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Plasmid Transfer between Marine Bacteria in the Aqueous Phase and Biofilms in Reactor Microcosms

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Plasmid transfer of broad-host-range plasmid RP1 from marine *Vibrio* sp. strain S14 to marine strain SW5 under optimum conditions on the surface of nutrient plates was improved 2 orders of magnitude by using the plasmid transfer process to select an SW5 recipient more efficient than the wild type in receiving and/or maintaining the plasmid. This recipient strain, SW5H, was used to form biofilms under flow conditions on the surfaces of glass beads in reactors. The S142(RP1) donor strain was introduced to the reactors after either 48 or 170 h of biofilm formation, and production of transconjugants in the aqueous phases and biofilms without selection pressure was assessed. Plasmid transfer to the recipient cells in the biofilm was detected for biofilms formed for 170 h but not in those formed for 48 h. The plasmid transfer frequency was significantly higher ($P < 0.05$) among cells attached to the bead surfaces in the biofilm than among cells in the aqueous phase.

There is a need to understand gene transfer between bacteria in natural environments. Research into this phenomenon is necessary because of a lack of knowledge of how bacteria acquire and disseminate genes in a natural situation and has been stimulated by concern about the impact that the eventual release of genetically engineered microorganisms, or their DNA, will have on natural environments.

Conjugal transfer of plasmids has been shown to occur in soil (19, 29, 30, 32), wastewater (22, 27, 30), and aquatic systems (13, 15, 27, 30, 32) in both laboratory microcosms and in situ (2, 4, 6, 26, 29). For example, Bale and coworkers (3–5) have shown plasmid transfer between natural freshwater bacteria in river epilithon. Fulthorpe and Wyndham (14, 15) studied the transfer of catabolic plasmid pBRC60, carrying a transposon that encodes 3-chlorobenzoate degradation, in flowthrough freshwater mesocosms with selection pressure for the plasmid and showed that transfer of the plasmid and the transposable element to members of the natural bacterial populations in the aqueous phase and sediment was possible. However, these studies generally did not examine the exchange of organisms from biofilms to the aqueous phase or effects the nature of surfaces may have had on the localization of donor and recipient cells.

There has been little research into plasmid transfer in the marine environment, and this has been restricted to laboratory paired matings between both marine bacteria (24, 28) and marine and terrestrial bacteria (16, 25, 28). Hence, there is a need to investigate gene transfer between marine bacteria first in laboratory microcosms and then in natural environments. The marine environment is mostly nutrient depleted, and as a result, a large proportion of the bacteria exist under oligotrophic conditions. At the solid/water interface, however, nutrients concentrate, leading to colonization by microorganisms, production of a polymer matrix, and eventually mature biofilm development (21). Hence, biofilms consist of cells immobilized in a polymer matrix, lying in close proximity to one another. This biofilm microenvironment would allow stable cell-to-cell contact and plasmid transfer. It is highly probable, therefore, that in aqueous environments, conjugation would be most likely to occur within biofilms.

A decrease in plasmid transfer frequencies among cells conjugated under laboratory conditions, in broth and on plates, compared with those existing under more natural environmental conditions has been reported (4, 26). Therefore, an initial aim of this study was to obtain a marine bacterial strain exhibiting high frequencies of plasmid transfer under laboratory conditions before proceeding with the main objective of investigating plasmid transfer between marine bacteria in the aqueous phase and in biofilms in laboratory microcosms. Interaction between cells in the two phases was studied by forming biofilms with a nonmotile, hydrophobic recipient, SW5H, and then introducing a motile, hydrophilic donor strain, *Vibrio* sp. strain S142(RP1). Transfer of broad-host-range plasmid RP1 (31) demonstrated an interaction between the two organisms. In addition, the effects of hydrophobic or hydrophilic substrata on gene transfer and colonization were investigated and the nature of cell attachment to the two different surfaces was determined. Thus, we aimed to establish whether we could detect plasmid transfer between marine bacteria in the absence of selection pressure under ideal microcosm conditions before proceeding with experiments under natural conditions. These ideal conditions included an effective marine plasmid donor strain, a known high-frequency-of-conjugation recipient strain, a broad-host-range self-transmissible plasmid, two defined substrata, and a defined artificial seawater with a defined energy source.

MATERIALS AND METHODS

Bacterial strains and plasmid. The marine bacterium SW5 is a nonmotile, gram-negative rod isolated from Cronulla Beach, New South Wales, Australia, by T. Neu. A streptomycin (Sm)-resistant recipient strain of SW5, namely, SW5S, was selected by spontaneous mutation of SW5 to resistance to 200 μg of Sm ml^{-1}. The motile marine bacterium *Vibrio* sp. strain S142 has been described previously (24), and S142(RP1) has been shown to be an effective donor in conjugation experiments (24). The cells were grown in the media used for the reactor studies (see below), and the cell

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surface hydrophobicity of the two strains was determined by the bacterial adhesion to hydrocarbons (BATH) test (23). It was found that 19% of the SW5 cells were left in the aqueous phase, compared with 88% of the S142(RP1) cells, indicating that the outer surfaces of SW5 and S142(RP1), under the conditions used in this study, were hydrophobic and hydrophilic, respectively. Donor strains *Escherichia coli* 803(RP1) (24) and S142(RP1) were resistant to rifampin (Rp) and harbored the conjugative broad-host-range IncP1 plasmid RP1, which encodes resistance to kanamycin (Km), tetracycline (Tc), and ampicillin (Ap) (31).

