APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1994, p. 1606–1613
0099-2240/94/$04.00+0

Vol. 60, No. 5

Survival of *lux-lac*-Marked Biosurfactant-Producing *Pseudomonas aeruginosa* UG2L in Soil Monitored by Nonselective Plating and PCR

C. A. FLEMMING,[1] K. T. LEUNG,[1] H. LEE,[1] J. T. TREVORS,[1]* AND C. W. GREER[2]

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1,[1] and Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P 2R2[2]

Received 10 November 1993/Accepted 6 March 1994

Two reporter systems, *lacZY* and *luxAB*, were stably integrated into the chromosome of *Pseudomonas aeruginosa* UG2, a biosurfactant-producing strain. Growth and rhamnolipid production of the UG2 wild-type and reporter gene-bearing UG2L strains were similar in liquid culture. A spontaneous rifampin-resistant derivative, UG2Lr, allowed antibiotic selection. Phenotypic characteristics were compared for usefulness in detecting UG2Lr colonies: morphology, fluorescent pigment production, light emission (*lux*), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) cleavage (*lac*), and rifampin resistance. Survival patterns of UG2, UG2L, and UG2Lr strains were similar in sandy loam soil microcosms over 12 weeks. The *lac* marker was not suitable for monitoring *P. aeruginosa* UG2Lr in soil since 20 to 42% of cultured, aerobic, heterotrophic soil microorganisms formed blue, lactose-positive colonies. The *lux* genes provided a stable and unequivocal reporter system, as effective as conventional antibiotic plating, for tracking microorganisms nonselectively at 10^3 CFU/g of soil. Three months after inoculation into oil-contaminated and uncontaminated soil microcosms, UG2Lr cells were recovered at 10^7 and 10^4 cells per g (dry weight) of soil, respectively. Detection by PCR amplification of part of the *luxA* gene confirmed a decrease in UG2Lr cell numbers in uncontaminated soil. In combination, antibiotic resistance, bioluminescence, and PCR analyses provided sensitive detection and quantitative enumeration of *P. aeruginosa* UG2Lr in soil.

It is probable that genetically engineered microorganisms (GEMs) will be used in environmental applications such as bioremediation and biological control (14, 34, 46). One concern with GEMs is the limited ability to monitor released microorganisms. Conventionally, specific phenotypic markers are used, allowing selective recovery or enumeration of bacteria from environmental samples (35). Other approaches include immunological techniques, DNA probing, and PCR (23).

Antibiotic resistance markers, commonly used for tracking microorganisms (7, 10, 18, 35, 44), are not entirely satisfactory. Widespread use of antibiotic markers may contribute to the antibiotic resistance gene pool (1), resistant strains may be less robust than parent strains (10), and stability of resistance markers in cells after release into soil may not be known, where spontaneous mutants are employed (25). Other phenotypic markers include color production in bacterial colonies (33, 35). Drahos et al. (15) cloned the *lacZY* genes from *Escherichia coli* into fluorescent pseudomonads, which resulted in blue, fluorescent colonies on selective media containing the β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Although some soil microorganisms display β-galactosidase activity (25), this activity is rare among fluorescent pseudomonads (15).

Bioluminescence is an effective reporter for detecting recombinant bacteria containing the *lux* operon genes (30, 38). Metabolizing bacteria containing the *luxAB* genes produce light in the presence of aldehyde substrate, and luciferase is constitutively expressed (25). Luminescence may be detected visually in a darkroom, with X-ray film (11, 28), scintillation counters, luminometers (20, 35), and highly sensitive charge-couple photon imaging devices (27, 29, 35). Using this marker, Grant et al. (19) enumerated *Erwinia carotovora* in soil and Shaw et al. (38) detected *Xanthomonas campestris* in field releases on cabbage plants and soil.

Approaches involving cultivation techniques underestimate bacterial populations, since bacteria can exist in a viable but nonculturable state in the environment (9, 45). With molecular techniques such as DNA probing and PCR, it is possible to detect nonculturable bacteria or specific DNA sequences in environmental samples (3, 12, 17, 21, 32, 40–42).

Pseudomonas aeruginosa UG2 produces two rhamnolipid biosurfactants (43) which can enhance removal of several aliphatic, aromatic, and chlorinated hydrocarbons from soil (4, 24, 37, 43). With its potential for soil bioremediation, it is necessary that reliable, rapid, and reproducible tracking methods be established for UG2. In the present study, *P. aeruginosa* UG2 was marked with the *luxAB* and *lacZY* genes, reporter genes recently used to monitor a 2,4-dichlorophenoxyacetic (2,4-D)-degrading bacterium in soils (28). Growth responses and biosurfactant production of UG2 and the genetically modified UG2L strains were compared in liquid culture and/or soil to determine the effects of *lux-lac* genes on these parameters. Phenotypic markers were compared for use in enumeration of UG2L strains. Survival of rifampin-resistant UG2Lr was investigated in uncontaminated and oil-contaminated sandy loam soil microcosms by selective and nonselective plating and PCR.

MATERIALS AND METHODS

Growth of *Pseudomonas aeruginosa* UG2. *P. aeruginosa* UG2 was originally isolated from an oil-contaminated soil (4). Strains were maintained on tryptic soy agar (TSA; Difco

* Corresponding author. Phone: (519) 824-4120 ext. 3367. Fax: (519) 837-0442. Electronic mail address: jtrevors@uoguelph.ca.

Laboratories, Detroit, Mich.) as described previously (43). Inocula, derived from single colonies, were grown for 18 to 24 h in tryptic soy broth (TSB; Difco) at 30°C with 200 rpm gyratory shaking. Portions of inoculum were washed in sterile 0.85% (wt/vol) saline by microcentrifugation (8,000 × *g*, 10 s), and 1% (vol/vol) inoculum was introduced into triplicate 250-ml Erlenmeyer flasks containing 50 ml of minimal salts-glucose medium (43). Cultures were incubated at 30°C with 200 rpm shaking until late-log phase (24 h).

