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Conjugal Transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* Strains in a Freshwater Environment

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Recent concern over the release of genetically engineered organisms has resulted in a need for information about the potential for gene transfer in the environment. In this study, the conjugal transfer in *Pseudomonas aeruginosa* of the plasmids R68.45 and FP5 was demonstrated in the freshwater environment of Fort Loudoun Resevoir, Knoxville, Tenn. When genetically well defined plasmid donor and recipient strains were introduced into test chambers suspended in Fort Loudoun Lake, transfer of both plasmids was observed. Conjugation occurred in both the presence and absence of the natural microbial community. The number of transconjugants recovered was lower when the natural community was present. Transfer of the broad-host-range plasmid R68.45 to organisms other than the introduced recipient was not observed in these chambers but was observed in laboratory simulations when an organism isolated from lakewater was used as the recipient strain. Although the plasmids transferred in laboratory studies were genetically and physically stable, a significant number of transconjugants recovered from the field trials contained deletions and other genetic rearrangements, suggesting that factors which increase gene instability are operating in the environment. The potential for conjugal transfer of genetic material must be considered in evaluating the release of any genetically engineered microorganism into a freshwater environment.

Environmental gene transfer, its ecological significance, and its importance in the release of genetically engineered microorganisms are relatively poorly understood, and understanding requires the development of a quantitative data base. While conjugative gene transfer is well known and widely used as a laboratory tool for genetic manipulation, information obtained in the laboratory may not be directly applicable to genetic transfer in situ. Many plasmids are readily transmissible to a large number of bacterial species, so that a recipient of a plasmid may be far removed, genetically and physiologically, from the donor (22). There are many factors that affect the survival of organisms and the transfer of genetic material in situ that cannot be adequately simulated in the laboratory.

Many studies of conjugation have been done in wastewater (2, 9, 14, 15, 17), but few have been done in fresh water. A limited number of in situ studies have been performed with both laboratory and naturally occurring strains. Grabow et al. (11) reported that indigenous coliforms can exchange genetic information in unsupplemented natural water. They detected transfer of naturally occurring R factors at low frequencies in dialysis bags containing river water immersed in the river and also in river water incubated in the laboratory. Low transfer frequencies of plasmids between introduced mating pairs of *Escherichia coli* K-12 were seen by Gowland and Slater (10) in sterile pond water in dialysis bags immersed in the pond. Schilf and Klingmüller (22), using the broad-host-range plasmids RP4 and pRD1 in *E. coli* hosts, detected plasmid transfer to the indigenous bacteria of pond water at low frequencies. Bale et al. (4) detected conjugal plasmid transfer between *Pseudomonas aeruginosa* strains attached to rocks immersed in a Welsh river. These transferred plasmids soon disappeared from the bacterial population.

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The purpose of this study was to determine the extent of conjugal transfer of the fertility factors R68.45 and FP5 between *P. aeruginosa* donor and recipient strains in a freshwater environment. Transfer was scored in both the absence and presence of the natural microbial community.

MATERIALS AND METHODS

Bacteria and plasmids. Strains and plasmids used are listed in Table 1.

Media and culture conditions. Bacteria were maintained in Luria broth (LB) (18) or on L-agar prepared by adding 12 g of agar (Difco) to 1 liter of LB. L-agar was supplemented with 250 μg of geneticin (G-418) per ml for the maintenance of RM2100 or with 40 μg of $HgCl_2$ per ml for the maintenance of RM2180. *Pseudomonas* minimal medium (18) was supplemented with glucose at 0.2% and with amino acids and nucleosides at the following concentrations: adenine at 20 μg/ml; histidine, isoleucine, and tryptophan at 50 μg/ml; lysine at 85 μg/ml; methionine at 110 μg/ml; proline and valine at 200 μg/ml. Antibiotics were used at the following concentrations in L-agar or in *Pseudomonas* minimal medium: carbenicillin at 500 μg/ml, G-418 at 250 μg/ml, nalidixic acid at 400 μg/ml, streptomycin at 1,000 μg/ml, and tetracycline at 250 μg/ml. $HgCl_2$ was added to L-agar at 40 μg/ml and to *Pseudomonas* minimal medium at 5 μg/ml.

Lake water was collected as described previously by Saye et al. (21). Water for laboratory simulations was shipped by overnight express and used within 1 to 2 weeks of collection. Lake water was sterilized by autoclaving.