Culture media and growth conditions. The SW5 strains were grown and maintained on tryptone soya agar (TSA) (tryptone soya broth [TSB] [Oxoid] with 15 g of Bi-Tek Agar [Difco] liter^{-1} supplemented with 20 g of NaCl liter^{-1}, 1 mM $MgCl_2$, and 300 μM $CaCl_2$). *E. coli* 803(RP1) and S142(RP1) were grown and maintained on Luria agar–1.5% NaCl (Luria broth, 15 g of NaCl liter^{-1}, prepared as described previously [24], containing 15 g of Bi-Tek agar liter^{-1}, 1 mM $MgCl_2$, and 300 μM $CaCl_2$). For recipients SW5S and SW5H, 200 μg of Sm ml^{-1} was added to each of the above-described media and 200 μg of Km ml^{-1} was added to maintain the RP1 plasmid in donors S142(RP1) and *E. coli* 803(RP1). The marine strains were grown at 30°C and stored at room temperature on plates sealed with Parafilm (American Can Co., Greenwich, Conn.) to prevent desiccation. The *E. coli* strain was grown at 37°C and stored on slants at 4°C. Long-term storage of all organisms was at −70°C in 40% glycerol plus half-strength nutrient broth.

The sensitivities of recipients SW5S and SW5H to Tc (10 μg ml^{-1}), Km (200 μg ml^{-1}), Ap (100 μg ml^{-1}), and Rp (100 μg ml^{-1}) and those of donors S142(RP1) and *E. coli* 803(RP1) to Sm (200 μg ml^{-1}) were determined by spread plating 100 μl of the respective overnight culture onto TSA supplemented with the appropriate filter-sterilized antibiotic (which was added aseptically to autoclaved medium cooled to about 50°C). The spontaneous mutation to resistance for each strain was $<10^{-8}$ for each of the appropriate antibiotics. When appropriate, antibiotics were added to the medium at the concentrations given above.

Selection and optimization of a mating pair. Conjugal plate matings were used to select the most efficient mating pair of marine bacteria for use in the flow reactors. An Sm-resistant strain of SW5 (isolated by spontaneous mutation to resistance to Sm at 200 μg ml^{-1}) was designated SW5S and used to produce high-frequency-of-conjugation strain SW5H (see ii, below).

(i) Conjugal plate matings. Recipients and donors were grown overnight, with shaking, in TSB at 30°C, and in the case of the donors, TSB-Km was used to maintain the plasmid. Aliquots of 20 μl of each were mixed with a further 60 μl of TSB and placed onto a sterile 0.22-μm-pore-size (25-mm-diameter) membrane filter (MFS, Dublin, Calif.) on a TSA plate which was then incubated for 24 h at 30°C. The cells were removed from the filter by vortex mixing the membrane in 1 ml of TSB for about 1 min. Control aliquots of donor and recipient cells were treated in the same manner. Serial dilutions were made, and transconjugants were selected on TSA-Sm-Km; Km counterselected the recipients, and Sm counterselected the donors. Recipients were selected on TSA-Sm, and donors were selected on TSA-Km. Transfer frequencies were recorded as the ratio of the number of transconjugants to the number of recipients or to the number of donors. Transconjugants were verified as originating from recipients, which were Rp^s, by patching to TSA-Rp. Possession of Km^r by transconjugants was shown to be due to the plasmid, since simultaneous acquisition of resistance to Ap and Tc was shown by patching. All conjugations were done in triplicate.

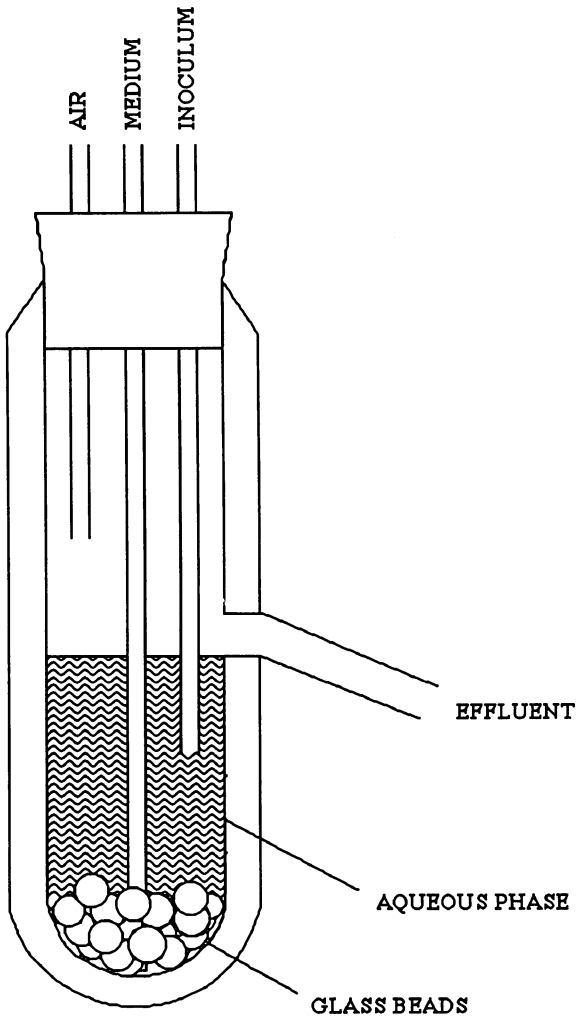

FIG. 1. Design of the reactors used in conjugation experiments.

