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Monitoring genetically engineered microorganisms in freshwater microcosms

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SUMMARY

The effectiveness of gene probe methods for tracking genetically engineered microorganisms (GEMs) in the environment was tested by inoculating nutrient-supplemented freshwater microcosms with *Alcaligenes* A5 (a naturally occurring 4-chlorobiphenyl degrader) or *Pseudomonas cepacia* AC1100 (a genetically engineered 2,4,5 T-degrader) and following the fates of the introduced bacterial populations. Colony hybridization of the viable heterotrophic bacterial populations and dot blot hybridization of DNA recovered from the total microcosm microbial communities showed persistence of both *Alcaligenes* A5 and *P. cepacia* AC1100 in the microcosms in the presence and absence of the xenobiotic substrates that these organisms biodegrade. Although there was a gradual decline in the added populations, both of the bacterial populations were still detected in the microcosms two months after their introduction into the microcosms. Addition of 2,4,5-T enhanced the survival of *P. cepacia* AC1100— and 4-chlorobiphenyl addition resulted in increased levels of *Alcaligenes* A5. The results indicate that both organisms may persist for very long periods in freshwater habitats.

INTRODUCTION

In order to evaluate the potential risks and to develop strategies for the successful deployment of genetically engineered microorganisms in the environment for biodegrading environmental pollutants, specific data is needed on the organism/gene survival, proliferation, and maintenance density, and the environmental factors that effect these parameters [4,7,21]. The adaptation of molecular analytical tools is necessary for strain and genotype specific analysis in environmental matrices and several molecular (gene probe) monitoring methodologies currently complement the battery of methods used in environmental microbiology for monitoring

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the fate of organisms that are deliberately released microorganisms [5,6,14,17,19,25]. Besides their potential use for monitoring genetically engineered microorganisms (GEMS) that enter the environment, the emerging molecular monitoring methods in conjunction with appropriate model ecosystems (microcosms) can aid in predicting the fate of GEMS in the environment and the factors that influence the persistence or disappearance of intentionally released organisms [15,19,20]. In this study we have evaluated the maintenance of two microbial genotypes by using freshwater microcosms and molecular probes for monitoring introduced populations. The study examined whether populations introduced into the environment for their abilities to degrade environmental pollutants rapidly disappear due to competition or other biological interactions, or whether such organisms persist under conditions that model freshwater microbial communities.

MATERIALS AND METHODS

Microcosms and test organisms

Microcosms were established by placing 19 l water samples (collected at a depth of 1 m from Fort Loudon Reservoir near Knoxville, Tennessee) into sterile 20 l glass carboys. Replicate microcosms were treated with either 4-chlorobiphenyl (4-CB; 25 mg/l, Alpha Products) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; 100 mg/l, Sigma) to model potential xenobiotic pollutants and then inoculated with either *Alcaligenes* A5 or *Pseudomonas cepacia* AC1100 as model bioremediation treatments. *P. cepacia* AC1100, which was created by plasmid assisted molecular breeding [10,11] is capable of degrading 2,4,5-T in pure culture and in soil [1,12,13]. *Alcaligenes* A5 contains the degradative plasmid pSS50 (53 kb) which mediates the mineralization of 4-chlorobiphenyl (3,20,26]. Control microcosms that were not treated with xenobiotics and/or not inoculated with *Alcaligenes* A5 or *P. cepacia* AC1100 were also included. Each microcosm also was supplemented with 100 mg/l glycose and a mineral salts solution containing per liter: 40 mg $NaNO_3$, 15 mg KH_2PO_4, 0.05 mg $FeCl_2$, 0.2 mg $MgSO_4$, 0.2 mg $CaCl_2$, 0.5 mg $NaHPO_4$ as model nutrient supplements that would likely be added during bioremediation to enhance the survival potential of the introduced microorganisms.

For microcosm inoculation, the bacterial strains were grown to early log phase. The *Alcaligenes* A5 was grown in YEPG broth [23]. The *P. cepacia* AC1100 was grown in basal salts medium [9] containing 1 mg/ml 2,4,5-T. The cells were collected by centrifugation, washed with sodium phosphate buffer (pH 6.8), and added to the microcosms to give final concentrations of 10^4 to 10^5 cells/ml. The microcosms were incubated in the dark at 25°C for 4 or 8 weeks with continuous stirring on stir plates. Each experimental treatment was tested in duplicate microcosms. Replicate experimental tests were run for 4 and 8 week incubation periods.