Mating methods. (i) Optimal mating conditions. Either RM2100 (R68.45$^+$) or RM2180 (FP5$^+$) was used as the donor strain and RM273 was used as the recipient strain. Donor and recipient cells were grown in LB (37°C with aeration) to mid-log phase (10^8 CFU/ml), pelleted by centrifugation (5,000 × *g* for 5 min), and suspended to the original volume in LB. For liquid matings, donors and recipients were mixed

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Plasmid	Genotype or relevant characteristics[a]	Source or reference
P. aeruginosa			
PAO303		*argB21*	18
PTO66		*his-4 ilv-1118 lys-12 pro-82 trpC6 recA102*	8
RM40		*lys-12 met-28 pur-600 trpC6 str-901*	18
RM273		*nalA901*	21
RM2100	R68.45	As for PTO66	This study
RM2116	FP5	As for PAO303	This study
RM2180	FP5	As for PTO66	This study
Plasmids			
R68.45		Cb^r Tc^r Km^r Tra^+	12
FP5		Hg^r Tra^+	16

[a] Genetic abbreviations used are consistent with the nomenclature of Bachmann (3). *pur*, Purine (adenine); *his*, histidine; *ilv*, isoleucine-valine; *lys*, lysine; *met*, methionine; *nal*, nalidixic acid resistance; *pro*, proline; *rec*, recombination deficient; *str*, streptomycin resistance; *trp*, tryptophan. Phenotype designations: Cb^r, carbenicillin resistance; Hg^r, mercury resistance; Km^r, kanamycin (G-418) resistance; Tc^r, tetracycline resistance; Tra^+, transfer proficient.

in a 1:1 ratio and incubated at 37°C for 2 h without shaking. For filter matings, donors and recipients were mixed at the same ratio and filtered onto a 0.22-μm-pore-size, 47-mm diameter nitrocellulose membrane (Nalgene). Filters were placed, bacteria side up, on the surface of an L-agar plate and incubated for 2 h at 37°C. Cells were then eluted from the filter and resuspended in 1/10 volume LB.

(ii) Laboratory experiments in lake water (in vitro). Factors affecting transfer were tested in laboratory simulations. Matings were done in sterile lake water rather than in LB. In specific experiments, donor-to-recipient ratio, total cell density, temperature, incubation time, method of sterilization, or marker selection was varied.

(iii) Field trials (in situ). Cells for in situ matings were prepared in the same manner as cells for in vitro mating. The resuspended cells were placed on ice for transport to the resevoir, where they were inoculated into test chambers as described previously (21) at the desired cell concentrations. Two 125-ml samples were aseptically removed from each chamber at 0.5, 12, 24, 48, 60, and 72 h after inoculation. One sample was used at the site to determine physical and chemical characteristics. The ranges for these parameters were: air temperature, 19.0 to 28.5°C; water temperature, 20 to 28°C; dissolved oxygen, 5.9 to 12.3 mg/liter; conductivity, 110 to 150 μS; pH, 6.74 to 9.94. The other sample was placed on ice and transported to the laboratory for immediate microbiological analysis.

Enumeration of bacteria. Following incubation, samples from in vitro and in situ matings were serially diluted and plated on various selective media for enumeration. When large volumes were required, 1.0 ml of the sample was spread on plates in three portions, and the colonies appearing on the three plates were counted and their numbers were pooled. Volumes of 10 or 100 ml were filtered through 0.2-μm (in vitro experiments) or 0.45-μm (in situ experiments) membrane filters (Millipore, Bedford, Mass.). Each membrane was transferred to the surface of an L-agar plate supplemented with the appropriate antibiotics and incubated at 37°C for 1 to 2 days.

A total viable cell count for each sample was determined by plating to L-agar. Donor bacterial concentrations were estimated by plating appropriate dilutions on L-agar containing carbenicillin (R68.45) or Hg^{2+} (FP5). Recipients were enumerated by plating on L-agar containing nalidixic acid. The number of transconjugants in each mating mix was determined by plating on L-agar containing carbenicillin and nalidixic acid (R68.45) or on L-agar containing Hg^{2+} and nalidixic acid (FP5). Just before plating for the recovery of transconjugants, nalidixic acid was added to the samples to a final concentration of 400 μg/ml to inhibit DNA metabolism of the Nal^S donor cells (5, 6). Preliminary and control experiments demonstrated that this treatment was effective in eliminating additional gene transfer on the filters and during outgrowth of the environmentally generated transconjugants. In addition to the above selections, lake water was plated to *Pseudomonas* isolation agar (Difco) to determine the number of pseudomonads present in the natural community.