(ii) Development of a high-frequency-of-conjugation strain. A method was adapted from that described for selection of high-frequency-of-transformation strains (12) to generate a recipient, SW5H, that was more efficient at conjugation than the parent strain. Transconjugants from the mating of S142(RP1) and SW5S were cured of the plasmid by repeated subculture without antibiotic selection for the plasmid. A cured strain, SW5H, was conjugated with the original S142(RP1) donor and with *E. coli* 803(RP1) by the method described above.

Reactor experiments. (i) Reactor design and operation. Each reactor (Fig. 1) consisted of a 20-ml mini chemostat (Quickfit) containing 25 acid-washed glass beads (with a surface area of 0.785 cm^2 each or 19.6 cm^2 in total). The reactors were sterilized by autoclaving. The glass beads were hydrophilic, and for comparison, sterile, washed beads were made hydrophobic by placement in sterile 2% dimethyldichlorosilane in 1,1,1-trichloroethane (Coatasil; Ajax Chemicals, Sydney, New South Wales, Australia) for 10 min before addition to sterile reactors.

The growth medium used in the reactors was a buffered

TABLE 1. Transfer frequencies from conjugal plate matings

Conjugation pair	Plasmid transfer frequency[a]	
	No. of transconjugants/recipient	No. of transconjugants/donor
SW5S × S142(RP1)	4.4×10^{-4}	7.2×10^{-5}
SW5H × S142(RP1)	3.1×10^{-2}	8.4×10^{-3}
SW5S × *E. coli* 803(RP1)	5.6×10^{-4}	8.8×10^{-4}
SW5H × *E. coli* 803(RP1)	5.8×10^{-2}	1.7×10^{-1}

[a] Mean transfer frequency of triplicate matings.

minimal medium, devised as an artificial seawater medium, MMM (24). For SW5H, this was supplemented with 20 mM glutamic acid (MMMglt) as a carbon and energy source in place of glucose. A peristaltic pump (LKB, Bromma, Sweden) was used to provide a flow rate of 10.5 ml h^{-1}. Filtered air was supplied by diffusion through the air inlet, and the medium, fed into the reactor through the medium inlet, was aerated by mixing (Fig. 1). The operating temperature of the reactors was between 25 and 27°C (ambient temperature).

(ii) **Recipient biofilm formation.** Cultures of SW5H were grown overnight with shaking in 20 ml of MMMglt at 30°C. The A_{600} of the cultures was determined and adjusted to between 0.3 and 0.4 by dilution in MMMglt. The reactors were inoculated with 15 ml of the diluted suspension through the inoculum inlet (Fig. 1) and left for 1 h, after which time flow of sterile MMMglt was started. To investigate the effects of time on biofilm formation and gene transfer, the recipient biofilms were formed under flow conditions for either 48 or 170 h.

(iii) **Donor addition.** The donor, S142(RP1), was grown overnight, with shaking, at 30°C in 20 ml of MMM-Km. The donor cultures were washed once before being resuspended in MMM (supplemented with glucose and glutamic acid) to an A_{600} of approximately 1.6. Following biofilm formation, the aqueous phase was removed by back pumping through the medium inlet (Fig. 1). A 15-ml volume of the donor, S142(RP1), suspension was pumped into each of the reactors through the medium inlet (Fig. 1) and left for 24 h under nonflow conditions to allow conjugation to occur.

(iv) **Sampling.** Following 24 h of incubation with the donor, 1 ml of the aqueous phase was sampled through the inoculum inlet (Fig. 1) by using a sterile syringe. The reactor was then emptied by back pumping, and the beads were sampled by removing the stopper at the top of the reactor and withdrawing 10 beads with sterile forceps. Cells were harvested from the beads by vortex mixing in 2 ml of MMM for 2 min. Scanning electron microscopy (using a Stereoscan 360 scanning electron microscope [Cambridge Instruments Ltd., Cambridge, England]) of vortex-mixed beads which had been air dried and sputter coated with a gold-palladium alloy (Polaron E 5000 sputter coater; Biorad Polaron Equipment, Watford, England) revealed that no cells were left on the beads after vortex mixing. Transconjugants were selected on TSA-Sm-Km, recipients were selected on TSA-Sm, and donors were selected on TSA-Km. Transfer frequencies were calculated by the following formula: number of transconjugants/(number of recipients × number of donors), as done by Jones et al. (18). Jones et al. (18) found, when investigating conjugative plasmid (R68-45) transfer among cells partitioning between the aqueous phase and the freshwater air/water interface that, because numbers of recipients, and particularly donors, were higher when the interface was nutrient enriched, expression of transfer frequency was better shown by the formula number transconjugants/(number of recipients × number of donors), rather than transconjugant/recipient or transconjugant/donor ratio alone. This was done to account for relative differences in the localization of the donor strain in relation to the recipient strain, since plasmid transfer depends upon both donor and recipient cell numbers. For standard conjugation experiments, in which donor and recipient strains are allowed to conjugate in rich nutrient broth or are held together on the surface of nutrient-rich agar plates, plasmid transfer frequency is conventionally expressed as the transconjugant/donor or transconjugant/recipient ratio. Usually, either expression yields the same result, since under such conditions cell numbers of donors and recipients are approximately equal. For example, in Table 1, the plasmid transfer frequencies showed the same 2-order-of-magnitude difference between the efficiencies of SW5S and SW5H as recipients, whether expressed as transconjugant/donor or the transconjugant/recipient ratio. However, in dynamic microcosms the localization of cells may be different, as can be seen in Table 2, where cell numbers of donors and recipients in different phases of the reactors (i.e., biofilms and the aqueous phase) were always different by up to 2 orders of magnitude. This is a direct consequence of differences in the nature and behavior of these donor and recipient strains in the microcosm environments. In such a case, expressing the plasmid transfer frequency as the transconjugant/donor or transconjugant/recipient ratio does not yield similar results.

