁴ to 10⁻¹) of *P. putida* 2440 in PBSA with 1% albumin was added per well; cells were then blocked by adding 100 μl of PBSA with 1% albumin. The cells were washed three times with PBST, and then 50 μl of ascitic fluid appropriately diluted in PBSTA with 1% albumin was added per well. All subsequent steps were as for the ELISA, except that PBS and PBST were always supplemented with 1% (wt/vol) albumin. The reaction was developed outside the device with 40 μg of 3,3'-diaminobenzidine and 40 μl of hydrogen peroxide per 100 μl of PBS and stopped with distilled water.

**Immunodetection of Proteins and LPS.** Electrophoretically separated polypeptides and promase-resistant cellular components were transferred to nitrocellulose paper by electroblotting (250 mA for 5 h for proteins and 3.5 h for LPS) (43). Blots were dried at room temperature, and only those containing LPS were baked at 80°C for 2 h. Subsequently, blots were blocked with PBSA with 2% albumin for 1 h and incubated with 10³-fold-diluted ascites fluids in PBSTA with 1% albumin for 2 h (or overnight at 4°C). After five washes in PBST for 6 min each, blots were incubated with 10³-fold-diluted peroxidase-conjugated rabbit anti-mouse immunoglobulins in PBSTA with 1% albumin for 2 h. The blots were then washed three times with PBST, once with PBS, and then once with TB. Western blots were developed by using 100 μg of 4-chloro-1-naphthol in 10 ml of 1-propanol and 250 μl of hydrogen peroxide per 100 ml of TB. LPS blots were developed by using either 4-chloro-1-naphthol or 50 μg of 3,3'-diaminobenzidine and 75 μl of hydrogen peroxide in 100 ml of TB. Reactions were stopped in TB.

**In situ analysis of *P. putida* by 4,6-diamidino-2-phenylindole (DAPI) staining and immunofluorescence.** The experimental design for release of monocultures of *P. putida* (PWW0) in...
mesocosms in a eutrophic lake (Plussee, northern Germany) is described elsewhere (6).

Bacterial cells were fixed after sampling with formaldehyde (final concentration, 2%). Cells were first stained with DAPI (Sigma, St. Louis, Mo.). DAPI staining was performed in the water samples with a final DAPI concentration of 6 ng/ml. The DAPI-stained cells were filtered on a black Nuclepore filter (25-mm diameter, 0.2-μm pore size; Nuclepore Corp.). For immunofluorescence staining on the filter, 0.5 ml of the primary antibody (MAb 7.3B) was applied for 1 h at a dilution of 1:100 in PBSA with 0.5% albumin. The FITC-conjugated anti-mouse immunoglobulin M antibody (Sigma Chemical Co.) was applied twice (0.5 ml each time) for 15 min at 1:120 dilution in PBS.

For microscopic observations, membrane filters were covered with a solution of 20% glycerol in 200 mM Tris-HCl, pH 8.3. To retard fading, 1% 1,4-diazabicyclo(2,2,2)octane (DABCO; Sigma Chemical Co.) was added to the mounting medium.

The cells were observed with a Zeiss epifluorescence microscope equipped with an HBO 50-W light source. Photographs of DAPI-stained cells were made with the Zeiss filter set at 01 (bandpass 365-370 nm exciting filter, FT 395 nm chromatic beam splitter, LP 370 nm barrier filter), FITC immunofluorescence photographs were made with the filter set at 09 (bandpass 450-490 nm exciting filter, FT 510 nm chromatic beam splitter, bandpass 515-565 nm barrier filter). Exposures with subsequent use of both filter sets enabled direct comparison of DAPI-stained and fluorescent-antibody-stained cells. In double-exposure photographs, DAPI- and FITC-stained cells are slightly displaced; therefore, the different staining versions of the same cells can be seen beside each other.

RESULTS

Isolation of MAbs against *P. putida* 2440 surface determinants and specificity profile. Mice were immunized with *P. putida* 2440 as described in Material and Methods. Supernatants from 366 hybridomas were screened by indirect immunofluorescence for production of immunoglobulins against whole cells of *P. putida* 2440. Supernatants from 11 wells gave a positive reaction.

