Detection and Enumeration of a Tagged *Pseudomonas fluorescens* Strain by Using Soil with Markers Associated with an Engineered Catabolic Pathway

INGYU HWANG^{1,†} AND STEPHEN K. FARRAND^{1,2,*}

Departments of Crop Sciences¹ and Microbiology,² University of Illinois
at Urbana-Champaign, Urbana, Illinois 61801

Received 19 August 1996/Accepted 3 December 1996

Previously we described a novel gene tagging method, using the *moc* (mannityl opine catabolism) region from the *Agrobacterium tumefaciens* Ti plasmid pTi15955, to identify microorganisms destined for release into the environment. Here, we used the engineered strain *Pseudomonas fluorescens* PF5MT12 carrying the *moc* region integrated into the bacterial chromosome to demonstrate the usefulness of the markers for detection and direct selection of marked organisms present in soil samples. Using this system, we routinely detected population levels as low as 10² CFU per g of soil sampled. In addition to direct selection, we developed an immunologically based assay using MOP cyclase, a unique enzyme associated with *moc*, as the epitope for detecting the tagged organism. The colony immunoblot assay proved to be highly specific and without any false-positive signals when used to identify organisms cultured from soil on nonselective medium. The numbers of colonies that were immunoreactive with the anti-MOP cyclase antibody were essentially equal to those that grew out on selection plates. This indicates that MOP cyclase can be used as a marker and that we can use nonselective medium to retrieve the marked genetically engineered microorganisms and then identify them by using colony immunoblot assays. These direct selection and colony immunoblot methods provide a sensitive and accurate strategy for identifying and enumerating marked organisms recovered from soil samples. We also developed a rapid assay for MOP cyclase that does not require cell permeabilization with toluene. This assay can be used to verify tagged organisms isolated by other methods or to screen large numbers of colonies for the tag following nonselective isolation.

The ability to detect genetically engineered microorganisms (GEMs) released into the environment is important for investigating the fate of released GEMs, as well as to determine their impact on the ecology of the release sites. Several methods to detect and identify specific microorganisms have been developed (1–4, 6, 10, 21–23). Markers for such purposes should have high specificity for the tagged organisms. They also should provide for direct selection on an appropriate culture medium. Furthermore, methods to detect these markers should be sensitive, amenable to processing large numbers of samples, and inexpensive. Antibiotic resistance determinants, which are the most widely used markers, are easy to use, but they may not provide the high specificity and sensitivity needed to detect released organisms (5). Moreover, such mutations to antibiotic resistance sometimes cause deleterious effects on the environmental fitness of the GEMs (18). Gene probes coupled with detection by PCR have relatively high sensitivity (1–4, 24, 25, 27) but lose specificity when similar organisms exist indigenously in the environment. Moreover, in most cases, PCR-based detection systems do not allow accurate enumeration of the target organisms. Immunological detection methods targeted to epitopes specific to certain strains of microorganisms also have been developed (6, 21). While these methods are sensitive, it can be difficult to obtain mono-

clonal antibodies with high affinity against appropriate epitopes.

Recently, we described a novel gene tagging strategy that uses, as markers, the mannityl opine catabolic (*moc*) genes from the Ti plasmid of *Agrobacterium tumefaciens* (17). Mannopine (MOP) and agropine (AGR), members of the mannityl opines, are found only in plant neoplasias induced by some pathogenic strains of *A. tumefaciens* and *A. rhizogenes* (9, 12, 26). The capacity to utilize these two opines as a carbon and nitrogen source is associated with certain strains of *Agrobacterium* spp. (7, 12). Genes for the catabolism of MOP and AGR are linked and are present on a 14-kb *Bam*HI fragment from the octopine-mannityl opine-type Ti plasmid, pTi15955 (8, 15). Furthermore, catabolism of AGR proceeds through MOP, with the first step being catalyzed by MOP cyclase (13). This enzyme, which is unique to certain *Agrobacterium* isolates, is encoded by *agcA*, and this gene is located within the 14-kb *Bam*HI fragment (15). The enzyme catalyzes the reversible lactonization of MOP to AGR (Fig. 1), and this reaction is very easy to monitor by high-voltage paper electrophoresis (HVPE) (14).

The *moc* genes confer utilization of MOP and AGR on a relatively broad range of nonenteric bacteria (8, 11, 28). We have shown that a *Pseudomonas fluorescens* strain into which we had inserted the Ti plasmid AGR catabolic genes utilizes AGR as the sole carbon source and produces easily detectable levels of MOP cyclase (17). Furthermore, the ability to utilize AGR provided a sensitive and selective method for recovering and enumerating the marked strain from a population dominated by closely related bacteria (17). We also developed a novel nucleic acid-based detection strategy that targets the unique fusion region between *Agrobacterium* DNA and

* Corresponding author. Mailing address: Department of Crop Sciences, University of Illinois at Urbana-Champaign, 240 Edward R. Madigan Laboratory, 1201 W. Gregory Dr., Urbana, IL 61801. Phone: (217) 333-1524. Fax: (217) 244-7830. E-mail: stephenf@uiuc.edu.