(v) **Washing.** The aqueous phases of two of the 170-h reactors, one containing hydrophilic beads, the other containing hydrophobic beads, were sampled immediately before and after donor addition. This was done to ascertain how many recipient cells were removed from the biofilm by pumping in the donor. The aqueous phases were sampled as described previously, and the recipients were selected on TSA-Sm. The remaining volume of the donor culture was left in the reactor to conjugate for 24 h. The aqueous phase was then sampled, and the reactors were emptied as described above.

To determine the number of cells loosely attached, compared with those firmly attached, in the biofilm, the reactors were washed with 40 ml of MMM (without a carbon source) through the medium inlet (Fig. 1) at 10.5 ml h^{-1}. The wash, simultaneously removed at the same flow rate through the inoculum inlet (Fig. 1), was collected in a sterile flask. The 40 ml of wash was centrifuged, the cells were resuspended to the original reactor volume (20 ml) in MMM (without a carbon source), and transconjugants, donors, and recipients were selected on TSA plates containing the appropriate antibiotics. The numbers of cells from the wash (loosely attached) and biofilm (firmly attached), expressed as CFU per square centimeter of bead surface, were added together to give the total number of cells in the biofilm for each strain. By expressing the cell numbers from the wash and biofilm as a percentage of the total number of biofilm cells for each

TABLE 2. Cell numbers and transfer frequencies for reactors set up to investigate conjugation in a marine environment

Reactor	Treatment	Source	No. of cells			Plasmid transfer frequency [10^{-14} transconjugants/(recipient × donor)]
			SW5H	S142(RP1)	Transconjugants	
1	Hydrophilic glass beads; biofilm formed for 170 h	Aqueous phase[a]	6.0×10^8	9.2×10^6	4.3×10^2	7.8
		Biofilm[b]	8.9×10^7	1.2×10^5	3.0×10^1	280
2	Hydrophilic glass beads; biofilm formed for 170 h; aqueous phase sampled immediately before and after donor addition; aqueous phase sampled before washing; reactor washed before biofilm sampled	Aqueous phase before donor addition[a]	1.4×10^7	NA[c]	NA	NA
		Aqueous phase after donor addition[a]	2.5×10^7	NA	NA	NA
		Aqueous phase before washing[a]	1.5×10^8	1.4×10^7	7.2×10^2	34
		Wash[b]	2.3×10^8	7.3×10^6	5.4×10^3	320
		Biofilm[b]	4.6×10^6	2.5×10^5	3.3×10^2	29,000
		Total biofilm (wash + biofilm)[b]	2.3×10^8	7.6×10^6	5.7×10^3	330
3	Hydrophobic glass beads; biofilm formed for 170 h; aqueous phase sampled immediately before and after donor addition; aqueous phase sampled before washing; reactor washed before biofilm sampled	Aqueous phase before donor addition[a]	1.4×10^7	NA	NA	NA
		Aqueous phase after donor addition[a]	5.2×10^7	NA	NA	NA
		Aqueous phase before washing[a]	4.6×10^7	1.5×10^8	2.5×10^2	3.6
		Wash[b]	1.4×10^8	5.1×10^6	8.5×10^2	120
		Biofilm[b]	2.1×10^7	2.1×10^5	4.8×10^1	1,100
		Total biofilm (wash + biofilm)[b]	1.6×10^8	5.3×10^6	9.0×10^2	110
Controls	Plate conjugation		8.3×10^8	1.7×10^8	ND[d]	NA
	Conjugation in aqueous phase	Still	1.0×10^7	1.3×10^7	ND	NA
		Stirred	7.0×10^8	4.8×10^8	ND	NA
Control reactor	Colonization by S142(RP1); nonflow for 24 h	Aqueous phase[a]	NA	4.0×10^{7e}	NA	NA
		Biofilm[b]	NA	1.9×10^{5f}	NA	NA

[a] CFU per ml.
[b] CFU per square centimeter of bead.
[c] NA, not applicable.
[d] ND, not detected.
[e] Mean of three experiments ($\log_{10}$ of x), 7.57; standard deviation, 0.21; coefficient of variation, 2.7%.
[f] Mean of three experiments ($\log_{10}$ of x), 5.27; standard deviation, 0.05; coefficient of variation, 0.9%.

strain, the numbers of loosely attached cells compared with firmly attached cells in the biofilm could be determined.