Growth was followed by optical density measurements at 600 nm with a Phillips spectrophotometer and by viable-cell counts, performed by spread-plating 0.1 ml of dilutions in sterile 0.85% (wt/vol) saline onto TSA plates. Colonies were enumerated after 48 h of incubation at 30°C.

Chromosomal integration of reporter genes. The *lux-lac* genes, described in detail elsewhere (28), were inserted into *P. aeruginosa* UG2 by using a broad-host-range shuttle system (2, 28). The element contained the *luxAB* genes from *Vibrio harveyi* (31), the *lacZY* genes from *E. coli*, and the Tn*7* termini, but it lacked the Tn*7* transposase genes. The final construct (28) contained an unstable transposase function and was introduced into UG2 by electroporation (13, 28). Following introduction of the final plasmid construct, the cell suspension was diluted into sterile water and incubated at 22°C overnight (starvation conditions). Cells were plated onto Oxoid Antibiotic Media no. 2 (Unipath Ltd., Nepean, Ontario, Canada) containing X-Gal without or with 30 μg of gentamicin per ml. No growth on gentamicin agar and abundant growth on nonantibiotic agar indicated that starvation conditions promoted transposition at high efficiency.

Antibiotic selection. A spontaneous rifampin-resistant *P. aeruginosa* UG2L strain, designated UG2Lr, was isolated and subsequently enumerated on solid media containing 50 μg of rifampin per ml and 50 μg of cycloheximide per ml.

Rhamnolipid analysis. Concentrations of rhamnolipid biosurfactants produced by UG2 were determined by a modified L-rhamnose assay (6) on 100-μl aliquots of culture supernatant acidified with a drop of 1 N HCl. Extractions were performed as described by Scheibenbogen et al. (37). L-Rhamnose content was determined by comparison with α-L-rhamnose standards.

Soil characteristics and microcosm design. A sandy loam soil (75% sand, 18% silt, 7% clay) was collected from field plots at the University of Guelph Cambridge Research Station and was stored at 4°C. Soil pH (1:1 soil-distilled water) was 7.8, field capacity (measured on disturbed soil at −1/3 bar) was 11%, and saturation point was 44% (wt/wt). Other soil characteristics were described previously (16). Fifteen grams of moist (13.9 g of oven-dry weight), sieved (2-mm pore diameter) soil was placed in sterile 59-ml French Square Bottles (Fisher Scientific Co., Toronto, Ontario, Canada). Oil-contaminated microcosms consisted of the sandy loam soil spiked with 5% (wt/wt) Esso virgin heavy atmospheric gas oil (refined from light Canadian crude oil; Esso Research Centre, Sarnia, Ontario) by mixing with a sterile spatula. Oil-treated soil microcosms were aged 4 months, prior to inoculation, under experimental conditions described below. Aging allowed time for indigenous microbial communities to more closely represent those of a contaminated field soil.

Soil in microcosms was adjusted to a bulk density of 1.2 g/cm^3 and was inoculated with 1 ml of bacterial cell-water suspensions at final densities of 10^8 to 10^9 CFU/g (dry weight) of soil. Suspensions were added dropwise and were spread by capillary action (24), raising soil moisture content to 15% of soil dry weight. Equivalent amounts of water were added to noninoculated controls. In oil-contaminated microcosms, bacterial suspensions were added by mixing with a sterile spatula and were readjusted to 1.2 g/cm^3. Microcosms were incubated at 22°C in dark, moist, sealed bags at 100% relative humidity. Periodic mass measurements over time indicated minimal water loss.

Enumeration of microorganisms from soil. Triplicate microcosms were sacrificed to enumerate microorganisms. Sterile sodium pyrophosphate solution (0.1% wt/vol, pH 6.9) was added to microcosms, producing a final dilution of 1:2 (soil dry mass-suspension volume). Microcosms were shaken for 40 min at 200 strokes per min in a straight-action shaker (Eberbach Corp., Ann Arbor, Mich.) at 22°C. Some aliquots (1.0 ml) of soil suspension were removed and frozen at −20°C for DNA extraction and PCR analyses; others (0.2 ml) were diluted in sterile saline. Cell recovery from microcosms ranged from 96 to 105% when this method was used; mechanical dispersants were unnecessary.

Aliquots (0.1 ml) were spread-plated on TSA, King's B agar (16), and/or minimal salts medium with 2% (wt/vol) glucose and 1.5% agar (wt/vol) (43). Rifampin and cycloheximide were added to selective media at 50 μg/ml each. Plates were incubated for 2 days at 30°C. Fluorescent colonies, on King's B plates, were enumerated under UV light.

Enumeration of lactose-positive colonies. Lactose-positive colonies, exhibiting β-galactosidase activity, turned blue in the presence of X-Gal (15). Lactose-positive colonies were assessed on media with and without 2% (wt/vol) lactose, 6 to 24 h after being lightly sprayed with X-Gal (20 mg/ml in formamide).

Enumeration of photon-emitting colonies. Bioluminescent colonies were enumerated on spread plates containing vaporous n-decanal (Sigma), by collecting photons emitted over 10 s. Photons were collected by using a Biomedical Image Quantifier (BIQ; Bioview Image Research, Cambridge, United Kingdom) equipped with an intensified, charge-coupled camera device interfaced with an IBM-compatible processor.

Data analyses. Analysis of variance and Duncan's multiple-range tests were carried out by using CoStat Statistical Package software (CoHort Software, Berkeley, Calif.) at a 95% confidence level.

DNA extraction and purification. The DNA extraction procedure was adopted from Leung et al. (26) with modifications. One-milliliter portions of soil suspension in sodium pyrophosphate (described above) or 1.0-g portions of soil in 450 μl of sterile deionized water were used for direct DNA extraction. Five milligrams of lysozyme in 50 μl of Tris buffer (250 mM, pH 8.0) was added, and samples were shaken at 400 rpm for 30 min at 30°C. After a 30-min incubation at 37°C, 100 μg of Proteinase K (Sigma) per ml (final concentration) was added, and samples were incubated for 30 min. Fifty-microliter portions of filter-sterilized 20% sodium dodecyl sulfate solution were added, and samples were incubated for 30 min at 85°C. Soil residue was removed by centrifugation (14,000 × *g*, 10 min), and one-half volume of 7.5 M ammonium acetate was added to supernatant to precipitate proteins and humic substances. Crude DNA extract was purified by a 1-min centrifugation (Hermle Z320) at 100 × *g* through a 5-ml Sephadex G75 (Sigma) column. Sample DNA was precipitated in ethanol (36) and resuspended in 100 μl of sterile deionized water. DNA extracts were purified twice with glass milk according to Geneclean instructions (Bio101 Inc., La Jolla, Calif.) and were resuspended in 100 μl of sterile ultrapure water.