† Present address: 111 Koshland Hall, Department of Plant and Microbial Biology, University of California at Berkeley, Berkeley, CA 94720.

FIG. 1. Reactions catalyzed by catabolic MOP cyclase. The enzyme, which has been purified to homogeneity (14), catalyzes the reversible lactonization of mannopine (MOP) to agropine (AGR). While the lactonizing reaction is dominant and extremely easy to detect, the lactonase reaction is that which is required for the catabolism of agropine (13). The two compounds can be separated from each other by HVPE and detected with an alkaline silver nitrate reagent, all as described in Materials and Methods.

Pseudomonas DNA (17). Strains marked with this fusion were easily detected by hybridization. Coupling this marker to PCR amplification and hybridization with specific probes allowed us to detect as few as one or two marked organisms from mixtures containing a large excess of closely related bacteria (17).

In addition to direct selection with AGR and specific detection of the fused DNA region by PCR, we considered the possibility of targeting MOP cyclase to detect tagged organisms. This enzyme is easily assayable and is found in nature only in certain strains of *Agrobacterium* spp. Furthermore, the enzyme is extremely stable (14). These properties of MOP cyclase could provide for the rapid and sensitive detection of GEMs by using enzyme assays or immunological probes.

In this study, we demonstrate that the capacity to utilize AGR can be used to quantitatively recover the marked bacterium from soil samples containing indigenous microflora. We describe here two methods for detecting bacterial isolates that are expressing MOP cyclase. For the first, we present a rapid qualitative assay for detecting bacterial colonies that contain the active enzyme. For the second, we demonstrate that colony immunoblots, using polyclonal antisera against MOP cyclase, can be used to identify isolates expressing the *agcA* gene. Finally, we show that both techniques can be used to screen bacteria recovered from soil samples to identify those isolates expressing the marker enzyme. We also demonstrate that direct selection and immunoblot assays on colonies isolated after otherwise nonselective recovery from soil samples give accurate and comparable values for population sizes of viable marked organisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids and their relevant characteristics are listed in Table 1. Strains of *P. fluorescens* and *P. putida* were grown in L broth (LB) (19) or on Nutrient Agar (NA) plates (Difco Laboratories, Detroit, Mich.) at 28°C. *Escherichia coli* strains were grown in LB or on L agar plates at 37°C. When required, tetracycline was added at a final concentration of 10 µg/ml for *P. syringae* and *P. putida* strains, and ampicillin was added at a final concentration of 100 µg/ml for *E. coli*. For experiments involving growth on medium with specific carbon sources, *P. fluorescens*, *P. syringae*, and *P. putida* strains were grown on *Pseudomonas* minimal medium (PM) (17) solidified with Noble agar (Difco Laboratories) at 28°C. MOP was synthesized as described by Dessaux et al. (7). AGR was produced from MOP enzymatically by using lysates of *E. coli* DH5α(pUC18PSS) (14). The recombinant plasmid pUC18PSS expresses *agcA*, and the *E. coli* strain produces large amounts of enzymatically active MOP cyclase (14).

Treatment of soil samples and plating for colonies. Drummer silty clay loam soil samples that had been planted to soybeans were taken from a 5-cm depth at the Agronomy/Plant Pathology Farm, University of Illinois, Urbana. Soil samples were inoculated with bacteria as follows. *P. fluorescens* PF5MT12 was grown in LB overnight, and suspensions were prepared in 0.9% saline that contained

approximately 4×10^5 , 4×10^4 , 4×10^3 , and 4×10^2 CFU per ml, as determined by dilution plating on NA. A 100-µl volume of each of these cell suspensions was added to 1 g of soil, and the inoculated samples were mixed and incubated at room temperature for 3 h. This gave samples containing inoculum loads of the marked *P. fluorescens* strain of approximately 4×10^4 , 4×10^3 , 4×10^2 , and 4×10^1 CFU per g of soil. A 100-µl volume of sterile 0.9% saline was added to an additional sample of soil to serve as an uninoculated control. For recovery experiments, 2 ml of sterile 0.9% NaCl solution was added to 1 g of each of the inoculated or control soil samples, the slurries were vortexed vigorously for 1 min, and the soil particles and other debris were removed by centrifugation at $170 \times g$ in an HR4 rotor of a Sorvall GLC-2B centrifuge for 2 min at room temperature. The supernatants were diluted 10-fold, and 100-µl volumes of this dilution from each soil sample were spread onto two sets of plates. One set contained NA to select and enumerate the total cultivatable organisms, including the marked pseudomonad, present in the soil. The second contained PM supplemented with 5 mM AGR (PM-AGR) to select for those bacteria able to utilize the opine as sole source of carbon. All plates were incubated at 28°C and examined at 24 and 48 h.