TABLE 2. Potential for conjugation under optimal mating conditions

Mating type	Mating medium	No. of transconjugants/donor[a]	
		R68.45	FP5
Liquid	LB	5×10^{-4}	9×10^{-5}
	Lake water	3×10^{-5}	1×10^{-6}
Filter	Lake water	5×10^{-2}	ND[b]

[a] All transfer frequencies are calculated as the number of transconjugants recovered at the sampling time divided by the number of donor bacteria present when mating was initiated.
[b] ND, Not done.

After 96 h of incubation, the field trial was terminated, and sections of the inner surface of the chambers were replica plated to selective medium for the enumeration of attached bacteria.

Genetic and molecular screening of the recovered cells. To verify that the cells recovered on selective medium were donor, recipient, or transconjugant cells, the colonies were patched to L-agar containing a single antibiotic (carbenicillin, G-418, Hg^{2+}, nalidixic acid, or tetracycline) to *Pseudomonas* minimal medium, and to *Pseudomonas* isolation agar. Only if appropriate markers were present were the isolates considered to be recovered donor, recipient, or transconjugant cells. Transconjugants containing FP5 were also tested for chromosome-mobilizing ability by the method of Stanisich and Holloway (23), with RM40 as the recipient strain in these crosses. The presence of R68.45 in the in situ isolates was confirmed by comparing restriction endonuclease digestion patterns of DNA isolated from these colonies with those of plasmid DNA isolated from laboratory stock cultures of RM2100. Plasmid DNA was isolated by rapid alkaline lysis (21), digested with *Sma*I or *Kpn*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as recommended by the manufacturer, and subjected to electrophoresis on 0.7% agarose gels (21).

RESULTS

Potential for conjugation. The potential for conjugal transfer of the plasmids R68.45 and FP5 was first examined under optimal laboratory conditions. Liquid matings in LB or in sterile lake water showed that plasmid transfer was approximately the same in both media when the temperature was optimal, the donor-to-recipient ratio was approximately 1, and the total cell density was 10^8 CFU/ml (Table 2). As Haas and Holloway (12) had reported that transfer of R68.45 occurs with higher frequency on a solid support, transfer of

TABLE 3. Factors affecting conjugation

Factor tested	Transfer frequency	
	No. of transconjugants/ml	No. of transconjugants/donor[a]
Temp (°C)		
37	4.3×10^3	2.5×10^{-5}
25	2.9×10^3	1.7×10^{-5}
16	2.2×10^3	1.3×10^{-5}
Total cell density (CFU/ml)		
10^9	8.9×10^4	1.5×10^{-3}
10^7	3.0×10^3	5.1×10^{-3}
10^5	1×10^1	1.7×10^{-3}
Method of sterilization		
Autoclaving	1.7×10^5	1.5×10^{-3}
Filtering[b]	8.5×10^4	7.1×10^{-4}
Donor-to-recipient ratio		
1:2	1.9×10^2	1.1×10^{-4}
1:50	2×10^1	2.8×10^{-4}
50:1	$<1 \times 10^1$	$<6.0 \times 10^{-6}$
Primary selection (contraselection)[c]		
Cb (Nal)	4.9×10^4	7.7×10^{-5}
G-418 (Nal)	7×10^1	1.1×10^{-7}
Tc (Nal)	5.0×10^2	7.8×10^{-7}

[a] All transfer frequencies are calculated as the number of transconjugants recovered at the sampling time divided by the number of donor bacteria present when mating was initiated.

[b] Lake water was sterilized by filtration through a 0.2-μm-pore-size membrane filter (Nalgene) as described previously (21).

[c] Cb, Carbenicillin; Tc, tetracycline; Nal, nalidixic acid.

this plasmid was also tested in filter matings. The results showed that this plasmid does transfer at a higher frequency in filter matings than in a liquid medium.

Factors affecting rates of plasmid transfer by conjugation. R68.45 was used to test the effects of nonoptimal conditions in lake water on frequencies of conjugal transfer (Table 3). No significant effect was observed in the frequency of transfer when the temperature of the mating or total cell density in the mating mixture was varied. Likewise, the method of sterilization of the lake water used as a mating medium had little effect on conjugal transfer.

The ratio of donors to recipients did significantly affect the number of transconjugants recovered. An excess of recipient cells in the mating mix increased the frequency of transconjugants recovered by as much as 100-fold. Similarly, it was found that the choice of antibiotic resistance marker used as the primary selection significantly affected the recovery of transconjugants. Selection for Cb^r resulted in the recovery of the greatest number of transconjugants.