(vi) Controls. Putative transconjugants were verified as being derived from the recipient, which was Rp^s, by patching to TSA-Rp. Km^r transconjugants were patched to Tc and Ap; simultaneous acquisition of Tc^r and Ap^r was assessed as being caused by acquisition and maintenance of the RP1 plasmid. Spontaneous mutation to resistance by the recipients to Km and the donors to Sm was tested at the time of inoculation of the reactors by spread plating 100 μl of each of the cultures onto TSA-Sm-Km. Spontaneous mutation to resistance for the respective antibiotics was found to be $<10^{-8}$ for each strain. To ensure that plasmid transfer was by conjugation occurring among cells in the reactors and not between cells spread onto the selective medium plates, 100-μl volumes of overnight cultures of donor and recipient cells were mixed on TSA-Sm-Km plates.

The extent of conjugation occurring in the aqueous phase was investigated by inoculating two 125-ml conical flasks with 9.5 ml of overnight MMM (supplemented with the appropriate carbon source and antibiotic)-grown cultures of SW5H and S142(RP1) whose optical densities had been adjusted to levels similar to those used in the reactors. The flasks were incubated at ambient temperature for 24 h; one was gently shaken on an orbital shaker to prevent the cells from settling, and the other was left standing. Transconjugant, recipient, and donor cell numbers were determined as described previously.

Three identical control reactors were run to investigate colonization of the beads by a pure culture of donor strain S142(RP1). An increase in colonization of the beads by the donor in the conjugation reactors, compared with the control reactors containing no recipient biofilms, would indicate enhancement of donor colonization of the beads by the recipient biofilm. Reactors containing no recipient biofilms were inoculated with 15 ml of an overnight MMM-Km culture of S142(RP1) adjusted to an A_{600} of approximately 1.6. The reactors contained hydrophilic beads and were incubated in the same manner as the test reactors (i.e., under nonflow conditions for 24 h at ambient temperature).

A transformation and transduction control was performed by filtering an overnight TSB-Km culture of the donor strain through a 0.22-μm-pore-size syringe filter (MFS, Dublin, Calif.) and then mixing the filtrate with an overnight culture of the recipient in the same manner as the plate mating experiments. It was assumed that any bacteriophages or naked DNA could pass through the filters.

Statistical analysis. For statistical analysis, transfer frequencies (10^{14}) and counts were transformed by the $\log_{10}$ of x. Mean plasmid transfer frequencies calculated for cells in the aqueous phase and for cells in the biofilms (reactors 1, 2, and 3) were compared by using the unpaired Student t test with a pooled variance estimate, as were also the mean numbers of S142(RP1) donors in the aqueous phases and biofilms of the three control reactors. For the three control reactors, the means, standard deviations, and coefficients of variation of the cell numbers localized in the different phases are given in Table 2.

RESULTS

Isolation of a high-frequency-of-conjugation strain. Transconjugants from the mating of SW5S and S142(RP1) were used to generate high-frequency-of-conjugation strain SW5H, which yielded transconjugants at a plasmid transfer frequency 2 orders of magnitude higher than that of the original recipient, SW5S, with both S142(RP1) and *E. coli* 803(RP1) donors (Table 1). Conjugation with the *E. coli* donor was tested to ensure that any difference in plasmid transfer frequency noted between either SW5S or SW5H and S142(RP1) was due to the different recipient strain and was not caused by the donor ability of S142(RP1).

Investigation of conjugation between marine strains in biofilms. The results from three flow reactor experiments and controls set up to investigate plasmid gene transfer in marine biofilms and the aqueous phase are presented in Table 2. Reactor 1 contained hydrophilic beads on which the recipient biofilm was formed for 170 h. Transfer of RP1 was detected among cells in both the biofilm and the aqueous phase (Table 2). When the recipient biofilm was allowed to form for only 48 h on the hydrophilic beads, however, no plasmid transfer was detected in the biofilm (data not shown). The recipient and donor numbers and frequency of plasmid transfer in the aqueous phase were comparable to those found in reactor 1 (Table 2). A reactor was set up to allow the recipients to form a biofilm on hydrophobic beads for 48 h. Compared with the reactor containing hydrophilic beads, there was a 1-order-of-magnitude increase in the numbers of recipients colonizing the hydrophobic beads, but again no transconjugants were detected in either the biofilm or the aqueous phase (data not shown).

Reactors 2 and 3 were run to study the effects of hydrophilic and hydrophobic substrata on conjugation, colonization by SW5H in the longer term (recipients exposed to beads for 170 h), recipient numbers in the aqueous phase as a result of pumping in the donor, and the nature of the biofilms formed. Transconjugants were detected among cells recovered from the aqueous phase, those in the wash (loosely attached cells), and those firmly attached in the biofilms of both reactors 2 and 3 (Table 2). Plasmid transfer frequencies were higher among cells firmly attached in the biofilms, by 1 and 2 orders of magnitude for the hydrophobic (reactor 3) and hydrophilic (reactor 2) beads, respectively, than among cells loosely attached. Further, the plasmid transfer frequencies were higher among cells loosely attached to the hydrophilic (reactor 2) and hydrophobic (reactor 3) bead surfaces than among cells in the aqueous phase by 1 and 2 orders of magnitude, respectively. Taking the plasmid transfer frequencies (10^{14}) among the cells in the aqueous phase in reactors 1, 2, and 3 as one group and those among the total cells in the biofilms (i.e., frequencies calculated for the numbers of loosely and firmly attached cells added together) as a second group, statistical analysis showed that the difference between the means of the two groups was significant ($P < 0.05$). This analysis shows that the plasmid transfer frequency among organisms in the biofilms was significantly higher than that among organisms in the aqueous phase.