Polyclonal antibodies directed against catabolic MOP cyclase. Mouse polyclonal antibodies directed against MOP cyclase, purified to homogeneity as previously described (14), were obtained from the Hybridoma Laboratory at the University of Illinois at Urbana-Champaign. Lipoproteins were removed from the crude ascites fluids as follows. First, 1.25 ml of 5% sodium dextran sulfate was added to 25 ml of crude ascites fluid, and the mixture was stirred gently on ice for 5 min. Then, 2.25 ml of an 11.1% solution of CaCl_2 was added with gentle stirring, the mixture was incubated on ice for 20 min, and the precipitated lipoproteins were removed by centrifugation at $40,000 \times g$ for 15 min. One-half volume of saturated ammonium sulfate was added slowly to the supernatants with gentle stirring, the mixture was incubated on ice for 15 min, and the precipitated proteins containing the antibodies were collected by centrifugation at $40,000 \times g$ for 15 min. The precipitate was redissolved in a small volume of phosphate-buffered saline (PBS) (19), aliquoted in 100-µl volumes to small vials, and stored at -80°C . The optimal dilution point of this polyclonal antibody preparation was determined by enzyme-linked immunosorbent assay using catabolic MOP cyclase protein purified as described previously (14).

Western blots. Cells were grown overnight in 2 ml of LB, and total proteins were prepared as described by Maniatis et al. (19). Lysates in $1 \times$ gel loading buffer (19) were diluted 100-fold before electrophoresis in 10% polyacrylamide gels containing 0.2% sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting as described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Membranes were dried overnight at room temperature and incubated with blocking solution (5% skim milk in PBS) in plastic bags for 2 h at room temperature with gentle shaking. The blocking solution was replaced with polyclonal anti-MOP cyclase antibodies diluted 1,000-fold in blocking solution, and the membranes were incubated for 2 h at 4°C with gentle shaking. After several washes with PBS, membranes were washed once with 10 mM Tris (pH 7.0)–150 mM NaCl solution (TN). The membranes were incubated for 1 h at room temperature with goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Sigma Co., St. Louis, Mo.) diluted 1,000-fold in blocking solution. The membranes were washed three times with TN and transferred to fresh substrate solution containing a mixture of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Gibco-BRL, Gaithersburg, Md.) dissolved in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl_2 , 100 mM Tris-HCl [pH 9.5]).

Colony immunoblots. Volumes of supernatants prepared from soil samples as described above were spread on NA plates, and the cultures were incubated for 24 h at 28°C. Nitrocellulose membranes (15-cm diameter; Bio-Rad) were auto-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i> S17-1	Pro ⁻ Res ⁻ Mod ⁺ <i>recA</i> ; integrated plasmid RP4-Tc::Mu-Km::Tn7	23
<i>P. fluorescens</i> 1855.344	Wild type	8
PF5MT12	Marker exchange mutant, AGR ⁺ MOP cyclase ⁺	17
<i>P. putida</i> B53	Wild type	Our collection
<i>P. syringae</i> Cit7	Wild type	28
Plasmid pYDH208	Broad-host-range cosmid carrying the <i>moc</i> region from pTi15955	8

^a Pro, proline; Res, restriction system; Mod, modification system.

TABLE 2. Utilization of AGR and expression of MOP cyclase by *Pseudomonas* strains

Strain	Growth on AGR ^a	MOP cyclase activity ^b
<i>P. fluorescens</i> 1855.344	—	—
<i>P. fluorescens</i> PF5MT12	+++	+++
<i>P. fluorescens</i> 1855.344(pYDH208)	+++	+++
<i>P. syringae</i> Cit7	—	—
<i>P. syringae</i> Cit7(pYDH208)	+++	+++
<i>P. putida</i> B53	—	—
<i>P. putida</i> B53(pYDH208)	—	+++

^a Growth was determined in liquid PM (17) containing 5 mM agropine as sole carbon source. +++, growth as good as in PM containing 0.2% glucose. —, no detectable growth.

^b Presence of MOP cyclase activity was determined by the toluene permeabilization method using cells grown in PM containing glucose as the sole carbon source, all as described in Materials and Methods. The substrate and reaction product were separated by HVPE and visualized by staining with Trevelyan's alkaline silver nitrate reagent as described in Materials and Methods. +++, >75% of the added MOP converted to AGR. —, no detectable AGR produced from the added MOP.

claved, laid onto fresh NA plates, and allowed to wet completely. Bacterial colonies appearing on the NA plates were replica plated onto the nitrocellulose filters on the fresh NA plates by using Accurant Replica Plater Pads (Schleicher & Schuell, Keene, N.H.). The replica plates were incubated for 24 h at 28°C, and the membranes were removed from the plates and soaked in lysis buffer (100 mM Tris-HCl [pH 7.8], 150 mM NaCl, 5 mM MgCl₂, 1.5% bovine serum albumin, 1 mg of pancreatic DNase I per ml) for 5 min. After lysis, the membrane filters were treated as described for the Western blots to detect MOP cyclase present in any of the lysed colonies.

