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Maintenance and Stability of Introduced Genotypes in Groundwater Aquifer Material

RAKESH K. JAIN,[1] GARY S. SAYLER,[1*] JOHN T. WILSON,[2] LAURA HOUSTON,[1] AND DAVID PACIA[1]

Department of Microbiology and The Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37996,[1] and R. S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, Oklahoma 74821[2]

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Three indigenous groundwater bacterial strains and *Pseudomonas putida* harboring plasmids TOL (pWWO) and RK2 were introduced into experimentally contaminated groundwater aquifer microcosms. Maintenance of the introduced genotypes was measured over time by colony hybridization with gene probes of various specificity. On the basis of the results of colony hybridization quantitation of the introduced organisms and genes, all introduced genotypes were stably maintained at approximately 10^5 positive hybrid colonies g^{-1} of aquifer microcosm material throughout an 8-week incubation period. Concomitant removal of the environmental contaminants, viz., toluene, chlorobenzene, and styrene, in both natural (uninoculated) and inoculated aquifer microcosms was also demonstrated. The results indicate that introduced catabolic plasmids, as well as indigenous organisms, can be stably maintained in groundwater aquifer material without specific selective pressure for the introduced genotypes. These results have positive implications for in situ treatment and biodegradation in contaminated aerobic groundwater aquifers.

Chemical contamination of groundwater aquifer environments represents a significant environmental health hazard. In situ biodegradation of groundwater contaminants has been suggested as a mechanism to alleviate these problems (8, 11, 15). The potential exists for the use of bacteria with chromosomally encoded, natural, or chimeric plasmid-encoded catabolic pathways to augment or initiate in situ biodegradation. This potential in part is dependent on the ability of the organisms or plasmids to be successfully maintained in the groundwater environment. However, very little is known about the maintenance, stability, and monitoring of specific introduced organisms or genes within a microbial community. Furthermore, such information is also important to provide the safety assurances relative to the environmental risk of such applications.

To date, the microbiology of groundwater environments has not been investigated extensively; however, with the advent of subsurface aseptic sampling techniques (19), it has been demonstrated that shallow groundwater aquifer environments harbor significant, physiologically stressed bacterial populations (18, 19). Many of the populations can be cultivated by conventional microbiological methods and could potentially be used for augmenting indigenous populations to promote in situ biodegradation. Recently, plasmids have been detected in subsurface bacteria with frequencies from <1 to 38% in pristine and chemically contaminated aquifer materials, respectively (10). Therefore, it is possible that plasmids are associated with in situ biodegradation. Environmental application of the DNA-DNA colony hybridization approach (14) indicated that detection and monitoring of groundwater organisms or plasmids are possible and could contribute to determining the maintenance of specific genotypes.

The present investigation was undertaken to monitor the maintenance and stability of introduced genotypes in groundwater aquifer material. Three groundwater bacterial strains and *Pseudomonas putida* carrying the plasmids TOL (pWWO) (toluene-xylene catabolism) and RK2 (kanamycin resistance) were successfully introduced and maintained in a groundwater microbial community. The role of environmental contaminants that may serve as substrates or cooxidative substrates was also examined as a selective pressure that may affect organismal and gene maintenance. The results indicate that introduced organisms or plasmid-borne catabolic genes can be successfully maintained in groundwater aquifer material regardless of the selective pressure.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* containing the plasmids TOL and RK2 was obtained from the filter mating of *P. putida* (TOL) with *Escherichia coli* PRC116 (RK2) (Table 1); selection for transconjugants was on minimal agar plates (4) containing *m*-toluate and kanamycin (100 μg ml^{-1}). Results were confirmed by plasmid isolation and agarose gel electrophoresis from transconjugants. Strains ABS10, AHS24, and AOS23 (Table 1) are indigenous groundwater bacteria isolated from pristine aquifer material in a previous study (10, 12); AHS24 and ABS10 are plasmid-free putative "*Arthrobacter*" sp. and "*Pseudomonas*" sp., respectively. Strain AOS23 is an additional putative "*Arthrobacter*" sp. which carries a large cryptic plasmid of approximately 100 kilobase pairs (kb) (data not shown), designated pOS23. Routine maintenance of strains used for microcosm inoculation was on yeast extract peptone glucose (YEPG) agar, 1/10 strength (13).