There was an increase in the numbers of recipients in the aqueous phase immediately after donor addition in both reactors 2 and 3 (Table 2). Over the following 24 h, recipient numbers increased by about an order of magnitude in the aqueous phase of the reactor containing hydrophilic beads (reactor 2) only. Washing revealed that the majority of SW5H and transconjugant cells were loosely attached to the bead surfaces (Table 2). When the numbers of recipients in the wash and the biofilm were added together, it could be seen that there was little difference in colonization between the hydrophilic and hydrophobic surfaces (2.3×10^8 and 1.6×10^8 CFU cm^{-2}, respectively). The donor, S142(RP1), similarly showed little change in total biofilm cell numbers in a comparison of the numbers from the hydrophilic beads with those from the hydrophobic beads (7.6×10^6 and 5.3×10^6 CFU cm^{-2}, respectively). By expressing the wash and biofilm cell numbers as percentages of the total number of cells in the biofilm for each strain, the loosely attached nature of the biofilm became apparent, with about 90% of the recipients, donors, and transconjugants being removed by the wash from both the hydrophobic and hydrophilic bead surfaces.

The ratios of the cell numbers in the aqueous phase compared with numbers in the biofilm for reactors 2 and 3 are shown in Table 3. For both bead surfaces, the ratio of cells in the aqueous phase to cells in the biofilm was higher for the donor, S142(RP1), than for the recipient, SW5H, and was consistent with the differences in the colonizing abilities of the two organisms (Table 2). For reactor 2, containing hydrophilic bead surfaces, the number of SW5H cells in the aqueous phase was about 30 times higher than that of cells firmly adhered to the hydrophilic surface and about one-half that of cells loosely attached. There were about 50 times more S142(RP1) cells in the aqueous phase than there were firmly attached, and there were twofold more cells in the aqueous phase than there were loosely attached (reactor 2). For the hydrophobic bead surfaces in reactor 3, there were about two-fold more SW5H cells in the aqueous phase than firmly attached and about one-third as many as were loosely attached. There were about 700 times more S142(RP1) cells in the aqueous phase than firmly attached and about 30 times more than were loosely attached (reactor 3).

TABLE 3. Ratio of cell numbers in the aqueous phase to numbers in the biofilm for reactors 2 and 3

Reactor	Duration of biofilm formation (h)	Surface type	Ratio of cell numbers in aqueous phase to cell numbers in biofilm	
			SW5H	S142(RP1)
2	170	Hydrophilic	33[a]	56[a]
			0.7[b]	2[b]
3	170	Hydrophobic	2[a]	714[a]
			0.3[b]	30[b]

[a] Ratio of aqueous-phase cell number to number of firmly attached bacteria.
[b] Ratio of aqueous-phase cell number to number of loosely attached bacteria.

Controls. Patching revealed that transconjugants were derived from recipients and had simultaneously acquired Km^r, Tc^r, and Ap^r, indicating receipt and maintenance of RP1. No transconjugants were detected when recipients and donors were plated together on transconjugant selection plates (plate conjugation in Table 2). Hence, any plasmid transfer detected had occurred in the reactors.

The aqueous-phase conjugation controls, designed to determine the source of transconjugants in the aqueous phase, yielded no detectable transconjugants, even though the numbers of donors and recipients in the stirred control were higher than those in the experimental reactors (Table 2).

Three control reactors (Table 2) were set up with pure cultures of S142(RP1) to determine whether the recipient biofilm had any effect on the colonizing abilities of the donor. Donor numbers in the biofilms covering the hydrophilic beads of the three reactors ranged from 1.7×10^5 to 2.1×10^5 CFU cm^{-2}, and in the aqueous phase they ranged from 2.2×10^7 to 5.5×10^7 CFU ml^{-1}. Both the biofilm and aqueous-phase counts were similar to those in the experimental reactors (1 and 2) containing hydrophilic beads (Table 2). It appeared, therefore, that there was little or no enhancement or exclusion of donor colonization on either hydrophilic or hydrophobic beads by the recipients in the biofilm. The localization of the cells between the aqueous phase and the biofilm was significantly different, since cell numbers in the aqueous phase were significantly ($P < 0.01$) higher than cell numbers in the biofilm.

A transformation and transduction control yielded no transconjugants. Any transfer of RP1 in the reactors, therefore, was likely to have been due to conjugation and not to transformation or transduction.

DISCUSSION

In the reactor microcosms used, plasmid transfer was significantly higher ($P < 0.05$) among cells in the biofilms attached to bead surfaces than among cells in the aqueous phase. It was necessary to select a recipient, SW5H, that showed a relatively high ability to receive and maintain the RP1 plasmid under optimum laboratory conditions so as to evaluate effects of the nature of the surfaces and the time allowed for conjugation on efficiency of gene transfer. As pointed out by Fernandez-Astorga et al. (10), if transfer efficiency is low to start with, then transfer under less than optimum conditions might not be detected at all. This may explain the lack of detectable plasmid transfer from *Pseudomonas aeruginosa*(R68-45) to the natural bacterial population in the work of Jones et al. (18).