DNA amplification and probing. An 894-bp fragment located at nucleotide positions 740 to 1633 of the *luxA* gene (8) was amplified by using two 27-mer primers (primer 1: 5′-CCACCTGAGCTATCTCAGACCGAAGTG; primer 2: 5′-CGCGATACACTCTTCAGGCGTCCCTAC). PCR was per-

TABLE 1. Background numbers of indigenous, lactose-utilizing, bioluminescent, rifampin-resistant, and fluorescent pigment-producing microorganisms in a sandy loam soil[a]

Treatment	Cell numbers[b] (10^5 CFU/g of dry wt soil) in:						
	TSA		King's B			All media[c]	Rifampin-amended medium[d]
	Total	Lac[e] (blue)	Total	Fluorescent	Lac		
Sandy loam soil	140 ± 14	57 ± 45	110 ± 3.2	1.0 ± 1.0	29 ± 3.2	<0.01[f]	0.73 ± 0.11
Oil-contaminated soil[g]	160 ± 40	33 ± 8.0	120 ± 7.0	2.0 ± 2.0	21 ± 2.0	<0.01	n.d.[h]

[a] Determined by spread-plating on various media.
[b] Means ± SD (n = 3).
[c] Luminescent microorganisms.
[d] Rifampin-resistant microorganisms.
[e] Lactose-utilizing microorganisims.
[f] Below the limit of detection.
[g] Sandy loam soil contaminated with 5% (wt/wt) Esso light reference oil.
[h] Not determined.

formed in a Temp · Tronic thermocycler (Barnstead/Thermolyne Co., Dubuque, Iowa) in 50-μl volumes. The PCR solution contained 1× PCR amplification buffer, 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.), 100 μM (each) dNTPs (Pharmacia LKB Biotechnology, Milwaukee, Wis.), 0.22 μM (each) primers, 2% formamide, and 0.5 μl of template DNA. The reaction mixture was overlaid with 50 μl of sterile mineral oil. The mix was subjected to a 4-min denaturation at 94°C followed by a 6-min annealing and extension at 70°C. Parameters for the subsequent 39 cycles were a 30-s denaturation and a 2-min annealing and extension, followed by a final 10-min extension. PCR product was analyzed by electrophoresis in a 1.2% (wt/vol) agarose gel containing ethidium bromide (0.2 μg/ml). A 20-mer internal probe (5′-GTCGTCGCGGAGTCAGCATC) targeting nucleotide positions 1220 to 1239 of the *luxA* gene was synthesized and labelled with digoxigenin-dUTP by using the oligonucleotide tailing kit (Boehringer Mannheim, Indianapolis, Ind.). The agarose gel product was transferred to a nylon membrane (Boehringer Mannheim) with a vacuum blotter (36). The membrane was soaked in prehybridization and hybridization solutions (5) at 60°C for 1 h. Chemiluminescent detection was performed in AMPPD solution according to the Boehringer Mannheim Genius System User's Guide (5) with a 1-h incubation at 37°C. Chemiluminescence was recorded with the Biomedical Image Quantifier described above.

RESULTS

Growth and rhamnolipid production of *P. aeruginosa* UG2 and UG2L. *P. aeruginosa* UG2 and UG2L showed similar growth profiles and final growth yields, as determined by optical density and viable-cell counts (data not shown). During early exponential growth (10 to 13 h) doubling time was 1.9 h for both strains. UG2 and UG2L also produced similar amounts of extracellular rhamnolipid biosurfactants in liquid culture over 10 days (1.5 to 1.75 g of L-rhamnose per liter) (data not shown).

Background incidence of selected phenotypes in soil. Five characteristics used to distinguish *P. aeruginosa* UG2Lr colonies from those of indigenous soil microorganisms were bioluminescence, β-galactosidase activity (X-Gal cleavage), rifampin resistance, fluorescent pigment production, and distinct colony morphology. Numbers of indigenous, aerobic heterotrophic microorganisms which expressed these phenotypes are summarized in Table 1, for both oil-contaminated and uncontaminated sandy loam soil. No background luminescent microorganisms were detected at any time. Rifampin-resistant microorganisms represented 0.5% of cultured, indigenous soil microorganisms. Indigenous fluorescent pigment producers were also low (1 to 5%) in sandy loam soil. The β-galactosidase-positive portion of the indigenous population ranged from 20 to 42% of aerobic heterotrophic microorganisms, including fungi, cultured on King's B agar (with lactose) and TSA, respectively (Table 1).

Stability of phenotypic markers in *P. aeruginosa* UG2Lr. The stabilities of rifampin resistance, luminescence, and β-galactoside hydrolysis phenotypes in *P. aeruginosa* UG2Lr were examined over a 1-year period. Routine comparison of CFU on selective and nonselective agar from mid- to late-log cultures showed statistically similar numbers of blue, bioluminescent colonies on nonselective and rifampin-amended media (data not shown). These characteristics were also retained by UG2Lr cells over 8 weeks in soil (Table 2).