Microcosm preparation. A composite sample (9CCG) of approximately 2 kg was aseptically obtained from subsurface core material at Lula, Okla. The aquifer sample zone is considered pristine (21), and samples were obtained from the saturated zone (approximately −7 m) immediately below a

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Plasmid complement(s) (size in kb)	Source or reference
Escherichia coli C600	pTS206 (5.2)	5
Escherichia coli JM107	pUC13 (2.3)	17
Escherichia coli DH1	p2MLO (8.2)	12, this study
Escherichia coli PRC116	RK2 (58)	16
Pseudomonas putida (*arvilla*) mt-2	TOL (117)	ATCC 23973
Pseudomonas putida (*arvilla*) mt-2	RK2 (58) and TOL (117)	This study
"*Pseudomonas* sp. strain ABS10"	—[a]	12
"*Arthrobacter* sp. strain AHS24"	—	12
"*Arthrobacter* sp. strain AOS23"	pOS23 (~100)	12, this study

[a] —, None.

plastic clay layer (Fig. 1). Weighed subsamples were aseptically transferred to sterile glass vials and experimentally inoculated, independently at approximately 10^6 cells g^{-1}, with one of the three indigenous groundwater bacterial strains ABS10, AHS24, or AOS23 or with a model genetically modified bacterium, *P. putida* (harboring the plasmids TOL and RK2). Bacterial inocula were prepared from 24-h cultures in YEPG broth. Cells were harvested, washed in 0.1 M sodium PP_i; (pH 7.0), and suspended in reverse-osmosis-prepared water immediately before inoculation with a 0.3-ml volume. A replicate group of natural control microcosms received an addition of heat-killed strain ABS10 at the same concentration as that of the inoculated samples to serve as a nutrient control for an inoculum effect. Replicated groups of inoculated samples were experimentally treated with a dose of trichloroethylene, toluene, chlorobenzene, and styrene in reverse-osmosis water to achieve an effective concentration of approximately 400 μg liter^{-1} for each chemical. Trichloroethylene was used as a carrier and nonbiodegradable internal standard for normalization of residual concentrations of the other contaminants. Both uninoculated and uncontaminated control samples groups were prepared.

The treated aquifer samples were sealed with Teflon (Du Pont Co.)-lined, screw-cap closures to create a microcosm environment and were incubated at 22°C for various time periods. At each time period, duplicate pairs of microcosms from each treatment group were sacrificed and analyzed for maintenance levels of the introduced genotype. Residual levels of the chemical contaminants were determined after 8 and 16 weeks of incubation.

Isolation of DNA. Large-scale isolation of plasmid DNA from AOS23 (pOS23) was by the method of Kado and Liu (6) with some minor modifications. Plasmid DNA was isolated from 250-ml overnight-grown culture in nutrient broth. The lysing buffer contained freshly prepared 3% (wt/vol) sodium dodecyl sulfate (Sigma Chemical Co.) in 50 mM Tris (Sigma); the pH of the buffer was adjusted to 12.6 with 2 N sodium hydroxide solution. After extraction with phenol:chloroform-isoamyl alcohol (1:1), the DNA was purified by banding in a cesium chloride-ethidium bromide density gradient run at 100,000 × *g* for 42 h. After collection of the plasmid DNA under ultraviolet light, ethidium bromide and CsCl were removed by extraction with cold (−20°C) *n*-butanol and dialysis with TE (20 mM Tris, 1 mM EDTA; pH 8.0) buffer in the presence of Dowex ion-exchange resin (Dow Chemical Company, Midland, Mich.), respectively. Isolation of DNA from ABS10 and AHS24 have been previously described (12).

Plasmid pTS206 (5, 9) was isolated on a large scale from 300 ml of chloramphenicol-amplified overnight nutrient broth culture by a Triton X-100 (Sigma) cleared lysate procedure (7) with some modifications. After being harvested by centrifugation, cells were suspended at 0°C in 6 ml of 50 mM Tris–20% sucrose (pH 8.0). This was followed by the addition of 1 ml of 10 mg of lysozyme solution ml^{-1} in TE buffer. Then 8 ml of 0.5% Triton X-100–50 mM Tris–25 mM EDTA (pH 8.0) was added and mixed immediately and thoroughly at room temperature. After 10 min, the lysate was centrifuged at 20,000 × *g* for 10 min, and the supernatant from the clearing spin was taken. The plasmid DNA was purified by CsCl-ethidium bromide density gradient centrifugation as described above. Large-scale isolation of RK2 plasmid DNA was done by the method of Anderson and McKay (1). DNA was isolated from a 1-liter overnight culture of *E. coli* PRC116 in Luria broth with 50 mg of kanamycin liter^{-1}. After precipitation in isopropanol, the plasmid DNA was purified by CsCl-ethidium bromide density gradient centrifugation as described above.