In an initial experiment (reactor 1), the number of transconjugants detected in the aqueous phase was about 10-fold higher than that of those detected in the biofilm. This result appeared to contradict the well-known phenomenon that conjugation of RP1, an IncP1 plasmid, is several orders of magnitude more efficient for cells held on a surface than for those in the aqueous phase (7). We have shown increased RP1 plasmid transfer at a surface among *Vibrio* sp. strain S14 isolates (24). Indeed, aqueous-phase conjugal-transfer controls in our study (i.e., reactors without beads or preformed biofilms) showed that with comparable numbers of donors and recipients, no conjugation could be detected in the aqueous phase. We agree with Jones et al. (18) that since plasmid transfer depends upon both donor and recipient cell numbers, plasmid transfer frequencies are better expressed as the ratio of transconjugants to the donor-recipient product when the localization of donors and recipients is different. In the experiments presented here, we expressed the plasmid transfer frequency in this way and found that the plasmid transfer frequency was about 30 times higher among cells in the biofilm than among those in the aqueous phase (Table 2, reactor 1). This result was consistent with those for reactors 2 and 3.

In both reactors 2 and 3, addition of donor cells resulted in an increase in SW5H numbers in the aqueous phase. After 24 h under nonflow conditions, the SW5H numbers in the aqueous phase were similar to the total numbers in the biofilm in most of the reactors. Any increases in SW5H cell numbers in the aqueous phase may have been due to growth of SW5H in the aqueous phase under nonflow conditions but could also have been due to exchange of loosely attached cells between the biofilm and the aqueous phase, as found previously by Hermansson and Marshall (17) for a loosely attaching bacterium. Washing of the beads showed that about 90% of the donor, recipient, and transconjugant cells in the biofilms in reactors 2 and 3 could be classified as loosely attached to both the hydrophilic and hydrophobic beads. It is possible, therefore, that transconjugants detected in the aqueous phase arose from cells which had been loosely attached in the biofilm and then moved to the aqueous phase.

The hydrophilic or hydrophobic nature of the bead surfaces appeared to make little or no difference to the occurrence of conjugation, since plasmid transfer was detected among cells attached to either type of bead surface. Whether the nature of the bead surface significantly affects plasmid transfer frequency is now under investigation, since it appeared that transfer was more efficient on the hydrophilic surface (Table 2). The mean plasmid transfer frequency among all of the cells in the biofilms of all three reactors was found to be significantly higher ($P < 0.05$) than that among cells in the aqueous phase. In controls which contained only aqueous-phase cells, plasmid transfer was not detected.

It appeared that the hydrophobic SW5H cells preferred the hydrophobic surface to the hydrophilic one (Table 3). This was not surprising, as the importance of hydrophobic bonding between a hydrophobic cell and a hydrophobic substratum has been well documented (1, 11, 20). In contrast, the hydrophilic S142(RP1) cells appeared to be repelled by the hydrophobic surface (Table 3) and it was clear that this strain was not as good a colonizer of either of the two surfaces as hydrophobic strain SW5H (Tables 2 and 3). Such results are supported by other work, which found that

hydrophobic bacteria adhere to solid surfaces to a greater extent than do hydrophilic bacteria (9, 33) and that these differences follow the thermodynamic model of adhesion, in which hydrophobic cells are excluded from the aqueous phase and, consequently, attracted to the solid/liquid interface, whereas hydrophilic cells tend to stay in the aqueous phase (1, 11, 20).

Detection of plasmid transfer was less successful after 48 h of recipient biofilm formation, and plasmid transfer was detected only in the aqueous phase of the reactor containing hydrophilic beads. We do not know why transconjugants were not detected in the biofilm of this reactor or in the reactor containing hydrophobic beads, but it may be that transconjugant numbers were below the detection limit of our system. Characklis (8) stated that mature biofilm development is time dependent, and thus, at 48 h for either a hydrophilic or a hydrophobic surface, there may have been too few recipient cells firmly attached to the surfaces for plasmid transfer to have been detected.

The present work is the first to report conjugative plasmid transfer between marine bacteria in biofilms, in which biofilms of a recipient strain were established and then a donor strain was introduced for a short time, with no selection pressure for the plasmid. Our study has also shown that a plasmid-bearing bacterial strain which is poor at forming a biofilm, or becoming part of one already established by another strain, can nevertheless have sufficient cell-cell interaction with the cells in the biofilm for plasmid transfer to occur. We are examining this phenomenon further by investigating what effects laminar flow, nutrient deprivation, and addition of other strains to the biofilm may have on the spread of plasmids in marine biofilm microcosms.