Mannopine cyclase assays and separation of opines by high voltage paper electrophoresis. Mannopine cyclase activity was assayed in toluenized cells as described by Hong et al. (15). Briefly, cells to be assayed were resuspended in a buffer containing 10 mM potassium phosphate (pH 7.5), 90 mM ethylene glycol, 1 mM dithiothreitol, and 10% (vol/vol) toluene. A solution of mannopine was added to the toluenized cells (final concentration, 3 mM) and the mixture was incubated at room temperature for 1 h. The conversion of mannopine to agropine was assessed by separating the substrate and product by HVPE as previously described (8). The opines were visualized on the paper electrophoretograms by staining with Trevelyan's alkaline silver nitrate reagent (8). Authentic samples of mannopine and agropine were included as mobility standards on all electrophoretograms.

RESULTS

Utilization of AGR and production of MOP cyclase by *Pseudomonas* strains. The general usefulness of the *moc* genes as an expressible marker was evaluated by examining expression of *agcA* in *Pseudomonas* spp. into which we had introduced a clone carrying the *moc* region. Wild-type strains *P. fluorescens* 1855.344, *P. putida* B53, and *P. syringae* Cit7 do not utilize AGR as the sole carbon source (Table 2). Nor do these strains contain detectable levels of MOP cyclase activity (Table 2). We introduced pYDH208, a cosmid clone that encodes a functional *moc* region, including *agcA*, into these three strains and examined the ability of these constructs to catabolize AGR. Strains *P. fluorescens* 1855.344(pYDH208), *P. syringae* Cit7(pYDH208), and *P. fluorescens* PF5MT12, in which the *moc* genes are integrated into the chromosome (17), utilized the opine and produced detectable levels of MOP cyclase activity (Table 2). Strain *P. putida* B53(pYDH208) did not utilize AGR but produced detectable MOP cyclase activity (Table 2). The levels of MOP cyclase activity present in *P. putida* B53(pYDH208) were similar to those detected in the opine-utilizing *P. fluorescens* and *P. syringae* derivatives. This indicates that the *moc* genes are expressed in these *Pseudomonas* strains and that MOP cyclase can be used as a marker even when an engineered strain cannot utilize AGR.

Detection of MOP cyclase using polyclonal anti-MOP cyclase antibodies. We examined the usefulness of MOP cyclase as an epitope for an immunological detection strategy. Our polyclonal anti-MOP cyclase antibody preparation is highly specific when reacted with extracts of *E. coli*(pUC18PSS), the recombinant strain from which the enzyme was purified. Only a single band was detected by the antiserum, and this band electrophoresed at the same position at which a sample of the purified 45-kDa MOP cyclase protein migrated (Fig. 2). No other cross-reacting proteins were detected. Moreover, the antiserum reacted strongly with *A. tumefaciens* strains expressing the enzyme but did not cross-react with other proteins present in these strains or with strains of *A. tumefaciens* that do not contain the enzyme (reference 17 and data not shown). The antiserum also did not react with lysates prepared from *P. fluorescens* 1855.344 (data not shown). We have sequenced the *agcA* gene encoding MOP cyclase (16), and the amino acid sequence of the enzyme deduced from the DNA sequence is not detectably similar to any other protein in the databases, including other lactonases. This suggests that MOP cyclase represents a novel class of lactonases. Thus, it is unlikely that this antibody preparation will cross-react with lactonases other than MOP cyclase present in indigenous soil microflora.

Quantitative detection of *P. fluorescens* PF5MT12 in soil samples using anti-MOP cyclase antibodies. We tested the utility of the antibody preparation to identify colonies of *P. fluorescens* PF5MT12 recovered in mixed culture from non-sterile soil. Soil samples containing various numbers of the tagged pseudomonad were prepared and extracted as described in Materials and Methods. The uninoculated soil sample contained 1.36×10^4 total cultivatable CFU per gram of soil as assessed by plating dilutions of an extract on NA as described in Materials and Methods (data not shown). Several morphologically distinguishable colony types arose (Fig. 3C), but we made no effort to identify these organisms. None of the colonies recovered on NA plates from the uninoculated soil sample reacted with the anti-MOP cyclase antibody preparation (Fig. 3D). Moreover, no colonies arose when the samples from this uninoculated extract were plated on PM-AGR medium (data not shown). The inoculated soil samples contained between 1.24×10^4 and 5.4×10^4 total cultivatable CFU per g depending upon the titer of the *P. fluorescens* that had been added to the sample. As shown in Table 3, the number of colonies recovered on NA plates that reacted with the anti-MOP cyclase antibody preparation paralleled the expected

FIG. 2. Immunoblot analysis of catabolic MOP cyclase. Purified MOP cyclase and total cell lysates containing the enzyme prepared from *E. coli* DH5 α (pUC18PSS) were electrophoresed in SDS-PAGE. Separated proteins were transferred to nitrocellulose filters, and these were probed with polyclonal antibody directed against purified MOP cyclase as described in Materials and Methods. Lanes: 1 and 2, total proteins from cell lysates; 3 and 4, 0.1 and 0.2 μ g of purified MOP cyclase protein, respectively.