Rapid isolation of plasmid DNA was by the method of Kado and Liu (6), and agarose gel electrophoresis with 0.7% agarose vertical slab gels was as previously described (10).

Probe preparation and DNA-DNA colony hybridization. The maintenance and monitoring of organisms was determined with the DNA-DNA colony hybridization approach (3, 14). Monitoring of ABS10 was performed with a whole chromosomal DNA probe of the ABS10 genome. For the

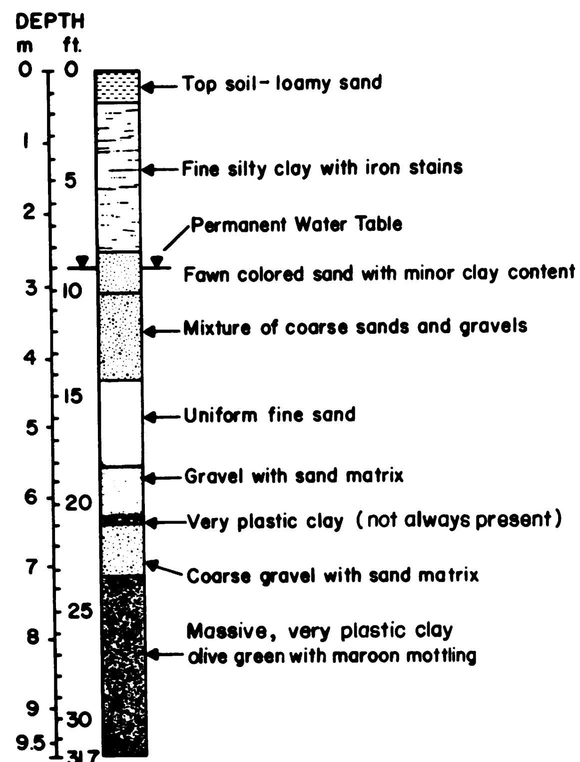

FIG. 1. Subsurface profile of the Lula, Okla., sample site. Aquifer material was recovered from the region approximately 7 m deep.

monitoring of AHS24, a DNA probe (p2MLO) (12) consisting of a 5.9-kb AHS24-specific chromosomal insert in the pUC13 cloning vector (17) was used. This specificity has been demonstrated by extensive cross-hybridization analysis with other isolates from the same sample site (12). AOS23 was monitored by a pOS23 plasmid DNA probe (Table 1). Plasmids TOL and RK2 carried by *P. putida* were monitored with plasmids pTS206 (5) and RK2 (16) as probe DNAs, respectively; pTS206 contains a 0.6-kb insert of the *xylR* gene fragment of TOL cloned into pMCR600 (9). All DNA probes were prepared by the nick translation method (2) with [^{32}P]dCTP (ICN Radiochemicals), and detection of positive hybrids was by autoradiography. Quantitative samples for hybridizations were obtained by dilution of samples from each microcosm in sodium PP_i (pH 7.0), by spread plate inoculation of YEPG agar plates, and then by incubation at 22°C for 3 to 4 days. After bacterial colony development, colonies were directly transferred to Biotrans nylon filters (ICN) by overlaying the filters on the agar surface and lifting off the colonies. Conditions for colony lysis, prehybridization, hybridizations with appropriate gene probes, washing, and autoradiography have been previously described (12, 14); hybridization washing conditions (low salt) were imposed at 95% stringency, thus allowing no more than a 5% mismatch of base pairs. Control hybridizations with the cloning vector alone and for background indigenous homologous genes in uninoculated samples were performed to normalize the data or are reported where appropriate. For data recording, both weak and strong colony hybridization signals were scored; for data reporting, only strong signals are reported.

Data on bacterial and gene maintenance were statistically analyzed by a four-way analysis of variance and correlation analysis. Tests of significance were conducted at a probability (P) level of $\geq$0.95.

Chemical analysis. At each time period, supernatant water was removed from the microcosms with a gas-tight syringe after centrifugation. The volume of water was noted, and an internal standard, fluorobenzene, was added to the syringe at a concentration of 25 μg liter^{-1}. The sample was injected into the purging vessel of a Tekmar LSC-2 purge-and-trap and analyzed according to Environmental Protection Agency Method 624. A Tenax gas chromatography (GC) trap was used, and the volatiles were chromatographed on a column (2 m by 2 mm) packed with 0.2% Carbowax 1500 on 80/100 Carbopack C (Union Carbide Corp.).