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REFERENCES

1. **Absolom, D. R., F. V. Lamberti, Z. Policova, W. Zingg, C. J. van Oss, and A. W. Neumann.** 1983. Surface thermodynamics of bacterial adhesion. Appl. Environ. Microbiol. **46:**90–97.
2. **Altherr, M. R., and K. L. Kasweck.** 1982. In situ studies with membrane diffusion chambers of antibiotic resistance transfer in *Escherichia coli*. Appl. Environ. Microbiol. **44:**838–843.
3. **Bale, M. J., M. J. Day, and J. C. Fry.** 1988. Novel method for studying plasmid transfer in undisturbed river epilithon. Appl. Environ. Microbiol. **54:**2756–2758.
4. **Bale, M. J., J. C. Fry, and M. J. Day.** 1987. Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. J. Gen. Microbiol. **133:**3099–3107.
5. **Bale, M. J., J. C. Fry, and M. J. Day.** 1988. Transfer and occurrence of large mercury resistance plasmids in river epilithon. Appl. Environ. Microbiol. **54:**972–978.
6. **Bentjen, S. A., J. K. Fredrickson, P. van Voris, and S. W. Li.** 1989. Intact soil microcosms for evaluating the fate and ecological impact of the release of genetically engineered microorganisms. Appl. Environ. Microbiol. **55:**198–202.
7. **Bradley, D. E., D. E. Taylor, and D. R. Cohen.** 1980. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. J. Bacteriol. **143:**1466–1470.
8. **Characklis, W. G.** 1990. Biofilm processes, p. 195–231. *In* W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley & Sons, Inc., New York.
9. **Fattom, A., and M. Shilo.** 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. Appl. Environ. Microbiol. **47:**135–143.
10. **Fernandez-Astorga, A., A. Muela, R. Cisterna, J. Iriberri, and I. Barcina.** 1992. Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Appl. Environ. Microbiol. **58:**392–398.
11. **Fletcher, M., and K. C. Marshall.** 1982. Are solid surfaces of ecological significance to aquatic bacteria? Adv. Microbial Ecol. **6:**199–236.
12. **Frischer, M. E., J. M. Thurmond, and J. H. Paul.** 1990. Natural plasmid transformation in a high-frequency-of-transformation marine *Vibrio* strain. Appl. Environ. Microbiol. **56:**3439–3444.
13. **Fry, J. C., and M. J. Day.** 1990. Plasmid transfer in the epilithon, p. 55–80. *In* J. C. Fry and M. J. Day (ed.), Bacterial genetics in natural environments. Chapman & Hall, Ltd., London.
14. **Fulthorpe, R. R., and R. C. Wyndham.** 1989. Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl. Environ. Microbiol. **55:**1584–1590.
15. **Fulthorpe, R. R., and R. C. Wyndham.** 1991. Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. Appl. Environ. Microbiol. **57:**1546–1553.
16. **Gauthier, M. J., F. Cauvin, and J. Breittmayer.** 1985. Influence of salts and temperature on the transfer of mercury resistance from a marine pseudomonad to *Escherichia coli*. Appl. Environ. Microbiol. **50:**38–40.
17. **Hermansson, M., and K. C. Marshall.** 1985. Utilization of surface localized substrate by non-adhesive marine bacteria. Microb. Ecol. **11:**91–105.
18. **Jones, G. W., L. Baines, and F. J. Genthner.** 1991. Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microb. Ecol. **22:**15–25.
19. **Klingmüller, W., A. Dally, C. Fentner, and M. Steinlein.** 1990. Plasmid transfer between soil bacteria, p. 133–151. *In* J. C. Fry and M. J. Day (ed.), Bacterial genetics in natural environments. Chapman & Hall, Ltd., London.
20. **Marshall, K. C.** 1986. Adsorption and adhesion processes in microbial growth at interfaces. Adv. Colloid. Interface Sci. **25:**59–86.
21. **Marshall, K. C.** 1992. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. ASM News **58:**202–207.
22. **McClure, N. C., J. C. Fry, and A. J. Weightman.** 1990. Gene transfer in activated sludge, p. 111–129. *In* J. C. Fry and M. J. Day (ed.), Bacterial genetics in natural environments. Chapman & Hall, Ltd., London.
23. **Neu, T. R., and K. C. Marshall.** 1991. Microbial "footprints"—a new approach to adhesive polymers. Biofouling **3:**101–112.
24. **Östling, J., A. Goodman, and S. Kjelleberg.** 1991. Behaviour of IncP-1 plasmids and a miniMu transposon in a marine *Vibrio* sp.: isolation of starvation inducible *lac* operon fusions. FEMS Microbiol. Ecol. **86:**83–94.
25. **Patt, T., K. Nealson, and C. Eberhard.** 1972. Promiscuous transfer of genetic information from non-marine to marine bacteria. Biol. Bull. **143:**473.
26. **Rochelle, P. A., J. C. Fry, and M. J. Day.** 1989. Plasmid transfer between *Pseudomonas* spp. within epilithic films in a rotating disc microcosm. FEMS Microbiol. Ecol. **62:**127–136.
27. **Saye, D. J., and R. V. Miller.** 1989. The aquatic environment: consideration of horizontal gene transmission in a diversified habitat, p. 223–259. *In* S. B. Levy and R. V. Miller (ed.), Gene transfer in the environment. McGraw-Hill Publishing Co., New York.
28. **Sizemore, R. K., and R. R. Colwell.** 1977. Plasmids carried by antibiotic-resistant marine bacteria. Antimicrob. Agents Chemother. **12:**373–382.
29. **Stotzky, G.** 1989. Gene transfer among bacteria in soil, p. 165–222. *In* S. B. Levy and R. V. Miller (ed.), Gene transfer in the environment. McGraw-Hill Publishing Co., New York.
30. **Stotzky, G., and H. Babich.** 1986. Survival of, and genetic

transfer by, genetically engineered bacteria in natural environments. Adv. Appl. Microbiol. **31:**93–138.
31. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.
32. **Trevors, J. T., T. Barkay, and A. W. Bourquin.** 1987. Gene transfer among bacteria in soil and aquatic environments: a review. Can. J. Microbiol. **33:**191–198.
33. **van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder.** 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. **53:**1893–1897.