The 11 hybridomas that produced immunoglobulins against *P. putida* 2440 were amplified in mice by inducing ascites tumors. Serial dilutions (500 to 5 × 10⁵) of the 11 ascites fluids were used in ELISAs to test the reactivities of the immunoglobulins against (i) several strains of *P. putida*, (ii) different bacteria belonging to the pseudomonad group, and (iii) different bacterial phyla, including cyanobacteria, flavobacteria, gram-positive eubacteria, and purple bacteria. Among the former, several bacteria belonging to the α (e.g., *Rhizobium* sp.), β (e.g., *Alcaligenes eutrophus*), and γ (enteric bacteria) subdivisions were considered (48). In all, about 30 species of different eubacteria were considered (see below for further details). Three ascites fluids were highly specific against *P. putida*, while the other eight ascites fluids produced cross-reactions with several bacteria and were discarded. The three hybridomas that produced highly specific antibodies were cloned by dilution, and the clones secreting MAbs were used for production of ascites fluids. These MAbs belonged to the immunoglobulin M class and were called 7.3B, 7.4D, and 7.5D.

In ELISAs, it was found that these three MAbs recognized *P. putida* 2440 with and without the TOL plasmid or recombinant TOL plasmids. *P. putida* 43 was recognized only by MAb 7.3B, and none of the MAbs showed cross-reactivity with *P. putida* 29. None of the MAbs recognized pseudomonads such as *Pseudomonas* sp. strain B13, *P. aeruginosa*, *P. fluorescens*, *P. syringae*, *P. oleovorans*, and *P. putida* 29.

These MAbs did not recognize other purple soil bacteria, such as *R. meliloti*, *R. leguminosarum*, *E. chrysanthemi*, *A. vinelandii*, *A. tumefaciens*, *A. calcoaceticus*, and *A. eutrophus*; members of the family *Enterobacteriaceae*, such as *E. coli*, *S. typhimurium*, *K. pneumoniae*, and *K. aerogenes*; or gram-positive eubacteria, such as *B. subtilis* and *Corynebacterium* sp.; cyanobacteria, such as *Synecocystis* sp. strain PPC 6803; and flavobacteria, such as *F. marinotypicum*.

In addition to the above-described ELISAs, immunofluorescence assays were also carried out to test the cross-reactivity of these MAbs. None of the above-mentioned bacteria gave a positive response, except *P. putida* 2440, which confirmed the high specificity of the MAbs against *P. putida* 2440.

The potency of the MAbs was tested against *P. putida* 2440 in ELISAs. It was found that the ascites fluids could be diluted up to 5 × 10⁴-fold before reaching background levels. Ascites fluids were routinely used at 1,000-fold dilutions.

**Solid-phase semiquantitative assay.** To establish the minimum number of *P. putida* 2440 bacteria that could be detected by the specific MAbs, a dot blot ELISA was developed. Serial dilutions of a culture of *P. putida* 2440 grown on LB medium were filtered through a nitrocellulose membrane so that the number of bacteria per well ranged from 10⁵ CFU to less than 1 CFU per well. The immunosay revealed that spots containing as few as 10³ CFU were consistently positive (Fig. 1). It is worth noting that in ELISAs, the minimum number of cells corresponded to about 10⁴ CFU added per well of microtiter plates for MAb 7.3B and about 10⁵ for MAbs 7.4D and 7.5D with 10³-fold diluted MAbs (Fig. 2).

**Presence of the antigen under different growth conditions, in soil, and in lake water samples.** The potential use of these MAbs as tracking reagents is based on expression and stable maintenance of the antigen under different growth condi-
We first studied expression of the antigen in *P. putida* 2440(pWW0) in different growth phases in rich media and minimal medium with different C sources by using microtiter plate ELISAs. With *P. putida* 2440(pWW0) grown on LB medium, it was found that regardless of the growth phase, cells were recognized by all three MAbs (Fig. 3A). However, with cells grown on minimal medium with glucose, succinate, and 3-methylbenzoate as a C-source, different patterns were found, depending on the MAb and the growth phase.

Cells of *P. putida* 2440(pWW0) grown on minimal media and in different growth stages (early and late exponential phase and stationary phase) reacted strongly with MAb 7.3B. In contrast, cells grown in 3-methylbenzoate were not recognized (or were weakly recognized) by MAbs 7.4D and 7.5D in the early exponential phase as well as in the stationary phase; in the late exponential phase, the OD$_{492}$ was about one-fourth of that of cells grown in LB. Cells grown in succinate were not recognized by MAbs 7.4D and 7.5D in the stationary phase (Fig. 3C).