Detection of microbial genotypes

Plate counts were performed weekly by diluting samples from each microcosm in 0.1 M sodium phosphate buffer and spread-inoculating them onto 0.5 strength YEPG agar plates [23]. Plates were incubated at 25°C for 7 days and 'total' heterotrophic viable cell numbers were determined. Both *Alcaligenes* A5 and *Pseudomonas cepacia* AC1100, as well as numerous other heterotrophic bacteria, grow and form colonies on YEPG agar under these incubation conditions.

Colony hybridization was used to detect specifically *Alcaligenes* A5 and *P. cepacia* AC1100. The gene probe used to detect *P. cepacia* AC1100 was a 1.3 kb repeat sequence (RS-1100-1) which is present in 15 to 20 copies on this organism's chromosome and plasmids (P. Tomasek, personal communication). *Alcaligenes* A5 was detected by using the entire pSS50 plasmid (53 kb) as a probe. The probes were radiolabeled by using a nick translation kit (BRL) and either [α-^{32}P]-dCTP or [α-^{32}P]-dATP. Colony hybridization was performed as previously described [16,24] after transferring the colonies to Biodyne A nylon membranes (Pall Ultrafine Filtration Corporation). A high stringency wash was employed to minimize nonspecific hybridization [24]. Autoradiography was performed by exposing the

filters to X-ray film (X-Omat AR, Kodak) for 4 h at −70°C as previously described [16].

At the end of each experiment (4 or 8 weeks), the cells from each microcosm were collected by using a Millipore Pellicon Cassette System (Millipore Products). The recovered cells were then lysed and their DNA recovered as described by Ogram et al. [18] with the following modifications: the lysates were brought to 2.5 M sodium acetate to precipitate non-DNA organics; after centrifuation to separate the ammonium acetate precipitated material, the DNA in the supernatant was precipitated with ethanol; hydroxyapatite column chromatography was not used for DNA purification. The recovered DNA was diluted in TE (10 mM tris-HCl, 1 mM EDTA, pH 8.0) and 0.001 μg to 1.0 μg from each sample was transferred to Biodyne A membranes by using a Minifold I microsample filtration manifold (Schleicher and Schuell). The DNA was then denatured by placing the membranes on filter paper pads saturated with 1.5 M NaCl and 0.5 NaOH for 5 min and then neutralizing them by placing them on pads saturated with 3 M sodium acetate (pH 5.5). The DNA was bonded to the membranes by baking them for 1 h at 80°C. The presence or absence of DNA from the test microorganisms was determined by hybridization analysis using the previously described gene probes followed by autoradiography.

RESULTS

The initial level of heterotrophic bacteria in the microcosms was 10^5/ml. In the control microcosms, which were not inoculated with *Alcaligenes* A5 or *P. cepacia* AC1100 and did not receive 4-chlorobiphenyl or 2,4,5.T, the total heterotrophic bacterial population did not increase in response to the addition of nutrients to the microcosms and gradually declined to 10^4/ml during the 8 week incubation (Fig. 1A). Neither the *Alcaligenes* A5 nor the *P. cepacia* AC1100 were detected in these control microcosms by colony hybridization. The detection level by colony hybridization (shown as a dotted line in Fig. 1) is about 3 orders of magnitude below the number of viable bacteria enumerated in the plate count procedure because when the target organism is present at levels below 0.1% of the total enumerated viable population the plates where the target population can be detected are totally overgrown by the non-target ('total' viable) population. Thus, either organism could have been present in For Loudon Reservoir water at a level of 10^2/ml or less and not been detected by this method.

Tests on lakewater samples and thousands of bacterial isolates from Fort Loudon Reservoir nev-