Laboratory simulations. Extended matings in lake water were conducted in the laboratory in preparation for field trials. Both sterilized and nonsterilized lake water was tested. In the absence of the natural microbial community (sterilized water), some dependence of the initial transfer frequencies of R68.45 on the total cell density was observed when the donor-to-recipient ratio was 1 (Fig. 1). Interestingly, the presence of an initial excess of donors had a significant effect only on the initial frequency of transfer events (Fig. 2). All matings reached the same plateau frequency. FP5 transfer frequency was affected by donor-to-recipient ratio (Fig. 3). When there was an initial excess of donor cells, transfer of the plasmid was significantly reduced.

When the natural microbial community was present, the frequency of plasmid transfer was generally lower than in

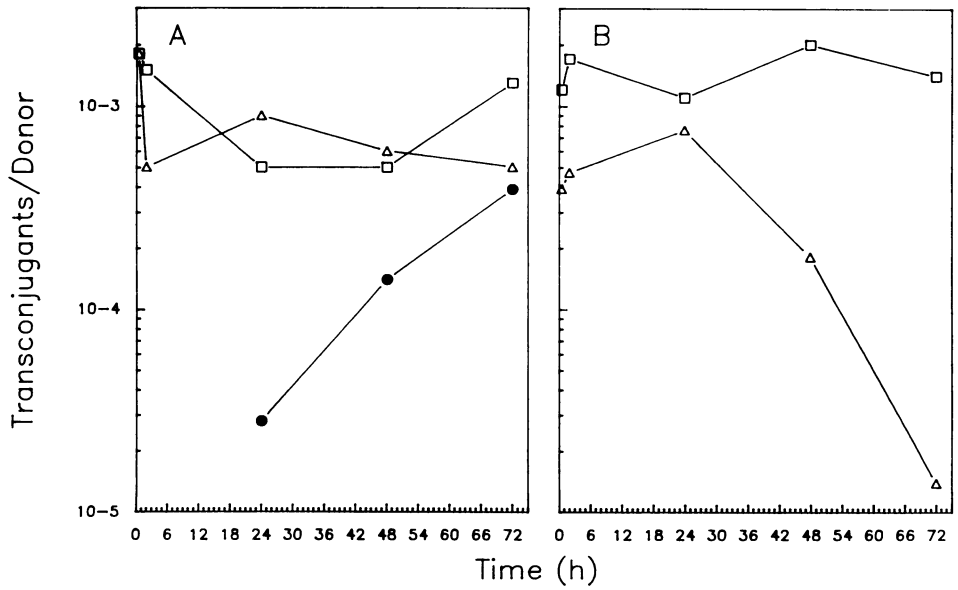

FIG. 1. Frequency of plasmid R68.45 transfer in laboratory simulations at various cell densities and a donor-to-recipient ratio of 1:1. Laboratory simulations were carried out in lake water as described in the text. (A) Natural microbial community not present (sterile lake water). (B) Natural community present (nonsterile lake water). Concentrations were 10^8 CFU/ml (□), 10^6 CFU/ml (△), and 10^3 CFU/ml (●). Points not plotted were below our level of detection (10^{-9} CFU/ml). Open symbols, Water collected in January 1987; solid symbols, water collected in June 1987. All transfer frequencies are calculated as the number of transconjugants recovered at the sampling time divided by the number of donor bacteria inoculated into the mating chamber at time zero.