The analyses were performed on a Finnigan model 4000 GC-mass spectrometry system interfaced to an INCOS 2300 data system. Before the samples were analyzed, a calibration standard of the compounds of interest at a concentration of 10 μg liter^{-1} was established for retention times and response factors. The microcosm samples were then analyzed, and the concentrations were determined with the INCOS quantitative software.

For direct GC analysis, the Tenax GC trap (Chemical Data Systems) was dried of water with N_2 at 40 ml min^{-1} for 2 min at 40°C. For desorption, the trap was backflushed with 20 ml of N_2 min^{-1} for 8 min at 200°C onto a cryogenically cooled GC column (0.25 mm by 30 m, DB5 thick film; J & W Scientific) at −80°C. After desorption, the column was brought to 0°C ballistically and then programmed from 0 to 105°C at 5°C min^{-1} with an N_2 carrier flow of 1 ml min^{-1} by a Hewlett-Packard 5880A GC with a flame ionization detector and 5880A series GC terminal for peak-area integration. The following operating parameters were used: detector, 105°C; injector, 250°C; and transfer line, 200°C.

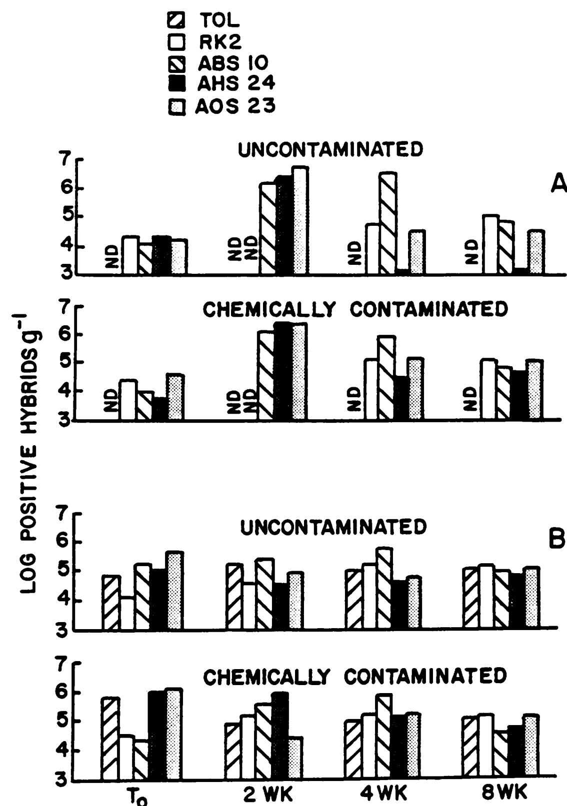

FIG. 2. Comparative population densities of positive colony hybridization genotypes for natural and inoculated groundwater aquifer microcosms. Shown are Natural (uninoculated) (A) and inoculated (B) microcosm samples. The inoculum was either *P. putida*(TOL, RK2), ABS10, AHS24, or AOS23 (natural microcosm samples [panel A] received an equivalent amount of inoculum of heat-killed ABS10 for an inoculum effect). T_0 denotes time immediately after microcosm construction or inoculation. ND, Not detected at an arbitrary detection limit of 10^3 CFU g^{-1}.

RESULTS

Bacterial and gene maintenance. The maintenance of introduced *P. putida*(TOL, RK2) and three indigenous groundwater aquifer isolates in uncontaminated and chemically contaminated Lula aquifer material is described in Fig. 2. These results were obtained by periodic plate counts of total viable cells and by colony hybridization with specific gene probes to detect those colonies representing the introduced genotype. As a control experiment, natural (uninoculated) aquifer samples were examined in an identical experimental design to determine the background of bacteria that contained homologous sequences to the specific gene probe used (Fig. 2A). Natural microcosms demonstrated a significant background density for ABS10, AHS24, and AOS23 genotypes, as expected, and for RK2 but not for the TOL genotype (Fig. 2A). There was no significant contaminant dose effect in the natural microcosms (Fig. 2A) except in the case of the AHS24 genotype, for which a significant increase in this population occurred, compared with the population size of the chemically uncontaminated microcosm, after a 2-week period. There was also a significant increase in the populations of ABS10-, AHS24- and AOS23-related genotypes after initial construction of the natural microcosms.