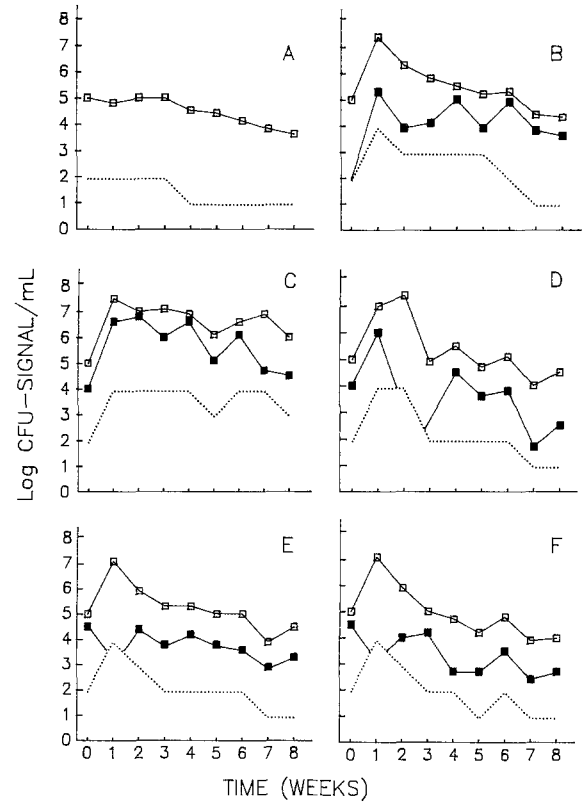

Fig. 1. Levels of organisms detected by viable plate count and colony hybridization procedures. The treatments for the microcosms are as follows: microcosm A, no organism and xenobiotic no chemical added; microcosm B, no organism and 4-chlorobiphenyl added; microcosm C, *Alcaligenes* A5 and 4-chlorobiphenyl added; microcosm D, *Alcaligenes* A5 and no xenobiotic chemical added; microcosm E, *Pseudomonas cepacia* AC1100 and 2,4,5-T added; microcosm F, *Pseudomonas cepacia* AC1100 and no xenobiotic chemical added. Dotted lines indicate levels of detection limits by colony hybridization. Open squares indicate viable plate count. Solid squares indicate colony hybridization detection of the specified genotypes *Alcaligenes* A5 (A–D) and *P. cepacia* AC1100 (E–F).

er detected *Pseudomonas cepacia* AC1100 or any other 2,4,5 T-degrader, strongly suggesting that *P. cepacia* AC1100 does not naturally occur in Fort Loudon Reservoir. This result is consistent with those of Chakrabarty and coworkers who have never detected the 'genetically engineered' 2,4,5 T-degrader *P. cepacia* AC1100 in natural systems (A. Chakrabarty, personal communication). In contrast, the *Alcaligenes* A5 pSS50 plasmid occurs naturally in the aquatic bacterial community of Fort Loudon Reservoir as evidenced by the significant level of this plasmid detected in microcosms one week after treatment with 4-chlorobiphenyl even though these microcosms had not been inoculated with *Alcaligenes* A5 (Fig. 1B). In these microcosms, the pSS50-bearing populations that were enriched for by addition of 4-chlorobiphenyl fluctuated between 10^4 and 10^5/ml throughout the experiment.

The levels of pSS50-bearing plasmid populations in the microcosms receiving only 4-chlorobiphenyl and no inoculum were about an order of magnitude lower than the microcosms to which both 4-chlorobiphenyl and *Alcaligenes* A5 were added (Fig. 1C). In both the inoculated and uninoculated microcosms with 4-chlorobiphenyl, the maintenance levels of the pSS50 bearing populations appear to be in excess of 10^4/ml or 5–50% of the 'total' viable heterotrophic microbial populations. Inoculation of the microcosms without the addition of 4-chlorobiphenyl resulted in wide population fluctuations of the *Alcaligenes* A5, with the organism dropping below detection limits at the 2 and 3 week sampling times, reappearing at significant levels between weeks 4 and 6, and beginning to disappear again between weeks 7 and 8 (Fig. 1D). Levels of *Alcaligenes* A5 after 2 months in the inoculated microcosms without 4-chlorobiphenyl were only 1% of those receiving both the organism and 4-chlorobiphenyl, indicating that the presence of 4-chlorobiphenyl greatly enhanced the level of persistence of the *Alcaligenes* pSS50-bearing population.

Inoculation with *P. cepacia* AC1100 with and without the addition of 2,4,5-T resulted in persistent levels of 10^3 to 10^4/ml of this organism during the 2 month incubation (Fig. 1E, 1F). The levels of *P. cepacia* AC1100 were slightly higher in the microcosms receiving both the organism and 2,4,5-T (Fig. 1E) than in those to which only the organism was added (Fig. 1F).