FIG. 3. Colony immunoblots of *P. fluorescens* PF5MT12 recovered from soil samples. The soil samples were extracted and processed for culturing on NA plates as described in Materials and Methods. A control soil, not inoculated with the pseudomonad, was processed in a similar manner. (A) Total culturable microorganisms present in 100 μ l of a 20-fold dilution of extract prepared from nonsterile soil inoculated with 4.25×10^4 CFU of *P. fluorescens* PF5MT12 per g. The soil sample contained a total of 5.4×10^4 CFU of culturable microorganisms per g (see text). (B) Colony immunoblot of the plate shown in panel A. (C) Total culturable microorganisms present in 100 μ l of a 20-fold dilution of extract prepared from the nonsterile soil sample which had not been seeded with *P. fluorescens* PF5MT12. The total number of culturable microorganisms present in this sample was 1.35×10^4 CFU per g (see text). (D) Colony immunoblot of the plate shown in panel C.

number as calculated from the input titer of the *P. fluorescens* PF5MT12 inoculum. Moreover, the difference between the total number of CFU recoverable from each sample and the number of recovered colonies that reacted with the antiserum was, in large part, attributable to the numbers of colonies representing the indigenous bacteria present in the nonsterile soil. In fact, each of these plates contained the expected number of variant colony types in addition to colonies showing the typical morphology of *P. fluorescens* PF5MT12 (for example, see Fig. 3A). Only those colonies showing the morphology typical of strain PF5MT12 reacted with the anti-MOP cyclase antibody (Fig. 3B). This indicates that the tagged bacteria recovered as colonies on nonselective medium can be detected and enumerated with high specificity by using this anti-MOP cyclase polyclonal antibody preparation.

Direct selection and enumeration of *P. fluorescens* PF5MT12 from soil samples. We reported previously that the ability to utilize AGR can be used to select and enumerate marked strains from mixed populations in liquid media containing a

large excess of closely related bacteria (17). Here we tested this trait for its usefulness in directly selecting and enumerating the marked strain present in nonsterile soil. Parallel 100- μ l volumes of the same extracts from uninoculated and inoculated soils used for the immunoblot screenings described above were spread onto PM-AGR plates as described in Materials and Methods. Following incubation, bacterial colonies growing on these plates were counted. As noted above, no colonies arose on the PM-AGR medium spread with extracts prepared from the uninoculated control soil sample. However, colonies did arise on plates inoculated with extracts from soil samples to which the tagged pseudomonad had been added. The numbers of colonies recovered on PM-AGR plates were very close to the expected number calculated from the input titer of the tagged bacterium (Table 4). All of the bacterial colonies growing on the PM-AGR plates were fluorescent under UV light and exhibited a morphology characteristic of *P. fluorescens* PF5MT12. No other bacteria, including agrobacteria, formed visible colonies on the PM-AGR plates (data not shown). In

TABLE 3. Detection and quantitation of *P. fluorescens* PF5MT12 by reactivity with anti-MOP cyclase antibodies following nonselective isolation^a

Total CFU recovered ^b	Total reactive CFU ^c		Efficiency of detection ^d	Fraction of total CFU reactive with the antibody	
	Expected	Observed		Expected ^e	Observed ^f
270	215	172	0.80	0.79	0.63
99	22	18	0.82	0.22	0.18
62	2	2	1.0	0.03	0.03
68	0	0			

^a Values presented are from one experiment which was repeated twice. While differing in absolute numbers, the data are representative of those obtained in all three experiments.

^b Numbers of colonies appearing on NA plates inoculated with 100 μ l of a 1/20 dilution of extract from the inoculated soils as described in Materials and Methods.

^c Colonies that reacted with anti-MOP cyclase antibodies following lysis on nitrocellulose filters as described in Materials and Methods.

^d Calculated by dividing the number of reactive colonies by the number of expected reactive colonies (based on input titers of strain PF5MT12) for each soil sample.

^e Calculated by dividing the number of total colonies recovered by the number of expected reactive colonies.

^f Calculated by dividing the number of total colonies recovered by the number of observed reactive colonies.

the experiments shown, we could reproducibly detect and enumerate *P. fluorescens* PF5MT12 by selecting directly on PM-AGR plates when the marked bacterium was present at population levels as low as 400 CFU per g of soil (Table 4). As described above, these soil samples contained cultivatable indigenous microflora at population levels as high as 1.3×10^4 CFU per gram. Thus, direct selection on PM-AGR plates allows specific detection and enumeration of *P. fluorescens* PF5MT12 cells present in soil samples even in the presence of a minimum 30-fold excess of indigenous cultivatable microorganisms.