TABLE 2. Proportions of genotypes maintained in natural and inoculated groundwater aquifer microcosms

Treatment of microcosm	Proportion of introduced genotype[b]				
	TOL	RK2	AHS24	ABS10	AOS23
None (natural)[a]	0.001	0.023	0.010	0.130	0.025
Natural, chemically contaminated[a]	0.001	0.030	0.046	0.065	0.073
Inoculated[c]	0.066	0.070	0.028	0.050	0.126
Inoculated, chemically contaminated[c]	0.098	0.108	0.094	0.095	0.083

[a] Supplemented with approximately 10^6 killed ABS10 cells g^{-1} for an inoculum effect.

[b] Ratio of total probe-positive CFU g^{-1} to total CFU g^{-1}; 0.001, arbitrary limit of detection. Means were determined for both natural and inoculated microcosms (respectively) for TOL (0.001 and 0.086), RK2 (0.026 and 0.089), AHS24 (0.029 and 0.061), ABS10 (0.097 and 0.072), and AOS23 (0.049 and 0.104). The total number of observations per genotype for each microcosm was eight (two observations for each of four time periods).

[c] Microcosms were inoculated with *P. putida* (TOL, RK2), ABS10, AHS24, or AOS23.

This increase may have been due to an inoculum-nutrient effect (see Materials and Methods).

The nutrient effect was not observed for inoculated microcosms (Fig. 2B), and furthermore, no statistically significant chemical contaminant effects were observed for populations present in these microcosms. The individual inocula of TOL-RK2, ABS10, AHS24, and AOS23 genotypes were readily detected at time T_0 (time of initial microcosm construction) well above the background bacterial density of the natural microcosms (cf. Fig. 2B with 2A). Although there was no significant difference in the total densities of inoculated genotypes over an 8-week period, AHS24 appeared to be maintained at higher concentration in chemically contaminated microcosms (similar to the effect observed for the natural microcosm as described above). Overall, there was stable bacterial or gene maintenance (mean approximation, 10^5 positive hybrid colonies g^{-1}) for all inocula over the 8-week incubation period, and this includes the TOL genotype, which was not detected in the natural microcosm samples. At the termination of the 8-week experiment, all introduced genotypes were stably maintained at equivalent or higher densities than those present in the natural microcosms.

The relative proportion (numbers of introduced genotypes/total viable cells) of the introduced genotype was significantly higher for inoculated microcosms, compared with the natural microcosms, except for ABS10 (Table 2). The proportions ranged from 0.001 (0.1% arbitrary detection limit) for TOL to 0.097 (9.7%) for ABS10 in natural microcosms, compared with 0.061 (6.1%) for AHS24 to 0.104 (10.4%) for AOS23 in inoculated microcosms. There was a general trend of higher proportions of introduced organisms in chemically contaminated microcosms; however, there was no statistically significant chemical contamination effect. The higher relative proportions of the introduced genotypes in uninoculated microcosms seem to be due not only to the ability of individual bacterial inocula to survive at high density but also to the fact that the natural microcosms maintained higher total viable cell densities during the 8-week incubation period (Fig. 3). Therefore, computationally, the higher total viable cells in natural microcosms would result in a lower relative proportion of genotypes testing positive for the individual gene probes. There was no significant difference in total viable cells for all microcosms at the termination of the experiment (Fig. 3), although in natural microcosms, a 1- to 2-order-of-magnitude increase was observed at the 2-week sampling time. This increase is similar to that observed for ABS10, AHS24, and AOS23 indigenous or related organisms in natural microcosms (cf. Fig. 2A and 3) and further supports the existence of a nutrient effect.

Comparative TOL-RK2 maintenance in *P. putida*. There is a relatively good proportional agreement of TOL and RK2 genotypes maintained in the inoculated microcosms (Table 2). Perfect correlation between the density of these two genotypes was not obtained for the uncontaminated inoculated microcosms (data not shown). The above results indicate that (i) plasmids TOL and RK2 were not stably comaintained under the experimental conditions, (ii) the cultivation medium (YEPG) selected against one or the other plasmid, or (iii) the background homology (Fig. 2) to the RK2 probe interfered with the analysis. To control for the influence of the enumeration medium in counterselection against either the TOL or RK2 plasmid, enumeration-hybridization of the TOL-RK2 genotypes was performed with nonselective YEPG medium, *m*-toluate selective medium, and YEPG-Kanamycin selective medium. The results of this analysis for the four experimental incubation times are presented in Fig. 4, which shows that on *m*-toluate minimal agar medium, both plasmids were comaintained over an 8-week period. This result was confirmed when both plasmids were detected by agarose gel electrophoresis in