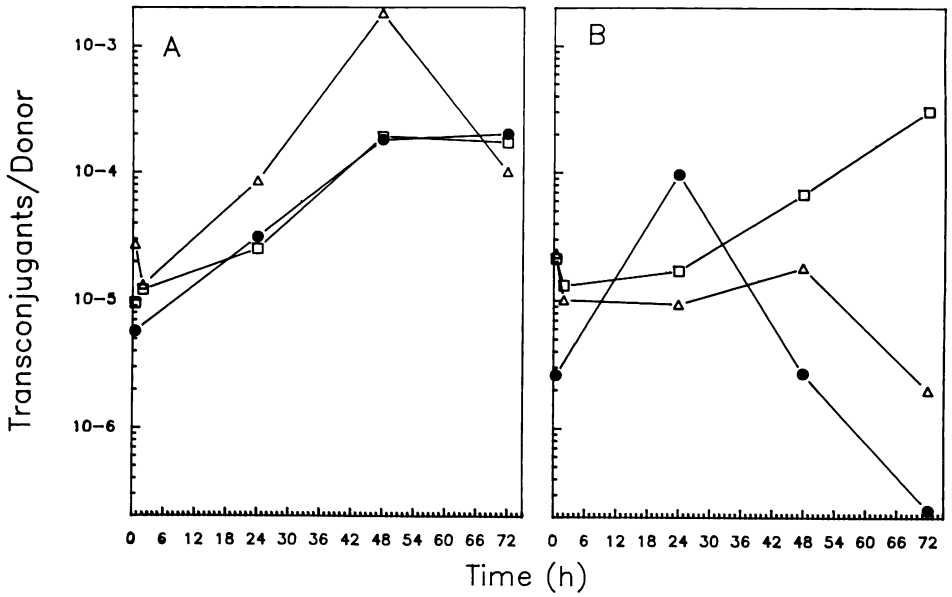

FIG. 2. Frequency of plasmid R68.45 transfer in laboratory simulations at various cell densities and a donor-to-recipient ratio of 1,000:1. Laboratory simulations were carried out in lake water as described in the legend to Fig. 1. (A) Natural community not present (sterile lake water). (B) Natural community present (nonsterile lake water). Concentrations were 10^8 donor and 10^5 recipient CFU/ml (□) or 10^6 donor and 10^3 recipient CFU/ml (△ and ●). Open symbols, Water collected in January 1987; solid symbols, water collected in June 1987.

comparable matings done in the absence of the natural microbial community (Fig. 1, 2, and 3). Transfer of FP5 at a 1:1 donor-to-recipient ratio remained below our level of detection ($<10^{-8}$ transconjugants per donor) throughout the experiment.

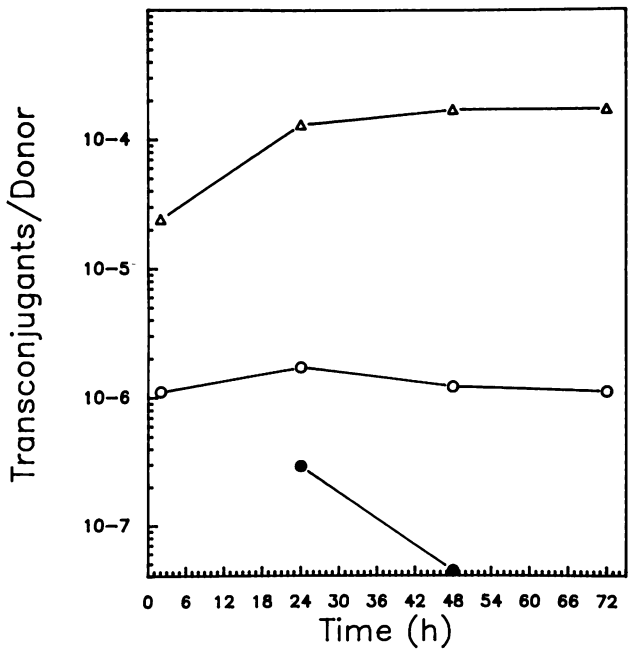

FIG. 3. Frequency of plasmid FP5 transfer in laboratory simulations. Laboratory simulations were carried out in lake water as described in the legend of Fig. 1. Concentrations were 10^6 donor and 10^3 recipient CFU/ml (○ and ●) or 10^3 donor and recipient CFU/ml (△). Open symbols, Natural community not present; solid symbols, natural community present.

Measurement of plasmid transfer in a natural freshwater environment. (i) In situ field trial 1 (October 1986). In the first field trial, only transfer of R68.45 was evaluated. Test chambers were incubated in Fort Loudoun Lake, Knoxville, Tenn. These in situ matings yielded much lower frequencies of transfer than similar matings conducted in the pretrial laboratory simulations (Fig. 1 and 2). Again, the frequency of detectable transfers was lower when the natural microbial community was present in the test chamber.

Interestingly, when confirmatory analysis of transconjugants isolated from in situ incubation chambers was carried out, it was found that gene rearrangements in transconjugants occurred more frequently in situ than in laboratory simulations with lake water as the incubation medium. Approximately 50% of the transconjugants isolated from in situ matings showed at least one rearrangement or deletion. No alterations were detected in laboratory experiments. These rearrangements were detected by nonconformity to antibiotic resistance patterns and confirmed by restriction analysis of the plasmids (Fig. 4). There were three patterns to the loss of antibiotic resistance: either Tc^r or G-418^r was lost or both Tc^r and G-418^r were lost. In addition, when these isolates were used as donors in laboratory matings with RM40, the transfer frequency of several of the plasmids contained in these isolates was reduced or eliminated. When the Tra^+ phenotype was retained, the resulting second-generation transconjugants showed the same antibiotic resistance patterns as the parent isolated from the in situ mating. Therefore, the loss of antibiotic resistance in the in situ isolates was not due to any chromosomal alterations.