We also tested whether expression of the antigen was maintained after introduction of *P. putida* 2440 bearing recombinant TOL plasmid pWW0-EB62 in soils. *P. putida*(pWW0-EB62) was grown on minimal medium with p-ethylbenzoate as the sole C source and introduced into a sandy loam-fluvisol soil for periods of about 1 month. In situ determinations were hampered by the soil background immunofluorescence and the smaller size of the cells kept in soils. Cells were then recovered from the soil on selective solid agar plates and grown in LB for ELISA and immuno-

**FIG. 2.** Counting of *P. putida* 2440 cells in ELISAs. Serial dilutions of a *P. putida* 2440(pWW0) culture grown on LB were set up. Cells were fixed to polyvinyl chloride microtiter plates, and ELISAs were done as described in Materials and Methods. A$_{492}$ was determined. Symbols: ○, MAb 7.3B; □, MAb 7.4D; Δ, MAb 7.5D; ●, control immunoglobulin M.

**FIG. 3.** Expression of *P. putida* 2440 cell surface antigen determinants under different growth conditions. *P. putida*(pWW0) cells were grown on LB (A) or minimal medium with glucose (B), succinate (C), or m-toluate (D). Cultures in the early exponential phase (E; OD$_{660}$ about 0.3), late exponential phase (L; OD$_{660}$ about 1.5), and stationary phase (S; OD$_{660}$ >3) were harvested and washed in PBS, and about $5 \times 10^6$ cells were fixed to a microtiter plate. After the ELISA, A$_{492}$ was determined. Symbols: ○, MAb 7.3B; □, MAb 7.4D; Δ, MAb 7.5D.
fluorescence assay, which showed that these cells were recognized by all three MAb.s.

The MAb.s raised against P. putida 2440 were successfully used to track the bacterium in aquatic ecosystems. We used MAb 7.3B to track P. putida(pWW0) after release in mesocosms installed in a eutrophic lake (Plussee) in northern Germany. Figure 4 shows well-fed P. putida cells just after introduction into lake water. In the double exposure, all bacteria in the lake water are shown as blue (DAPI-stained) cells; the P. putida cells are easily distinguishable from autochthonous bacteria by their FITC immunofluorescence staining. Figure 5 shows FITC-stained P. putida cells 1 week after introduction into the lake water mesocosms without nutrient addition. A morphological change from rod to small coccoid cells was observed, often with large aggregations like that shown in Fig. 5. Therefore, MAb 7.3B turned out to be a reliable tool for tracking of P. putida bacteria since it showed no unspecific binding to autochthonous bacteria.

Molecular identification of surface antigens which react with MAb.s. LPS prepared from P. putida 2440 were subjected to SDSPAGE and silver staining, which revealed a series of bands ranging from about 45 to 55 kDa (Fig. 6). In LPS blots, MAb 7.3B revealed the same banding pattern as that observed with SDSPAGE after silver staining (Fig. 6) while MAb.s 7.4D and 7.5D and a negative control mouse immunoglobulin M did not reveal any band (data not shown). These results suggest that MAb 7.3B was directed against P. putida 2440 LPS.

To determine which cell antigens were recognized by MAb.s 7.4D and 7.5D, total cell protein was prepared from cultures of P. putida 2440. After separation of the proteins by SDS-PAGE and Western blotting, the immunological assays revealed that MAb.s 7.4D and 7.5D recognized a single protein band with an apparent molecular mass of 40 kDa (Fig. 7). In some immunoblots, in addition to the 40-kDa band, a band of about 65 kDa was also observed, which may have resulted from association of the 40-kDa protein with another protein.

DISCUSSION

MAb.s provide an excellent tool for tracking of genetically engineered microorganisms in the environment. However, successful use of antibodies in the environment can be affected by a number of factors, namely, specificity and cross-reactivity, sensitivity, and loss of bacterial cell antigens under environmental conditions.

MAb.s against surface determinants of P. putida 2440, the host bacterium for recombinant TOL plasmid and other recombinant plasmids (4, 9, 35), were sought with a screening strategy that involved an anti-antibody coupled to FITC and intact microorganisms as the antigen source. To select highly specific MAb.s, a large battery of eubacteria was used in the screening. The screening included closely related bacteria, e.g., species belonging to three different Pseudomonadaceae family rRNA groups (31), soil bacteria found in the same habitats as P. putida, and a number of nonrelated bacteria belonging to four different phyla according to Woese's classification (48). Of the 11 MAb.s, 3 were highly specific for P. putida 2440 as they did not recognize bacteria other than P. putida. However, different degrees of stringency were found in these MAb.s: MAb.s 7.4D and 7.5D
recognized only *P. putida* 2440; in contrast, MAb 7.3B reacted with other *P. putida* strains, e.g., strain 43, although it did not react with *P. putida* 29. Therefore, these MAbs may be useful in establishing serotypes within *P. putida* species (28).