Rapid MOP cyclase assay. Our standard assay for MOP cyclase activity involves toluenizing cells in the presence of substrate and detecting conversion of MOP to AGR by HVPE as described in Materials and Methods. While this is a reasonably simple assay, it could be tedious and time-consuming, especially if used to screen large numbers of colonies. As an alternative screening method to identify tagged bacteria, we developed a rapid and simple assay for MOP cyclase activity.

TABLE 4. Detection and quantitation of *P. fluorescens* PF5MT12 from samples of nonsterile soil by direct selection for the capacity to utilize AGR

Total recoverable CFU ^a	<i>P. fluorescens</i> CFU recoverable on PM AGR		Efficiency of recovery ^d
	Expected ^b	Observed ^c	
270	215	184	0.86
99	22	19	0.86
62	2	2	1.0
68	0	0	

^a Determined by plating samples on NA as described in Table 3 and Materials and Methods.

^b Calculated from the input titer of *P. fluorescens* PF5MT12 for each soil sample as described in Materials and Methods.

^c Determined by plating 100- μ l samples of a 1/20 dilution of extract from each soil sample on PM minimal medium containing AGR as the sole carbon source as described in Materials and Methods.

^d Calculated by dividing the number of CFU observed on the PM-AGR medium by the expected number.

FIG. 4. Rapid MOP cyclase assay. Cells of *P. fluorescens* 1855.344 (lanes 1, 2, 4, 5, 6, and 10) and PF5MT12 (lanes 3, 7, 8, and 9), removed from colonies on NA plates, were mixed with 5 μ l of 100 mM MOP on the electrophoresis paper and incubated for 30 min at room temperature. The paper was subjected to high-voltage electrophoresis, and the opines were detected as described in Materials and Methods. Abbreviations: m, marker; AGA, agropinic acid; AGR, agropine; MOP, mannopine.

In this assay a 5- μ l volume of 100 mM mannopine was mixed with a small amount of bacterial cell mass, taken from a single colony grown on NA or on PM-AGR plates, directly on the origin line of Whatman no. 1 chromatography paper. These cell pastes were incubated at room temperature for 30 min to allow the cells to take up the MOP and convert it to AGR. The papers then were processed for HVPE by standard protocols without modifications (8). As shown in Fig. 4, colonies of *P. fluorescens* PF5MT12, which expresses MOP cyclase, produced a silver nitrate-positive spot that comigrated with AGR (lanes 3, 7, 8, and 9). Colonies of *P. fluorescens* 1855.344, which does not produce this enzyme, did not produce the comigrating spot (lanes 1, 2, 4 to 6, and 10).

DISCUSSION

Strategies for monitoring GEMs in the environment require certain characteristics. Most important, monitoring methods should have high specificity and sensitivity. In addition, markers should allow for direct selection to recover tagged organisms from environmental samples. Monitoring methods also should be easy, inexpensive, and amenable to screening large numbers of samples. We reported previously that a novel gene tag system with a nutritional marker, the *moc* genes, meets these requirements (17). The engineered organism containing the *moc* genes can catabolize AGR as a sole carbon source. We showed previously that this characteristic provides a selective marker that can be used for the direct recovery and enumeration of marked bacteria in mixtures containing closely related microorganisms (17). In this study, we used *P. fluorescens* PF5MT12 as the engineered organism to determine the usefulness of our detection strategies involving direct selection and colony immunoblots when applied to environmental samples.

Plating extracts of soil samples directly on PM-AGR medium yielded direct and quantitative recovery of *P. fluorescens* PF5MT12. Moreover, there was little, if any, growth of indigenous soil microorganisms on this medium over the 24- to 48-h culture times tested. Under the conditions of the experiment, the lowest level of detection afforded by selection on PM-AGR plates was approximately 10^2 CFU per g of soil. This probably could be lowered one order of magnitude; our soil extracts were very dark, colloidal, and viscous, necessitating inclusion of a single 10-fold dilution of the samples before plating. Omitting this dilution certainly would have increased the sensitivity. However with these particular samples, it might well have decreased the precision since measuring accurate volumes of