Monitoring UG2Lr by using various phenotypic traits. Table 2 presents *P. aeruginosa* UG2Lr cell numbers obtained from uncontaminated and oil-contaminated soil microcosms 8 weeks after inoculation. Colonies were enumerated on selective and nonselective media and were distinguished by mor-

TABLE 2. Comparison of media and phenotypic markers for enumerating *P. aeruginosa* UG2Lr from oil-contaminated and uncontaminated sandy loam soil microcosms[a]

Agar[b]	Phenotypic marker	Cell numbers[c] (10^5 CFU/g of dry wt soil)	
		Uncontaminated soil	Contaminated soil[d]
TSA	Colony morphology	3.3 ± 0.62[x]	70 ± 46[x]
	Bioluminescence	4.0 ± 1.7[x]	64 ± 34[x]
	X-Gal cleavage	41 ± 4.6[y]	84 ± 46[x]
KB	Bioluminescence	2.7 ± 0.80[x]	56 ± 58[x]
	X-Gal cleavage	21 ± 8.7[z]	78 ± 48[x]
	Fluorescent pigments	3.0 ± 0.80[x]	n.d.[e]
Rif	Bioluminescence	2.7 ± 2.1[x]	67 ± 39[x]
	X-Gal cleavage	n.d.	80 ± 56[x]

[a] Isolated 8 weeks after inoculation with 5 × 10^8 CFU/g dry weight soil.
[b] Tryptic soy agar (TSA), King's B (KB), and minimal salts agar with 50 μg of rifampin (Rif) per ml and 50 μg of cycloheximide per ml.
[c] Means ± standard deviation (n = 3). Superscript x, y, and z: Values followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05) within each soil type. Least significant difference values were 4.1 × 10^5 and 100 × 10^5 for uncontaminated and oil-contaminated soils, respectively.
[d] Contaminated with 5% (wt/wt) Esso light reference oil.
[e] Not determined.

phology, bioluminescence, β-galactosidase activity, fluorescent pigment production, and rifampin resistance. There was no significant difference between UG2Lr cell numbers distinguished by colony morphology (and color) on TSA, fluorescence on King's B agar, luminescence on all media, or rifampin resistance on selective medium. These enumeration methods yielded similar mean cell numbers (Table 2).

Enumeration of UG2Lr cells by counting β-galactosidase-positive colonies resulted in a significant (10-fold) overestimation of UG2Lr cells in the sandy loam soil (Table 2), since microorganisms exhibiting β-galactosidase activity were abundant (Table 1). Therefore, X-Gal cleavage was not an effective marker for *P. aeruginosa* UG2Lr in this soil. Drahos et al. (15) successfully used the *lac* reporter system in combination with fluorescent pigment production to track recombinant pseudomonads in soil. Intense dye production and extensive diffusion of the water-soluble fluorescent pigments made enumeration of fluorescent, β-galactosidase-positive colonies difficult. In this soil, with a low fluorescent pseudomonad population (Table 1), a second phenotypic marker was not necessary to detect UG2Lr (Table 2). Hence, wild-type UG2 colonies could be detected by fluorescent pigment production alone.

Nonselective media were adequate to enumerate UG2Lr at soil dilutions of 10^{-3}. For lower dilutions (e.g., 10^{-1}), selective plating was effective for enumerating UG2Lr because indigenous rifampin-resistant microorganisms were few in soil (Table 1) and UG2Lr colonies grew rapidly, avoiding overgrowth by indigenous microorganisms. Numbers of UG2Lr colonies on rifampin-amended plates were similar to those on nonselective plates, even when the nonselective plates were crowded with indigenous soil microorganisms (500 to 1,000 CFU per plate).

Enumeration by photon imaging. Figure 1 shows the appearance (A) and photon image (B) of a rifampin-amended agar plate supporting both indigenous soil microorganisms and UG2Lr colonies isolated from soil at a low dilution (1/30). The small, obscured UG2Lr colonies (Fig. 1A) were easily detected beneath overlying fungal colonies and were distinguished from soil microorganisms by bioluminescence (Fig. 1B). Combining rifampin resistance and bioluminescence facilitated enumeration of UG2Lr colonies on plates at numbers as low as 10 CFU/g (dry weight) of soil (data not shown).

In general, enumeration based on photon imaging of light-emitting colonies, as compared with direct colony counts, underestimated CFU. Cell numbers were underestimated by 0 to 5% on pure culture plates with less than 75 CFU and by 1 to 20% on plates with more than 75 CFU. Colonies on plates containing greater than 200 CFU were difficult to discriminate by photon counting (data not shown). Grant et al. (19) described a similar reduction in counting efficiency with increasing colony number. Poor resolution was due to colony crowding. Furthermore, photon emissions decreased substantially with colony age. On crowded plates, better resolution was achieved with photon images collected from 18- to 24-h-old colonies.

Survival of UG2, UG2L, and UG2Lr strains in soil. Survival patterns of *P. aeruginosa* UG2, UG2L, and UG2Lr were compared in sandy loam soil microcosms over 12 weeks (Fig. 2). Nonluminescent UG2 colonies were distinguished by fluorescent pigment production on King's B agar. Although the inoculum density of UG2Lr was significantly higher than the inoculum densities of the other strains, numbers of cells surviving in soil were similar among strains after 2 weeks. After 7 weeks, UG2 cell numbers were higher than those of the reporter-gene-bearing strains, but this difference was not statistically significant (Fig. 2). The population densities established in soil after 12 weeks were similar among the three strains.

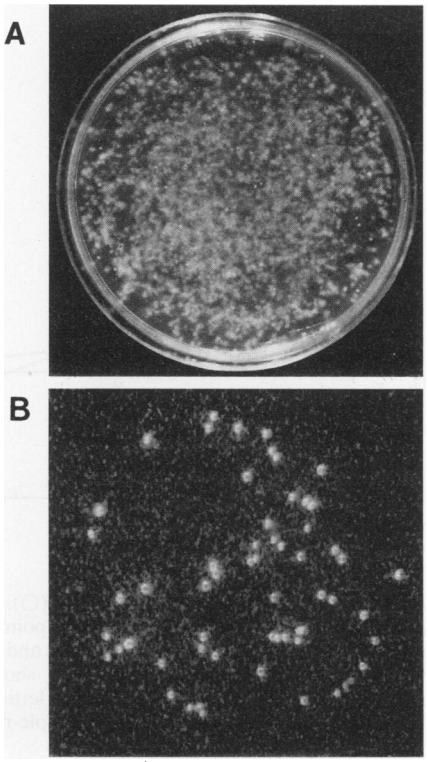

FIG. 1. Microbial growth on rifampin-amended minimal salts-glucose agar plate containing *P. aeruginosa* UG2Lr and indigenous soil microorganisms (A) and computer-generated photon image of the 59 bioluminescent *P. aeruginosa* UG2Lr colonies from the same plate (B). Bioluminescent colonies appear as bright spots against the lightly speckled background. Microorganisms were isolated from soil at a 1/30 dilution.