Our findings with Western and LPS blots elucidated the molecular nature of the *P. putida* antigens recognized by the MAbs. LPS and a 40-kDa protein constitute the antigenic components of the cell surface recognized by MAb 7.3B (Fig. 6) and by 7.4D and 7.5D (Fig. 7), respectively. The typical ladder of pronase-resistant cellular material observed in Fig. 6 has been identified in other gram-negative bacteria as a population of LPS molecules whose heterogeneity is due to a variable number of repeating O-antigen units (7, 11, 13, 18, 32, 38, 39) that probably make up the antigen recognized by MAb 7.3B. MAbs 7.4D and 7.5D both recognized a 40-kDa protein. Our studies did not allow us to discern whether these MAbs recognized different epitopes of the molecule, although the protein seems to be a major one in nondenaturing gels and is apparently highly antigenic, as 2 of 11 MAbs recognized the same target. We are currently cloning the gene that encodes this antigen.

For risk assessment studies, the sensitivity as well as the specificity of MAbs is important. We have developed a semiquantitative dot blot immunoassay for bacteria in liquid media. This allowed us to detect as few as 100 cells per spot by using peroxidase-conjugated antibody against the antibody that recognized *P. putida* 2440 (Fig. 1). An intrinsic limitation of this technique is the turbidity of the samples, which may limit maximum assay volume. Prefiltration to eliminate particles in suspension may overcome this limitation. This assay is also of limited use for bacteria introduced into soils or sediments because of intrinsic fluorescence backgrounds. However, selective enrichments similar to the immunocapture methods described by Morgan et al. (27) and Wipat et al. (47) may be useful for MAb-based quantification of genetically engineered microorganisms in soils. In addition, the combination of MAb technology with flow cytometry may improve the sensitivity of genetically engineered microorganism detection (2, 3).

The usefulness of MAbs as tracking reagents depends on expression of the antigen under environmental conditions. In...
the laboratory, we studied the expression of the antigens recognized by MAbs 7.3B, 7.4D, and 7.5D under different growth conditions. The titer of the 40-kDa protein antigen recognized by MAbs 7.4D and 7.5D varied depending on culture conditions and growth phase. The 40-kDa antigen was easily detectable when *P. putida* was grown on LB medium or minimal medium with glucose, but it was less readily detected in cells grown on minimal medium with succinate and methylbenzoate as a C source and became coccoid and tended to distinguish autochthonous bacteria from *P. putida* under incubation in the laboratory (41). Many cases of cell protein expression are affected by growth conditions (33), and this could indeed be the case for this protein. We isolated Tn5::phoA mutants of *P. putida* 2440 which exhibit the insertion in a gene that encodes a protein involved in LPS synthesis. The 40-kDa protein was synthesized in these *P. putida* Tn5::phoA mutants, as the protein was detected in Western blots, although it is undetectable in vivo (36). In contrast to the antigen recognized by MAbs 7.4D and 7.5D, the fittest of MA7.3B was always expressed at high levels: (i) its detection was independent of the bacterial growth phase and the nutrient used for culture (Fig. 3), (ii) dot blot assays consistently allowed us to detect as few as 100 cells per spot (Fig. 1), and (iii) immunofluorescence assays gave a strong signal; hence, these results were appropriate for flow cytometry studies. Therefore, we conclude that MAb 7.3B is a useful tool for tracking *P. putida* in natural settings.

We used MAb 7.3B to track *P. putida*(pWW0) released in lake mesocosms. The double staining procedure based on DAPI staining and indirect immunofluorescence allowed us to distinguish autochthonous bacteria from *P. putida* (pWW0) (Fig. 4). The indigenous bacteria appeared after DAPI staining as coccoïd organisms, while the well-fed *P. putida*(pWW0) bacteria appeared as rods. After 48 h in a nutrient-poor environment, the *P. putida*(pWW0) bacteria became coccoïd and tended to form clumps like those shown in Fig. 5. At this stage, our CFU counts revealed that the number of *P. putida*(pWW0) bacteria was similar to the number after introduction (6). The shrinkage of *P. putida*(pWW0) cells under starvation and their relatively high surface-to-volume ratio may offer survival advantages at very low concentrations of limiting substrates (23). The clump structure tended to be maintained after prolonged incubation in the nutrient-poor environment. This confirms the usefulness of MAB 7.3B not only as a tracking reagent but also as a tool for studies of population structure in a natural setting.

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REFERENCES


22. Kooistra, J., B. Vosman, and G. Venema. 1988. Cloning and characterization of a *Bacillus subtilis* transcription unit involved...