(ii) In situ field trial 2 (June 1987). As in the first field trial, the transfer frequency of R68.45 in situ (Fig. 5) was lower than or equal to the frequency observed in parallel chambers incubated in the laboratory (Fig. 1 and 2) whether or not there was an initial excess of donor cells in the mating mix. Unlike the first field trial (Table 4), no transfer of R68.45 was detected in the presence of the natural microbial community.

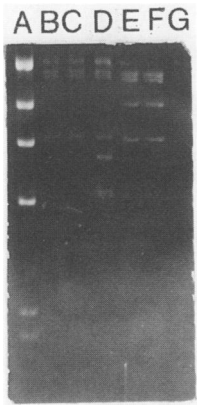

FIG. 4. Examples of *Sma*I restriction patterns of R68.45 DNA found in in situ transconjugants. (A) *Hin*dIII digestion of lambda DNA, (B) R68.45 from RM2100, (C) an in vitro transconjugant, (D to F) R68.45 with altered restriction patterns from in situ transconjugants, (G) R68.45 with normal restriction pattern from an in situ transconjugant.

The frequency of transfer of FP5 was also lower in situ (Fig. 6) than in laboratory simulations (Fig. 3). Transfer in the absence of the natural community occurred at higher frequencies when the initial donor-to-recipient ratio was close to 1 than when there was an initial excess of donor cells. At a ratio of 1,000:1, transfer was detected in only the 12- and 72-h samples and was close to the lower level of detection (10^{-9} transconjugants per donor) in both cases.

Transfer in the presence of the natural community was detected (1×10^{-9} to 2×10^{-9} transconjugants per donor) in the 12- and 24-h samples only when the chambers were inoculated with an excess of donors. Because of the low total cell density in chambers with a 1:1 donor-to-recipient ratio, it is very likely that if transfer did occur it would have been below our level of detection.

TABLE 4. Frequency of plasmid transfer in situ at a donor-to-recipient ratio of 1,000:1[a] (field trial 1)

Incubation time (h)	No. of transconjugants/donor[b] when natural microbial community:	
	Absent	Present
0.5	$<3.0 \times 10^{-6}$	$<3.5 \times 10^{-6}$
2	1.8×10^{-7}	7.0×10^{-9}
12	N.O.[c]	3.5×10^{-9}
36	N.O.	N.O.
60	3.0×10^{-9}	4.2×10^{-8}
84	1.2×10^{-8}	N.O.

[a] Donor cells were inoculated at a concentration of 10^6 CFU/ml. Recipients were inoculated at 10^3 CFU/ml.

[b] All transfer frequencies are calculated as the number of transconjugants recovered at the sampling time divided by the number of donor bacteria inoculated into the mating chamber at time zero.

[c] N.O., Not observed at the limit of our detection methods ($<3 \times 10^{-9}$ transconjugants per ml).

With few exceptions, the number of recoverable donors and recipients as well as the total cell density decreased with time of in situ incubation (Table 5). The decrease was greater in chambers containing the natural microbial community than in parallel chambers without the natural community. The terminal cell densities in in situ mating chambers approached the cell density of the natural microbial community in Fort Loudoun Lake water.

Conjugal transfer between the introduced organism and the natural community. Numerous colonies (200 to 300 clones