It is important to demonstrate that GEMs compete and survive as well as parent strains in a particular environment, because marker genes may affect host fitness (35). Compeau et al. (10) found that some rifampin-resistant *Pseudomonas fluorescens* strains exhibited slower growth rates in culture and higher population decay rates in soil. The relative fitness of the genetically altered UG2L and UG2Lr strains appeared to be unaffected in soil over 3 months (Fig. 2).

Enumeration of UG2Lr in oil-contaminated microcosms by spread-plating. Mean numbers of *P. aeruginosa* UG2Lr cells and indigenous microorganisms in uncontaminated and oil-contaminated sandy loam soil microcosms were determined over time in UG2Lr-inoculated and uninoculated soil (Fig. 3). At 5 and 8 weeks, the number of cultured indigenous aerobic heterotrophic microorganisms was significantly higher in oil-contaminated than in uncontaminated soil microcosms. This increase in the natural population likely resulted from crude oil serving as a carbon source.

In the first 2 weeks after inoculation, UG2Lr cell numbers dropped almost 2 log units in both the oil-contaminated and uncontaminated soil microcosms. After 5 weeks, the number of viable UG2Lr in oil-contaminated soil was higher than that in uncontaminated soil (Fig. 3). This difference, although not statistically significant, was notable at 8 weeks and pronounced at 12 weeks. Large standard deviations were obtained in all recovery experiments (e.g., Fig. 3) and resulted from high

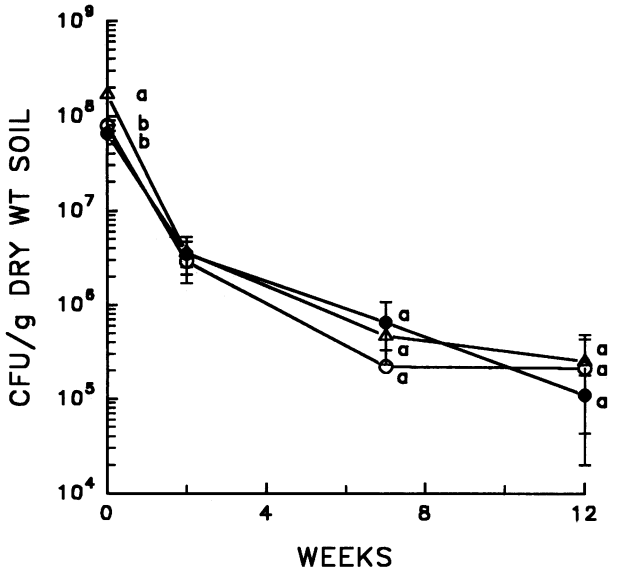

FIG. 2. Survival of *P. aeruginosa* UG2 (●), UG2L (○), and UG2Lr (△) in sandy loam soil microcosms over time. Data points represent mean CFU/g of dry weight soil determined for UG2L and UG2Lr (on TSA) and UG2 (on King's B agar). Error bars show standard deviations (n = 3). Data points followed by different letters (a and b) are significantly different according to Duncan's multiple-range test (P = 0.05).

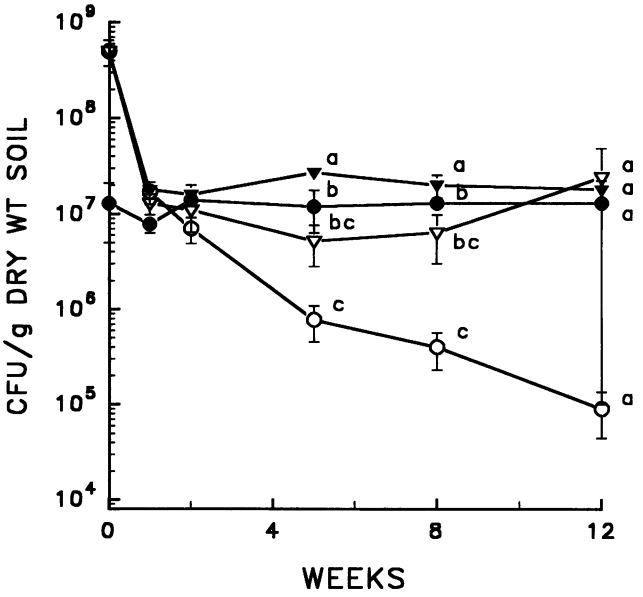

FIG. 3. Numbers of indigenous microorganisms (▽) and *P. aeruginosa* UG2Lr cells (○) in uncontaminated (hollow) and oil-contaminated (filled) sandy loam soil microcosms. Each data point represents mean CFU/g of dry weight soil determined on TSA plates from triplicate microcosms. Error bars show standard deviations (n = 3). Data points followed by different letters (a, b, and c) are significantly different according to Duncan's multiple-range test (P = 0.05).

variation in cell numbers among soil microcosms. Whole microcosms were sacrificed to minimize variation due to heterogeneity within a soil sample. Counts were consistent between replicate plates but varied largely among microcosms in some treatment groups.

Numbers of UG2Lr cells appeared to stabilize in oil-contaminated soil after 2 weeks, but they continued to drop about 2 log units in uncontaminated sandy loam soil over 12 weeks. Final UG2Lr populations comprised 60% of the total cultured, aerobic, heterotrophic microbial community in oil-contaminated sandy loam soil and only 0.7% of that in uncontaminated soil (Fig. 3). It is not surprising that *P. aeruginosa* UG2Lr survived well in oil-contaminated soil since the wild-type strain was originally obtained from an oil-contaminated soil and was selected for its high extracellular emulsifying activity (4). Low densities of UG2Lr cells, e.g., 10^4 CFU/g, were easily enumerated from uncontaminated soil by nonselective plating, and colonies were distinguished by their bioluminescence.