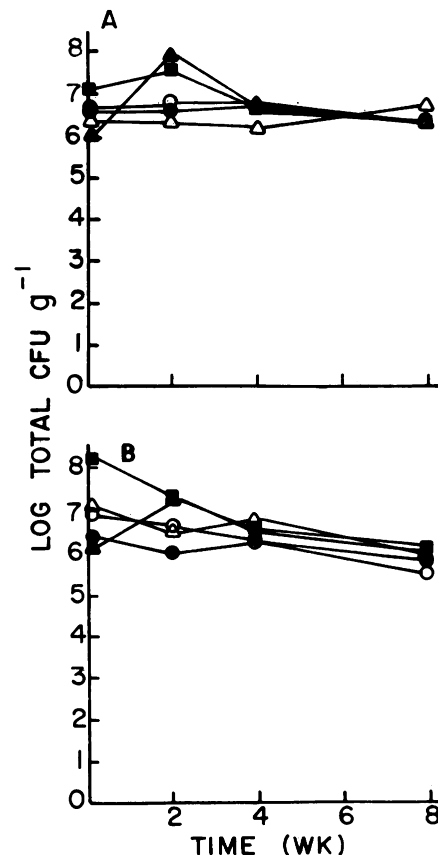

FIG. 3. Total bacterial population densities in natural and inoculated groundwater aquifer microcosms. (A) Uncontaminated; (B) chemically contaminated (see text). ▲, Natural microcosm (supplemented with killed ABS10 for an inoculum effect). Microcosms were inoculated with *P. putida*(TOL, RK2) (●), ABS10 (■), AHS24 (○), or AOS23 (△).

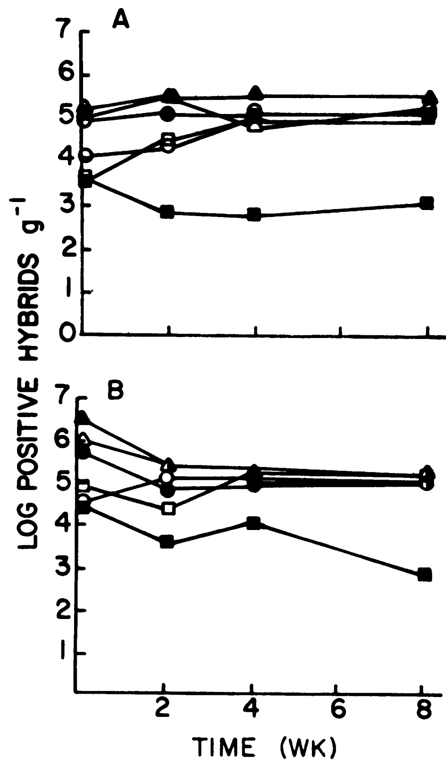

FIG. 4. Comparative selection of TOL and RK2 in *P. putida*(TOL, RK2)-inoculated groundwater aquifer microcosms. (A) Uncontaminated; (B) chemically contaminated. The densities of the TOL$^+$ populations are shown for the selection medium used: YEPG agar (●), *m*-toluate minimal agar (▲), or YEPG agar supplemented with kanamycin (100 μg ml^{-1}) (■). pTS 206 was used as the probe DNA. The densities of the RK2 population are indicated for the selection medium used: YEPG agar (○), *m*-toluate minimal agar (△), or YEPG agar supplemented with kanamycin (100 μg ml^{-1}) (□). RK2 was used as the probe DNA.

approximately 60 colonies, 30 each from uncontaminated and contaminated inoculated microcosms. The agarose gel electrophoresis of plasmids isolated from some of these colonies is represented in Fig. 5. Furthermore, it should be noted that TOL and RK2 are not in the same incompatibility group. Therefore, these findings suggest that the cultivation medium used is responsible for the selection of one or the other plasmid. This was indicated by the observation of a reduction of at least 1 to 2 orders of magnitude in TOL genotype in uncontaminated and contaminated inoculated microcosms when YEPG agar containing kanamycin was used as the cultivation medium (Fig. 4). In addition, 5 to 7% of colonies lost plasmid TOL, as shown by the plasmid isolation from approximately 40 colonies in each case (representative agarose electrophoresis results are shown in Fig. 5B). Although no significant difference in the population densities carrying TOL and RK2 occurred when YEPG agar was used as the cultivation medium (Fig. 4), plasmid isolation from a total of 50 colonies revealed that 8 to 10% of colonies lost plasmid RK2 (representative agarose gel electrophoresis of plasmids isolated from some of these colonies is shown in Fig. 5C).