In contrast to the colony hybridization results, which indicated the presence of the pSS50 plasmid at the endpoints of the 4 and 8 week replicate runs, dot blots on DNA extracted from the microbial community gave variable results for the detection of *Alcaligenes* A5 (Table 1). The failure to detect the pSS50 plasmid indicates limited sensitivity of this method using a very large (53 kb) gene probe. Although sometimes failing to detect the *Alcaligenes* A5 when it clearly was present, the dot blots consistently confirmed the presence of the *P. cepacia* AC1100 in microcosms where this organism was added and its absence in the other microcosms (Table 1). The gene probe analysis of the DNA recovered from the total microbial community for the presence of *P. cepacia* AC1100 appeared to give sensitive and reliable results. The difference between

Table 1

Dot blot hybridization using 1 microgram of DNA recovered from the total bacterial community in each microcosm

Microcosm					
A	B	C	D	E	F
Probe for *Alcaligenes* A5 pSS50					
		4 week incubation			
+	+	−	+	−	−
		8 week incubation			
+		+	+	+	+
Probe for *P. cepacia* AC1100					
		4 week incubation			
−	−	−	−	++	++
		8 week incubation			
−		−	−	++	++

The treatments for the microcosms are as follows: A, no organism and no chemical added; B, no organism and 4-chlorobiphenyl added; C, *Alcaligenes* A5 and 4-chlorobiphenyl added; D, *Alcaligenes* A5 and no chemical added; E, *Pseudomonas cepacia* AC1100 and 2,4,5-T added; F, *Pseudomonas cepacia* AC1100 and no chemical added. The probe for the *Alcaligenes* A5 pSS50 plasmic was 53 kb. The probe for *Pseudomonas cepacia* AC1100 was 1.3 kb. The signals for the dot blots were scored based upon visual intensity as follows: − = no signal; + = weak positive signal; ++ = strong positive signal.

the sensitivity of detection of *Alcaligenes* A5 and *P. cepacia* AC1100 may be due to the different sizes of the probes (the 1.3 kb probe used to detect *P. cepacia* AC1100 is likely to give better hybridization results than the 53 kb probe used to detect *Alcaligenes* A5), the number of copies of the target sequence (the target of the 1.3 kb *P. cepacia* AC1100 probe occurs as a repeat sequence), or real population survival differences between the two introduced populations.

DISCUSSION

The current study shows that specific microbial genotypes can be monitored in freshwater ecosystems using gene probe methods. However, caution must be used in interpreting results when the organism, or the gene for which the probe is targeted, is not detected. Clearly both the colony hybridization and community DNA-dot blot hybridization procedures sometimes failed to detect the presence of the introduced (targeted) organism when it was still present within the microcosm. It is possible that multiple methodologies will be required to monitor the fate of genetically engineered microorganisms in the environment. Future developments in detection technology should attempt to detect organisms present in small numbers, relative to the total population, and organisms present in a non-culturable state. Efficient DNA recovery methods [28] and the polymerase chain reaction (PCR) method [27], which amplifies a target DNA sequence may provide the necessary sensitivity for detection of genetically engineered microorganisms that are deliberately released into the environment.

Even using the present sensitivity limits imposed by the colony hybridization and dot blot procedures, we were able to establish that the bacterial populations added to freshwater microcosms can persist for long periods of time and may in fact become part of the autochthonous microbial community. Both the naturally occurring *Alcaligenes* A5 and the genetically engineered *P. cepacia* AC1100 remained at significant population levels within the microcosms, especially if the specific growth substrates for the organisms were also added. There may be a carrying capacity for the added organisms as indicated by lack of a significant negative slope to the survival curves.

Our findings differ from those that have were observed when *P. cepacia* AC1100 was introduced into soil and its persistence monitored using viable plate count methods [12]. In that study populations of *P. cepacia* AC1100 rapidly declined and became undetectable, but reappeared after addition of 2,4,5-T as a growth substrate to the soil indicating that the sensitivity of the viable plating procedure used was inadequate to detect the continued persistence of *P. cepacia* AC1100 in soil. Our finding of genotype persistence in freshwater microcosms is similar to those of Jain et al. [8], who observed that several plasmid-bearing bacterial strains were maintained in groundwater-aquifer microcosms without specific selective pressure for the organism or the plasmid. Devanas and Stotzky [2] also reported that populations of *E. coli* HB101 containing either pBR322 or pC357 persisted in soils supplemented with nutrients, although the same populations declined rapidly in non-sterile soils not supplemented with nutrients.

Our results refute the contention that deliberately released genetically engineered microorganisms will always rapidly disappear from natural communities in the absence of a specific selective pressure because of competition and predation. An important implication of our findings is that genetically engineered organisms intentionally added to freshwater environments potentially will survive long enough to permit horizontal gene exchange.

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