Detection of UG2Lr in soil microcosms by PCR. The detection limit of PCR was determined by analyzing soil samples spiked with a dilution series of UG2Lr cells. About 2 × 10^3 CFU/g (dry weight) of soil could be routinely detected (Fig. 4A). The amplified DNA fragment also hybridized to the 20-mer internal probe of the *luxA* gene (Fig. 4B).

Samples for PCR were taken from uncontaminated and oil-contaminated soil microcosms concurrently with those used for the viable-cell counts presented in Fig. 3. PCR was performed on UG2Lr-inoculated and control samples at weeks 0, 1, 5, and 12. Amplification of the *luxA* marker in the DNA extracts was equally successful in oil-contaminated and uncontaminated soils (Fig. 5, lanes 2 and 7). A successive decrease in the amount of amplified *luxA* marker DNA was observed in uncontaminated soil samples over 12 weeks (Fig. 5, lanes 2 to 5). This suggests that reduction in viable-cell numbers over 12 weeks (Fig. 3) resulted from cell disintegration rather than from cells being present in a nonculturable state. In oil-contaminated soil samples, the amount of target DNA after PCR amplification decreased markedly during the first week and then remained relatively constant for 1 to 12 weeks after inoculation (Fig. 5, lanes 7 to 10), a pattern that corresponded

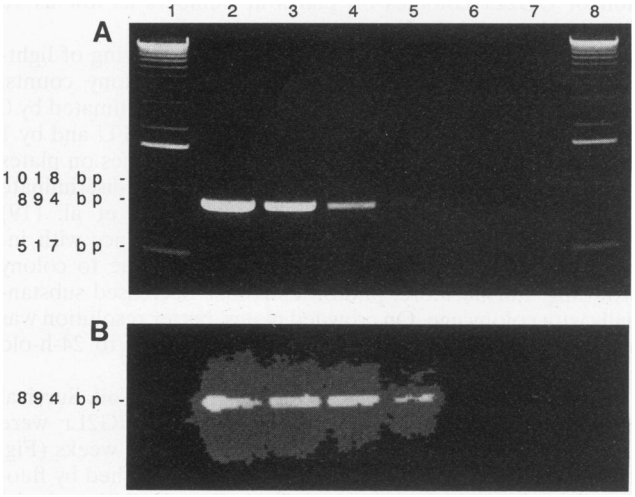

FIG. 4. (A) Agarose gel illustrating detection sensitivity by PCR of an 894-bp *luxA* fragment from *P. aeruginosa* UG2Lr. Lanes 1 and 8 were 1-kb DNA ladder marker; lanes 2 to 6 were loaded with DNA extracted from 2 × 10^6, 2 × 10^5, 2 × 10^4, 2 × 10^3, and 2 × 10^2 CFU/g of dry weight soil, respectively; lane 7 was the uninoculated control. (B) Photon image showing chemiluminescence detection of the amplified *luxA* fragment by using a digoxigenin-labelled internal probe.

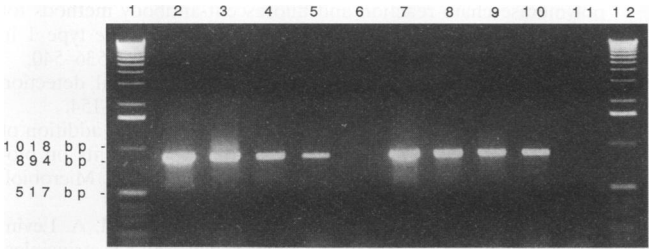

FIG. 5. Agarose gel illustrating PCR products of DNA extracts from uncontaminated (lanes 2 to 6) and oil-contaminated (lanes 7 to 11) soil samples taken at 0, 1, 5, and 12 weeks (first four lanes of each set, respectively) after inoculation with *P. aeruginosa* UG2Lr. Lanes 1 and 12 show the 1-kb DNA ladder; lanes 6 and 11 are uninoculated controls.

to the viable-cell counts over this period (Fig. 3). These data confirmed that viable-cell counts by nonselective culture techniques were accurate, and they demonstrated PCR to be an effective, semiquantitative technique when applied to the soil environment.

DISCUSSION

A reliable, rapid system to monitor GEMs in the environment is essential if these microorganisms are to be used for biotechnological purposes such as on-site remediation of contaminated soils. *P. aeruginosa* UG2L contained chromosomally integrated *luxAB* and *lacZY*, which allow detection of viable cells by light emission and blue colony color in the presence of *n*-decanal and X-Gal, respectively. The unique and artificial arrangement of DNA fragments in close proximity (28) also provided a means for unequivocal detection by PCR amplification.

The present study showed that *luxAB* reporter genes are suitable for tracking a GEM in soil because they are stable in soil, potentially innocuous to the indigenous gene pool, assayed easily and rapidly, unique to the GEM, and pose little metabolic burden to host cells. Other marker systems do not meet these criteria. For example, the *lacZY* (15) and *xylE* (33, 47) reporter genes for lactose and catechol utilization, respectively, are common in soil environments (25). Antibiotic resistance genes, which have been used to track microorganisms for the past few decades, may contribute to the indigenous antibiotic resistance gene pool, be unstable, and adversely affect competitive fitness of the host microorganism (10, 35). It was convenient, however, to use an antibiotic marker when numbers of UG2Lr became critically low in soil (10^2 CFU/g (dry weight) of soil).

Although genetic markers require some metabolic input from host cells (1, 10, 35), engineered UG2L, rifampin-resistant UG2Lr, and wild-type UG2 strains of *P. aeruginosa* survived equally well in soil over 12 weeks. UG2L and UG2 also exhibited similar growth and rhamnolipid production in liquid culture. England et al. (16) found no significant differences in respiratory activity or survival of wild-type and recombinant *lac*-modified *Pseudomonas aureofaciens* strains in soil. Masson et al. (28) also demonstrated that selected biological and physiological properties of a *lac-lux*-bearing *Pseudomonas cepacia* strain were not altered. Using the *luxAB* portion of the operon avoids light emission in the absence of substrate and circumvents the full metabolic burden posed by the *luxCDABE* operon (20).