these undiluted extracts was difficult. Comparison with the expected numbers of recoverable colonies derived from plating the original inoculum on nutrient agar indicated that selection on medium containing agropine as the sole carbon source did not significantly underestimate the numbers of marked cells present in the soil sample (Tables 3 and 4). The approximate 15 to 20% discrepancy between the expected and the actual cell numbers (Tables 3 and 4) most likely is due to tight adsorption of some of the bacteria to soil particles removed by the low-speed centrifugation step. This suggests that direct selection and enumeration of marked bacteria from soil samples using PM-AGR plates is as efficient and sensitive as any of the other systems reported to date (17). Some natural isolates of *Pseudomonas* species utilize some opines (20). However, there have been no reports of organisms, other than certain isolates of *Agrobacterium*, that can utilize AGR as the sole carbon source. The rarity of this trait ensures a very low probability of growth of other microorganisms on the selection plates during the recovery. Others have suggested that opine utilization markers might be compromised by the presence of opine-utilizing agrobacteria in the samples. Surprisingly, we did not detect any agropine-utilizing agrobacteria in our soil samples. This suggests that this *moc* marker may be very useful in the analysis of GEMs released into many soil samples. The apparent paucity of agropine utilizers in our soil is consistent with our observation that most mannopine-utilizing isolates of *Agrobacterium* spp. lack MOP cyclase activity and consequently do not utilize agropine (11). The rare nature of the agropine utilization trait provides high specificity to our detection system compared to other metabolic markers. For example, the relatively sensitive and selectable marker conferred by the *lacZY* genes from *E. coli* (10) becomes much less selective when indigenous lactose-catabolizing microorganisms are present in the samples being analyzed.

In addition to direct selection based on opine utilization, the novel enzyme, MOP cyclase, provides our system with an easily screenable marker. Our polyclonal antibody preparation directed against the enzyme clearly identified bacterial colonies expressing this protein apparently without any false-positive signals (Fig. 3). This allows us to use nonselective medium to recover all culturable microorganisms and then to specifically identify and quantify the released organism by using the immunoscreen for MOP cyclase. Furthermore, the numbers of immunoreactive colonies identified in the screen corresponded well with the numbers of colonies that grew out on the PM-AGR plates. Thus, the *moc* system can be used as an unbiased nonselective screen as well as for direct selection. Similar immunoblot assays have been described for the strain-specific quantitative analysis of commercial *Rhizobium* inoculants (21). This immunoassay for *Rhizobium* strains is highly strain specific, rapid, and reliable (21). In another case, monoclonal antibodies directed against *Bacteroides vulgatus* and *B. distasonis* were used to detect and enumerate these organisms in fecal samples (6).

We included two other *Pseudomonas* strains in this study to evaluate the applicability of the system to other microorganisms. While *P. syringae* Cit7 carrying pYDH208 utilizes AGR as the sole carbon source, *P. putida* B53 carrying the same plasmid cannot utilize this opine. However, *P. putida* (pYDH208) does produce MOP cyclase. Thus, at least one of the *moc* genes is expressed in this bacterium. We do not know why this isolate carrying pYDH208 cannot catabolize AGR. However, even though *P. putida* (pYDH208) cannot utilize the opine, we still can use MOP cyclase as a screening marker for identifying isolates of this organism expressing the tag.

Our detection and enumeration strategies require live and

culturable cells. This may underestimate the real population size in the environmental samples; some fraction of the marked organism may remain alive but in the dormant, viable but nonculturable state (5). Presumably, we should be able to detect these nonculturable cells by using our PCR-based assays (17). However, our direct selection and colony immunoblot assays are much more accurate for enumeration than DNA hybridization and PCR methods, even though the latter two methods provide considerably greater detection sensitivities.

The selection and screening assays based on *moc* are easy, reasonably rapid, and economical. Agropine can be prepared enzymatically from MOP, which is commercially available (7). MOP cyclase is easy to assay and simple to purify (14), and large quantities of polyclonal antibodies directed against the enzyme are available in our laboratory. In addition, our *in situ* assay for MOP cyclase allows rapid screening of large numbers of colonies.

This work shows that the *moc* gene tag system can be used to detect specifically marked organisms present in nonsterile soil samples and that MOP cyclase can be used as a marker even when the engineered microorganisms cannot utilize AGR as a sole carbon source. The system provides certain advantages over other monitoring methods. Most notably, we can use nonselective medium to recover released GEMs coupled with the immunoassay or the rapid MOP cyclase assay to identify the target organism. Furthermore, our gene tagging system uses nutritional markers which are presumably not harmful to the environment. Combining this strategy with our previously described detection methods using PCR and DNA hybridization (17), we believe that the *moc* system meets all of the criteria required for a safe, noninvasive marker system for tagging microorganisms designed for release into the environment.

ACKNOWLEDGMENT

This work was supported by a grant from the Biotechnology Research and Development Corporation to S.K.F.