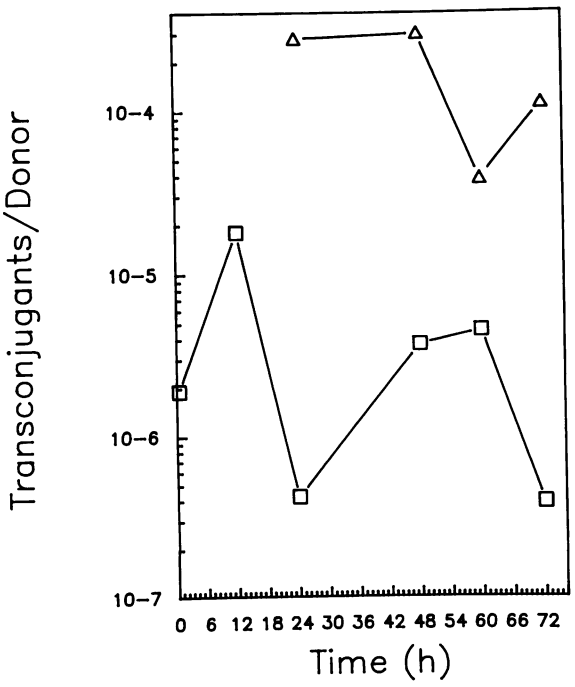

FIG. 5. Frequency of plasmid R68.45 transfer in in situ field trial 2. Incubation and sampling were carried out as described in the text and the legend to Fig. 1. Concentrations were 10^6 donor and 10^3 recipient CFU/ml (□) or 10^3 donor and recipient CFU/ml (△).

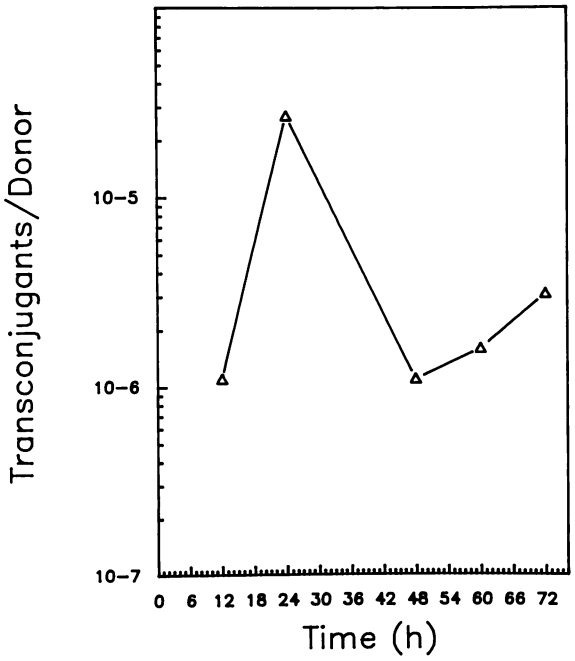

FIG. 6. Frequency of plasmid FP5 transfer in in situ field trial 2 at a cell density of 10^4 CFU/ml and a donor-to-recipient ratio of 1:1. Incubation and sampling were carried out as described in the text and the legend to Fig. 1. The frequency of transfer in the 0.5-h sample was below the level of detection ($<10^{-9}$ transconjugants per donor) and was not plotted.

TABLE 5. Donor, recipient, and total cell densities from in vitro chambers

Chamber no.	Donor plasmid	Natural microbial community	Cell density (CFU/ml)					
			Donor		Recipient		Total	
			Initial	Final	Initial	Final	Initial	Final
1	R68.45	Absent	1.8×10^7	$<10^{-2}$	1.4×10^4	2.4×10^3	1.8×10^7	3.3×10^4
2	R68.45	Present	6.0×10^6	$<10^{-2}$	1.1×10^4	16	6.1×10^6	95
3	R68.45	Absent	7.1×10^3	50	1.0×10^4	800	1.8×10^4	1.3×10^4
4	R68.45	Present	1.6×10^4	$<10^{-2}$	1.5×10^4	68	1.6×10^4	81
5	FP5	Absent	3.3×10^7	3.0×10^3	7.7×10^3	250	3.5×10^7	1.4×10^3
6	FP5	Present	7.5×10^6	45	6.2×10^3	17	6.8×10^6	120
7	FP5	Absent	1.9×10^4	2.4×10^2	6.9×10^3	380	2.9×10^4	1.0×10^4
8	FP5	Present	1.7×10^4	12	8.9×10^3	4.0×10^{-2}	2.7×10^4	170
9	R68.45	Present	1.9×10^7	$<10^{-2}$	—[a]	—	1.6×10^7	69
10	FP5	Present	2.9×10^7	42	—	—	2.9×10^7	32
11	None	Present			7.0×10^3	11	7.2×10^3	6
12	None	Present			—	—	1.2×10^3	120

[a] —, No recipient cells were added to the chamber.

per chamber) from samples of the in situ incubation chambers containing an R68.45 donor and the natural community were picked from nonselective medium. These colonies were analyzed for antibiotic resistance and plasmid restriction patterns. No transfer of R68.45 to members of the indigenous microbial community was detected within the limits of this assay.

A *Pseudomonas* sp. isolate was selected from the indigenous community present at the field site and used as a recipient in laboratory matings with an R68.45 donor strain. Transfer was detected at a frequency of 10^{-7} transconjugants per donor. The transconjugants, selected for Cb^r, also expressed Tc^r and Km^r phenotypes.