Occurrence of homologous sequences to plasmid RK2 in natural aquifer material. An interesting and confounding observation in the present work is the background occurrence of genes homologous to plasmid RK2 in the natural microcosms (Fig. 2A). When plasmids were isolated from those colonies which showed positive hybridizations with plasmid RK2 (Fig. 2A), approximately 20% carried plasmids. Agarose gel electrophoresis showed the presence of plasmid(s) in the above colonies, and the size of plasmids varied from approximately 50 to an estimated 250 kb (representative plasmid profiles are shown in Fig. 6); precise estimation of plasmid sizes was not carried out. These results indicate that background RK2-positive signals may result from RK2-related sequences that are either chromosomally or plasmid maintained.

Fate of environmental contaminants. Although the primary goal of the present work was to investigate the maintenance of introduced bacteria in groundwater aquifer microcosms, some preliminary data were also obtained on the removal of toluene, chlorobenzene, and styrene from natural aquifer material compared with that from sterile aquifer material.

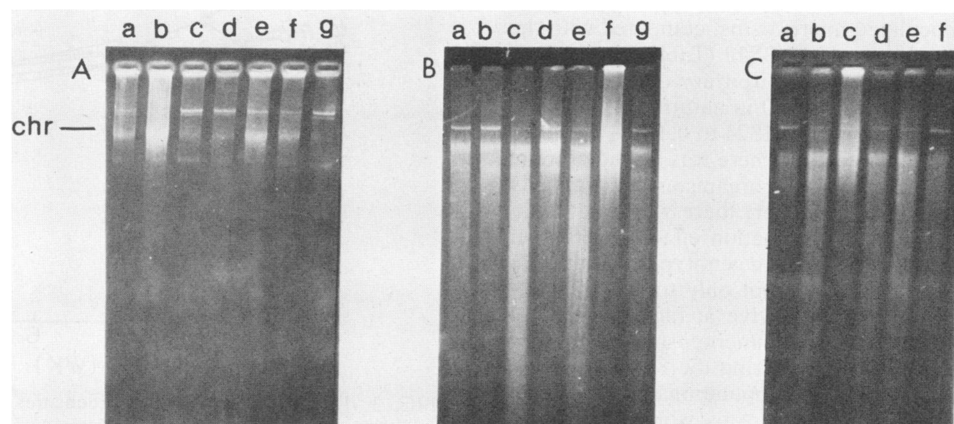

FIG. 5. Agarose gel electrophoresis of plasmid DNA in TOL-RK2-positive hybrids. (A) *P. putida* carrying plasmids TOL and RK2 as standard (a) and colonies picked from *m*-toluate minimal agar (b through g); (B) *E. coli* carrying RK2 as standard (a) and colonies picked from YEPG agar medium containing kanamycin (b through g); (C) *P. putida* carrying plasmids TOL and RK2 as standard (b) and colonies picked from YEPG agar medium (a and c through f). The lowermost band in Fig. A and B is the linear form of RK2. Chr, Chromosome.

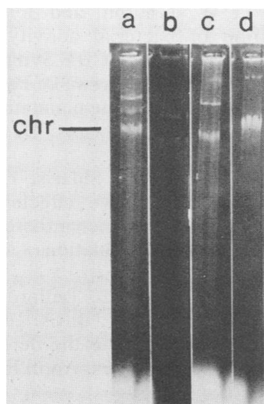

FIG. 6. Agarose gel electrophoresis of representative plasmid DNA of RK2-positive hybrids from natural aquifer material. (a) *P. putida* carrying plasmids TOL and RK2 as standard; (b through d) representative colonies picked from YEPG agar medium. Chr, Chromosome.

The *P. putida*-inoculated microcosm, over an 8-week period, demonstrated up to 92, 60, and 86% removal of toluene, chlorobenzene, and styrene, respectively (Table 3). However, the natural and other inoculated microcosms also showed equally significant removal of the contaminants over an 8- and 16-week period (Table 3). Further detailed studies are required to document that this process is mediated in part by the introduced microbial populations.