Luminescence is advantageous as it indicates living cells only, is rare in soil, and requires no selective media or knowledge of colony morphology. Only one terrestrial luminescent microorganism, *Xenorhabdus luminescens*, has been reported (20). Light emission varies with bacterial species (25, 35) and is unstable in some marine bacterial species (39). Although the usefulness of these genes may be limited to certain microorganisms, *lux* markers have been used successfully with numerous pseudomonads and other environmental isolates (11, 28, 35). The main disadvantage of using the *lux* genes was the requirement for costly photon imaging devices. X-ray film may be used to image light-emitting colonies (28, 35) but with reduced image quality and colony resolution.

Bioluminescence markers have been used to detect and enumerate microorganisms directly in soil (19, 27) and on roots (11, 38) with sensitivities of 10^7 CFU/g for soil and 10^4 CFU/cm for roots, respectively. Detection in the range of 50 to 10 CFU/g of soil (28, 38) has been achieved by using some form of antibiotic selection. Masuko et al. (29) detected single bacteria on filters with an ultra-high-sensitivity TV camera. Mahro et al. (27) detected single cells of a *Photobacterium phosphoreum* strain in liquid culture by using low-light video microscopy. With charge-couple-device image-enhanced microscopy, in situ detection of microcolonies and single cells of a marked population is possible (35).

Nonculturable cells have been reported in both terrestrial and aquatic systems (9, 12, 22, 32, 40). Smalla et al. (40) showed that DNA of a *Pseudomonas fluorescens* inoculant was recovered and amplified from soil after 5 months, while viable inoculant was not recovered. These data suggested that nonculturable cells or intact marker DNA was present. Similar observations were reported by Morgan et al. (32) for *Aeromonas salmonicida* in an aquatic environment. In the present study, the decline of culturable UG2Lr cells in soil microcosms over 12 weeks was accompanied by a decrease in PCR product of the *luxA* marker. Substantial portions of the UG2Lr population genuinely died off and cells were disintegrated. Given the PCR detection limit of about 2×10^3 cells/g (dry weight) of soil, however, the existence of nonculturable UG2Lr cells below this limit remains undetectable.

P. aeruginosa UG2Lr successfully established itself over 3 months in soil microcosms contaminated with a light crude oil, with populations remaining at 10^7 CFU/g (dry weight) of soil. Ongoing studies in our laboratory indicate that UG2Lr also survives well in a creosote-contaminated soil from a wood treatment facility. In microcosms containing this soil, a UG2Lr inoculum of 3×10^8 CFU/g was recovered at 5×10^6 CFU/g (dry weight) of soil after 3 months (unpublished data). The persistence of UG2Lr in hydrocarbon-contaminated soils demonstrates the potential of *P. aeruginosa* UG2Lr for use in bioremediation of contaminated soils.

ACKNOWLEDGMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Ontario Ministry of Environment and Energy, and the Institute for Chemical Science and Technology. We thank Luke Masson for preparing the final plasmid delivery system, Anca Mihoc for screening of UG2L, and R. Ian Lovegrove for analyzing L-rhamnose and assisting with growth studies. We also thank Mansel Griffiths, Department of Food Science, University of Guelph, for kindly allowing use of the Biomedical Image Quantifier and Esso Research Centre for providing the virgin heavy atmospheric gas oil.

REFERENCES

1. **Atlas, R. M.** 1992. Detection and enumeration of microorganisms based upon phenotype, p. 29–43. *In* M. A. Levin, R. J. Seidler, and M. Rogul (ed.), Microbial ecology. McGraw Hill, Inc., New York.
2. **Barry, G. F.** 1988. A broad-host-range shuttle system for gene