REFERENCES

1. Bej, A. K., R. J. Steffan, J. Dicesare, L. Haff, and R. M. Atlas. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Appl. Environ. Microbiol.* **56**:307-314.
2. Bej, A. K., M. H. Mahbubani, J. L. Dicesare, and R. M. Atlas. 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Appl. Environ. Microbiol.* **57**:3529-3534.
3. Bej, A. K., and M. H. Mahbubani. 1992. Applications of the polymerase chain reaction in environmental microbiology. *PCR Methods Appl.* **1**:151-159.
4. Chaudhry, G. R., G. A. Toranzos, and R. Bhatti. 1989. Novel method for monitoring genetically engineered microorganisms in the environment. *Appl. Environ. Microbiol.* **55**:1301-1304.
5. Colwell, R. R., C. Somerville, I. Knight, and W. Straube. 1988. Detection and monitoring of genetically-engineered microorganisms, p. 47-59. In M. Sussman, G. H. Collins, F. A. Skinner, and E. E. Stewart-Tall (ed.), *The release of genetically engineered microorganisms*. Academic Press, London, United Kingdom.
6. Corthier, G., M. C. Muller, and R. L'Haridon. 1996. Selective enumeration of *Bacteroides vulgatus* and *B. distasonis* organisms in the predominant human fecal flora by using monoclonal antibodies. *Appl. Environ. Microbiol.* **62**:735-738.
7. Dessaux, Y., P. Guyon, S. K. Farrand, A. Petit, and J. Tempé. 1986. *Agrobacterium* Ti and Ri plasmids specify enzymatic lactonization of mannopine to agropine. *J. Gen. Microbiol.* **132**:2549-2559.
8. Dessaux, Y., J. Tempé, and S. K. Farrand. 1987. Genetic analysis of mannitol opine catabolism in octopine-type *Agrobacterium tumefaciens* strain 15955. *Mol. Gen. Genet.* **208**:301-308.
9. Dessaux, Y., A. Petit, and J. Tempé. 1993. Opines in *Agrobacterium* biology, p. 109-136. In D. P. S. Verma (ed.), *Molecular signals in plant-microbe communications*. C.R.C. Press, Boca Raton, Fla.
10. Drahos, D. J., B. C. Hemming, and S. McPherson. 1986. Tracking recombinant organisms in the environment: β -galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. *Bio/Technology* **4**:439-443.
11. Farrand, S. K. Unpublished results.

12. Guyon, P., M.-D. Chilton, A. Petit, and J. Tempé. 1980. Agropine in "null-type" crown gall tumors: evidence for generality of the opine concept. *Proc. Natl. Acad. Sci. USA* **77**:2693–2697.
13. Hong, S.-B., and S. K. Farrand. 1994. Functional role of the Ti plasmid-encoded catabolic mannopine cyclase in mannityl opine catabolism by *Agrobacterium* spp. *J. Bacteriol.* **176**:3576–3583.
14. Hong, S.-B., and S. K. Farrand. 1996. Purification and characterization of catabolic mannopine cyclase encoded by the *Agrobacterium tumefaciens* Ti plasmid pTi15955. *J. Bacteriol.* **178**:2427–2430.
15. Hong, S.-B., Y. Dessaux, W. S. Chilton, and S. K. Farrand. 1993. Organization and regulation of the mannopine cyclase-associated opine catabolism genes in *Agrobacterium tumefaciens* 15955. *J. Bacteriol.* **175**:401–410.
16. Hong, S.-B., I. Hwang, Y. Dessaux, P. Guyon, K.-S. Kim, J. Tempé, and S. K. Farrand. Adaptation in microbe-host interactions: a T-DNA gene required for opine biosynthesis by transformed plants is structurally, functionally, and evolutionarily related to a Ti plasmid gene required for catabolism of that opine by *Agrobacterium*. Submitted for publication.
17. Hwang, I., and S. K. Farrand. 1994. A novel gene tag for identifying microorganisms released into the environment. *Appl. Environ. Microbiol.* **60**:913–920.
18. Lee, S. W., and G. Edlin. 1985. Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. *Gene* **39**:173–180.
19. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. Nautiyal, C. S., and P. Dion. 1990. Characterization of the opine-utilizing microflora associated with samples of soil and plants. *Appl. Environ. Microbiol.* **56**:2576–2579.
21. Olsen, P. E., and W. A. Rice. 1989. *Rhizobium* strain identification and quantification in commercial inoculants by immunoblot analysis. *Appl. Environ. Microbiol.* **55**:520–522.
22. Shaw, J. J., F. Dane, D. Geiger, and J. W. Kloepper. 1992. Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. *Appl. Environ. Microbiol.* **58**:267–273.
23. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:37–45.
24. Steffan, R. J., and R. M. Atlas. 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.* **54**:2185–2191.
25. Steffan, R. J., and R. M. Atlas. 1991. Polymerase chain reaction: applications in environmental microbiology. *Annu. Rev. Microbiol.* **45**:137–161.
26. Tempé, J., and A. Goldmann. 1982. Occurrence and biosynthesis of opines, p. 427–449. In G. Stall and J. Schell (ed.), *Molecular biology of plant tumors*. Academic Press, N.Y.
27. Tsai, Y.-L., and B. H. Olson. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**:754–757.
28. Wilson, M., M. A. Savka, I. Hwang, S. K. Farrand, and S. E. Lindow. 1995. Altered epiphytic colonization of mannityl opine-producing transgenic tobacco plants by a mannityl opine-catabolizing strain of *Pseudomonas syringae*. *Appl. Environ. Microbiol.* **61**:2151–2158.