DISCUSSION

Results obtained in the present investigation show that introduced indigenous organisms and catabolic genes can be stably maintained in groundwater aquifer material and may have potential for in situ removal of environmental contaminants. However, caution should be taken in regard to the relative specificity of DNA probes used to detect positive hybrid colonies which represent the original inoculum or related indigenous organisms. The ABS10 genotype was monitored by a whole chromosomal gene probe that lacks specificity and therefore can detect a variety of related genotypes (12). AHS24, TOL, RK2, and AOS23 genotypes were monitored with plasmid probes of different specificity. AHS24 was monitored with a small cloning vector (pUC13; Table 1) containing a 5.9-kb chromosomal fragment of AHS24 (p2MLO; Table 1) that is highly specific (12). Plasmid pTS206, which carries a 0.6-kb *xylR* fragment of TOL cloned into vector pMCR600 (9), was used to monitor the TOL genotype; the *xylR* gene is specific for the upper pathway of toluene-xylene catabolism by *P. putida*.

TABLE 3. Removal of environmental contaminants in natural and inoculated groundwater aquifer microcosms

Inoculum	Contaminant removal (%)[a]		
	Toluene	Chlorobenzene	Styrene
Natural microcosm	91.5	76.7	ND[b]
P. putida(TOL, RK2)	92.5[c]	50.0	86.4
	94.5	60.8	ND
ABS10	34	41.1	93
AHS24	89.8	64.7	ND
AOS23	83.8	77.8	ND

[a] Relative to sterile controls.
[b] ND, Not determined.
[b] Incubation of 8 weeks; all other datum points for incubations of 16 weeks.

Because of their smaller size and the lack of demonstrated homology of parent vectors pUC13 and pMCR600 with other groundwater indigenous bacteria, the probes p2MLO and pTS206, as predicted, appear to be very specific. This fact is reinforced by the absence of positive hybrid colonies in natural microcosms, with pTS206 as probe for TOL (Fig. 2A). RK2 and AOS23 genotypes were monitored with large plasmids RK2 and pOS23, respectively (Table 1). The specificity of these plasmid probes remains uncharacterized, and consequently these findings may indicate other indigenous related genes in the groundwater communities. It should be noted that no evidence of cross-hybridization was observed with the above probes.

As a result of the different specificity for each probe, it is difficult to make an absolute comparison of the relative maintenance of the introduced organisms or genes. However, over the 8-week experimental period in the present study, it does appear that specific organisms or plasmids could be maintained at high levels (up to 10% of the total culturable aerobic heterotrophs; Table 2) within the aquifer microbial community.

Interestingly, a significant density of populations in natural aquifer material carrying homologous sequences to plasmid RK2 was detected (Fig. 2A). This observation and the lack of perfect correlation between the proportion of colonies maintaining TOL and RK2, independently probed in the same samples (Fig. 4), suggest either the independent maintenance or the possible gene transfer of RK2 or related sequences among populations in the natural aquifer environment. Plasmid RK2 is a broad-host-range plasmid found in many aerobic gram-negative bacteria, is approximately 60 kb in size, contains Tn*1* transposon sequences, and has identity with plasmid RP4 (16). Since a plasmid(s) in this size range has been reported for the Lula site and because 28 to 36% of the cultured organisms previously examined were gram-negative (10), the potential exists that plasmid RK2 (or RP4), the Tn*1* transposon, or a plasmid with a common ancestor exists in the natural population.

Preliminary data on contaminant removal indicated that, under all conditions, natural or introduced organisms were able to remove toluene, chlorobenzene, and styrene. This observation included the microcosms inoculated with the genetically modified *P. putida* strain which was able to persist at high frequencies in the presence of the above-mentioned environmental contaminants. The relatively rapid rate of contaminant removal can be attributed in part to a nutrient effect in microcosms that received no inoculation, since these microcosms received an amendment of a heat-killed bacterial suspension, ABS10. It should be noted that biodegradation has been previously examined in this zone of Lula samples (which is pristine in nature) and that degradation was very slow (20, 21).

In conclusion, results obtained in the present investigation show that within a community the introduced genotypes can successfully be maintained and that they can be detected and quantitatively monitored by using the highly sensitive DNA-DNA colony hybridization technology. The maintenance of introduced genotypes, specifically TOL$^+$, did not require selective pressure imposed by toluene in the environment for at least the 8-week period of incubation used in this study. Whether these populations are dynamic or static but physiologically active is not clear. However, it is apparent that introduced genotypes can be successfully maintained in groundwater aquifer material, which may contribute to fu-

ture biotechnical applications for in situ treatment of contaminated groundwaters.

ACKNOWLEDGMENTS

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