insertion into the chromosomes of Gram-negative bacteria. Gene **71:**75–84.
3. **Bej, A. K., J. L. DiCesane, L. Haff, and R. M. Atlas.** 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. **57:**1013–1017.
4. **Berg, G., A. G. Seech, H. Lee, and J. T. Trevors.** 1990. Identification and characterization of a soil bacterium with extracellular emulsifying activity. J. Environ. Sci. Health **A25:**753–764.
5. **Boehringer Mannheim.** 1992. The Genius System user's guide for filter hybridization. Boehringer Mannheim Corporation, Indianapolis, Ind.
6. **Chadrasekaran, E. V., and J. N. BeMiller.** 1980. Constituent analysis of glycosaminoglycans. *In* R. L. Whistler and J. N. BeMiller (ed.), Methods in carbohydrate chemistry, vol. 8. Academic Press, New York.
7. **Chao, W. L., and R. L. Feng.** 1990. Survival of genetically engineered *Escherichia coli* in natural soil and river water. J. Appl. Bacteriol. **68:**319–325.
8. **Cohn, D. H., A. J. Mileham, M. I. Simon, K. H. Nealson, S. K. Rausch, K. Bonam, and T. D. Baldwin.** 1985. Nucleotide sequence of the *luxA* gene of *Vibrio harveyi* and the complete amino acid sequence of the α subunit of bacterial luciferase. J. Biol. Chem. **260:**6139–6146.
9. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but nonculturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3:**817–820.
10. **Compeau, G., B. Jadoun Al-Achi, E. Platsouka, and S. B. Levy.** 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. **54:**2432–2438.
11. **De Weger, L. A., P. Dunbar, W. F. Mahafee, B. J. J. Lutenberg, and G. S. Sayler.** 1991. Use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. Appl. Environ. Microbiol. **57:**3641–3644.
12. **Dijkmas, R., A. Jagers, S. Kreps, J.-M. Collard, and M. Mergeay.** 1993. Rapid method for purification of soil DNA for hybridization and PCR analysis. Microb. Releases **2:**29–34.
13. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
14. **Drahos, D. J.** 1991. Field testing of genetically engineered microorganisms. Biotechnol. Adv. **9:**157–171.
15. **Drahos, D. J., B. C. Hemming, and S. McPherson.** 1986. Tracking recombinant organisms in the environment: B-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Bio/Technology **4:**439–443.
16. **England, L. S., H. Lee, and J. T. Trevors.** 1993. Recombinant and wild-type *Pseudomonas aureofaciens* strains in soil: survival, respiratory activity and effects on nodulation of whitebean, *Phaseolus vulgaris* L., by *Rhizobium* species. Mol. Ecol. **2:**303–313.
17. **Fredrickson, J. K., D. F. Bezdicek, F. J. Brockman, and S. W. Li.** 1988. Enumeration of Tn*5* mutant bacteria in soil by using a most-probable-number—DNA hybridization procedure and antibiotic resistance. Appl. Environ. Microbiol. **54:**446–453.
18. **Genthner, F. J., J. Upadhyay, R. P. Campbell, and B. R. Sharak Genthner.** 1990. Anomalies in the enumeration of starved bacteria on culture media containing nalidixic acid and tetracycline. Microb. Ecol. **20:**283–288.
19. **Grant, F. A., L. A. Glover, K. Killham, and J. I. Prosser.** 1991. Luminescence-based viable cell enumeration of *Erwinia carotovora* in the soil. Soil Biol. Biochem. **23:**1021–1024.
20. **Hill, P. J., C. E. D. Rees, M. K. Winson, and G. S. A. B. Stewart.** 1993. The application of *lux* genes. Biotechnol. Appl. Biochem. **17:**3–14.
21. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54:**703–711.
22. **Islam, M. S., M. K. Hasan, M. A. Miah, G. C. Sur, A. Felsenstein, M. Venkatesan, R. B. Sack, and M. J. Albert.** 1993. Use of the polymerase chain reaction and fluorescent-antibody methods for detecting viable but nonculturable *Shigella dysenteriae* type 1 in laboratory microcosms. Appl. Environ. Microbiol. **59:**536–540.
23. **Jackman, S. C., H. Lee, and J. T. Trevors.** 1992. Survival, detection and containment of bacteria. Microb. Releases **1:**125–154.
24. **Jain, D. K., H. Lee, and J. T. Trevors.** 1992. Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. J. Ind. Microbiol. **10:**87–93.
25. **Kado, C. I.** 1992. Lux and other reporter genes. *In* M. A. Levin, R. J. Seidler and M. Rogul (ed.), Microbial ecology: principles, methods and applications. McGraw-Hill Inc., New York.
26. **Leung, K. T., J. T. Trevors, and J. D. van Elsas.** Extraction and amplification of DNA from the rhizosphere and rhizoplane of plants. *In* J. T. Trevors and J. D. van Elsas (ed.), Nucleic acids in the environment, in press. Springer-Verlag, Heidelberg, Germany.
27. **Mahro, B., A. Petermann-Hermann, S. Walenta, W. Muller-Klieser, and V. Kasche.** 1992. Non-extractive localization and imaging of luminescent bacteria in liquid and soil samples by luminescence microscopy. Microb. Releases **1:**79–85.
28. **Masson, L., Y. Comeau, R. Brousseau, R. Samson, and C. Greer.** 1993. Construction and application of chromosomally integrated *lac-lux* gene markers to monitor the fate of a 2,4-dichlorophenoxyactic acid-degrading bacterium in contaminated soils. Microb. Releases **1:**209–216.
29. **Masuko, M., S. Hosoi, and T. Hayakawa.** 1991. A novel method for detection and counting of single bacteria in a wide field using an ultra-high-sensitivity TV camera without a microscope. FEMS Microbiol. Lett. **81:**287–290.
30. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
31. **Miyamoto, C. M., M. Boylan, A. F. Graham, and E. A. Meighen.** 1988. Organization of the *lux* structural genes of *Vibrio harveyi*. J. Biol. Chem. **263:**13393–13399.
32. **Morgan, J. A. W., G. Rhodes, and R. W. Pickup.** 1993. Survival of nonculturable *Aeromonas salmonicida* in lake water. Appl. Environ. Microbiol. **59:**874–880.
33. **Morgan, J. A. W., C. Winstanley, R. W. Pickup, J. G. Jones, and J. R. Saunders.** 1989. Direct phenotypic and genotypic detection of a recombinant pseudomonad population released into lake water. Appl. Environ. Microbiol. **55:**2537–2544.
34. **Morgan, P., and R. J. Watkinson.** 1989. Hydrocarbon degradation in soils and methods for soil biotreatment. Crit. Rev. Biotechnol. **8:**305–332.
35. **Prosser, J. I.** 1994. Molecular marker systems for detection of genetically engineered micro-organisms in the environment. Microbiology **140:**5–17.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Scheibenbogen, K., R. G. Zytner, H. Lee, and J. T. Trevors.** 1994. Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemical surfactants. J. Chem. Technol. Biotechnol. **59:**53–59.
38. **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.
39. **Silverman, M., M. Martin, and J. Engebrecht.** 1989. Regulation of luminescence in marine bacteria, p. 71–86. *In* D. A. Hopwood and K. F. Chater (ed.), Genetics of bacterial diversity. Academic Press, London.
40. **Smalla, K., N. Cresswell, L. C. Mendonca-Haglar, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74:**78–85.
41. **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.
42. **Tsai, Y. L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
43. **Van Dyke, M. I., P. Couture, M. Brauer, H. Lee, and J. T. Trevors.**

1993. *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactants: structural characterization and their use in removing hydrophobic compounds from soil. Can. J. Microbiol. **39:**1071–1078.

44. **Van Elsas, J. D., J. T. Trevors, D. Jain, A. C. Walters, C. E. Heijnen, and L. S. Van Overbeek.** 1992. Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. Biol. Fertil. Soils **14:**14–22.
45. **Van Overbeek, L. S., J. D. Van Elsas, J. T. Trevors, and M. E. Starodub.** 1990. Long-term survival of plasmid stability in *Pseudomonas* and *Klebsiella* species and appearance of nonculturable cells in agricultural drainage water. Microb. Ecol. **19:**239–249.
46. **Wilson, M., and S. E. Lindow.** 1993. Release of recombinant microorganisms. Annu. Rev. Microbiol. **47:**913–944.
47. **Winstanley, C., J. A. W. Morgan, R. W. Pickup, and J. R. Saunders.** 1991. Use of a *xylE* marker gene to monitor survival of recombinant *Pseudomonas putida* populations in lake water by culture on nonselective media. Appl. Environ. Microbiol. **57:**1905–1913.