Plasmid transfer among attached subpopulations. In situ test chambers inoculated with an excess of donor cells were sacrificed after 96 h of incubation, and the inner surfaces were tested for bacteria. No transconjugants were recovered from the surface of any of the sacrificed chambers in either the presence or the absence of the indigenous population. However, introduced and indigenous bacteria were recovered in varying quantities from the sacrificed chambers (1 to 10 CFU/cm^2).

DISCUSSION

Conjugal transfer of laboratory plasmids R68.45 and FP5 between *P. aeruginosa* strains in a freshwater environment was detected at moderate frequency in the absence of the natural community and at low frequency in the presence of the natural microbial community. It appears that transfer under simulated natural conditions in the laboratory is not an adequate or reliable indicator of conjugal gene transfer in situ. Similar effects of natural microbes on the rate of plasmid transfer were observed by Bale et al. (4) in a freshwater river. This suggests that the indigenous microbial population affects either the transfer to the plasmids or the detection of transconjugants. The natural community also exerted an influence on the number of recoverable donor cells, which decreased more when the natural community was present than when it was absent; thus, it is likely that the indigenous population primarily affects the recovery of introduced bacteria rather than the potential for transfer of the plasmids. Grazing protozoa have been implicated in the decline in the number of introduced bacteria in sea water and in soil (1) and may exert a similar influence in fresh water. The effect of the indigenous population was more pronounced in matings performed in situ, where the only conjugal transfer detected in the presence of the natural community occurred during the first 24 h in the chamber containing an excess of FP5 donor cells. This may be an example of nonbiological factors in situ acting synergistically with the indigenous microbial population to affect the detection of transconjugants. The survival of introduced organisms in a natural sample may be affected by competition for available nutrients with the indigenous microbial population. Conditions optimal for the growth of an organism may be transient, and bacteria need to be capable of exploiting nutrients when they are abundantly available and have strategies for survival when nutrients are very limited.

The frequency of transfer of R68.45 in standard matings was 100 times higher when mating occurred on a filter placed on lake water solidified with 1.2% agar than in parallel liquid matings. Since it has been demonstrated previously that R68.45 transfers at higher frequencies on a solid support than in liquid medium (12), transfer in lake water could be occurring in cell aggregates on the surfaces of suspended particulate matter.

Use of Tc^r or G-418^r instead of Cb^r for primary selection of transconjugants decreased the number of transconjugants which could be recovered. The frequency of transconjugants recovered by using carbenicillin selection was several hundred times greater than the number of transconjugants recovered with either tetracycline or G-418 selection. Olsen has suggested that the discrepancy in the number of transconjugants recovered is not due to the instability of the markers, but rather to the specific mechanism of drug resistance, which may not allow sufficient expression of the resistance determinants in time for the bacteria to survive exposure to the antibiotic (R. Olsen, personal communication).

One explanation for the detection of less transfer in situ than in vitro may be the increased genetic instability of plasmid DNA observed in field trials. Gene rearrangement was observed more frequently in situ than in vitro and may be a result of environmental stress imposed by biological or physical factors found only in the environment. R68.45 is known to be unstable after conjugal transfer (7, 13). This instability does not necessarily result in the loss of the whole plasmid; only portions of the plasmid may be deleted (14). Deletion of all or part of the Cb^r determinant in in situ

transconjugants would decrease the number of transconjugants recovered when carbenicillin is used for selection.

Our data suggest that increased frequencies of gene rearrangement, perhaps due to elevated rates of transposition or recombination in situ over those observed in laboratory studies, may result in accelerated evolution of plasmid DNA in the environment. If so, an evaluation of the risk associated with the introduction of an engineered genetic element into the environment that is based on rates of genetic change measured in the laboratory may not be valid. Virtually nothing is known about the rates of recombination and transposition in microorganisms in situ. These phenomena must be investigated in the environment.

The studies reported here demonstrate that conjugal transfer of plasmids such as R68.45 and FP5 can and does occur under conditions found in nature. When considered in light of previous demonstrations of transmission of both chromosomal (19) and plasmid (21) DNA by generalized transduction, it is clear that a significant potential for gene transfer among bacteria exists in freshwater environments. Dissemination of genetic elements mediated by either conjugation or transduction may occur in the aquatic milieu. Both of these mechanisms of gene transfer must be considered in evaluating the potential risk associated with the environmental release of genetically engineered microorganisms.

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