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Conjugative Plasmid Transfer between Bacteria under Simulated Marine Oligotrophic Conditions

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Marine *Vibrio* S14 strains and an *Escherichia coli* strain were starved in artificial seawater (NSS) with no added carbon, nitrogen, or phosphorus. The broad-host-range plasmid RP1 was transferred between the starving S14 strains and also from the *E. coli* donor to the S14 recipient under oligotrophic conditions, in which mixtures of donor and recipient cells were held on Nuclepore filters either floated on NSS or held such that NSS flowed through the filter. Transconjugants were obtained from S14 donors and recipients starved for at least 15 days before being mixed together for conjugation, whereas transconjugants were recovered from the *E. coli* donor and S14 recipient for up to 3 days of prestarvation, but not after 5 days. Transconjugants were obtained when there were as few as about 10^5 and 10^4 cells of starving S14 donors and recipients, respectively, per ml held on the filters. Starved donor and recipient mixtures incubated at 4 or 26°C, as well as those allowed to mate for 2, 5, or 24 h, all yielded numbers of transconjugants which were not significantly ($P > 0.05$) different.

Open ocean waters are characteristically oligotrophic, with dissolved organic carbon concentrations of about 1 mg/liter (3), and although the available carbon in some marine and most estuarine environments may exceed this level, it is possible that a "fast and famine" existence may be the norm for most marine microorganisms (29). These microorganisms have developed a complex physiological adaptation strategy that allows them to remain viable over long periods of nutrient deprivation (23, 25). Enteric bacteria, introduced into this environment in wastewater effluents, can survive the high salt concentrations and nutrient deprivation for long periods but may not be able to multiply (2, 13, 28, 31, 33). The degree of survival depends on the strains of enteric bacteria involved (24, 34). Maintenance of plasmids in starved enterics and pseudomonads also depends on both the particular plasmid and the host strain (5).

These phenomena have implications in the potential dissemination of novel genetic information in the marine environment. Two areas are of immediate concern: first, the introduction of plasmids carrying antibiotic resistances to marine bacteria and, second, the dissemination of novel gene combinations from genetically manipulated microorganisms used in agriculture, food production, and bioremediation processes. Coliform bacteria containing transmissible R plasmids have been isolated from many marine and freshwater environments (7, 32, 33, 35). Sizemore and Colwell (32) have proposed that such plasmids were introduced to the marine environment via terrestrial bacteria, as similar plasmids were found in bacteria from the septic tanks and bilge water on their research vessel. The spread of genes encoding multiple resistances to antibiotics, antiseptics, and disinfectants among *Staphylococcus aureus* strains and enteric bacteria in hospitals over the past two decades has been attributed to mobile genetic elements, particularly transposons and insertion sequences (4). This spread represents an elegant, if undesirable, example of molecular evolution over a very short time scale. Only limited knowledge is available on the spread of genetic material to and among natural microbial communities and on the impact such processes may have on these communities. Gene transfer by conjugation, transformation, or transduction has been demonstrated in both laboratory and in situ microcosms using bacteria isolated from soil (36, 39), wastewater (21, 37), freshwater (10, 15, 26), and marine (9) environments. Most of these investigators have noted that gene transfer is not detectable in microcosms unsupplemented by nutrients. More recently, however, Fernandez-Astorga et al. (8) and Jones et al. (15) have demonstrated plasmid transfer by conjugation between *Escherichia coli* and *Pseudomonas aeruginosa* strains, respectively, taken from growing cultures directly into freshwater-type microcosms with no added nutrients.

The aims of the study reported here were to determine whether a plasmid could transfer from an enteric strain (*E. coli*) to a marine strain (*Vibrio* sp. strain S141) when both strains were already starving; whether the plasmid could transfer between marine strains under starvation conditions; and whether the respective strains could retain their functions as donors or recipients of genetic information following starvation for extensive periods. To achieve these aims, we used an ideal marine oligotrophic model system including the use of pure cultures of strains known to be efficient plasmid donors and recipients, a broad-host-range self-transmissible plasmid, a defined artificial seawater medium with no added carbon substrate, and a defined substratum.

MATERIALS AND METHODS

Bacterial strains and plasmid. The strains S141 and S142 were derived from the marine *Vibrio* sp. strain S14 as streptomycin-resistant (Sm^r) and rifampin-resistant (Rp^r) mutants, respectively (27). The physiological response of S14 to starvation, as well as its capacity to survive starvation, has been described in detail by Nyström et al. (25). Strain S141 was the recipient in all conjugation experiments. The donor strains *E. coli* 803(RP1) (27) and S142(RP1), both rifampin resistant, harbored the conjugative broad-host-

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range IncP1 plasmid RP1 (which is equivalent to RP4, RK2, R68, and R18) encoding kanamycin (Km^r), tetracycline (Tc^r), and ampicillin (Ap^r) resistance (38). S14 strains are naturally ampicillin resistant (Ap^r).

Culture media. Both *Vibrio* and *E. coli* strains were grown in VNSS liquid medium for marine bacteria (40) which consisted of the following (grams per liter): peptone, 1.0; yeast extract, 0.5; glucose, 0.5; soluble starch, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; and Na_2HPO_4, 0.01 g in NSS (nine-salt solution). The $FeSO_4 \cdot 7H_2O$ and Na_2HPO_4 were filter sterilized and added separately. NSS consisted of the following (grams per liter): NaCl, 17.6; Na_2SO_4, 1.47; $NaHCO_3$, 0.08; KCl, 0.25; KBr, 0.04; $MgCl_2 \cdot 6H_2O$, 1.87; $CaCl_2 \cdot 2H_2O$, 0.41; $SrCl_2 \cdot 6H_2O$, 0.01; and H_3BO_3, 0.01. NSS was used for the starvation regimen, for all dilutions, and for resuspending cells from filters. The water used was ultrapure deionized water (MilliRO, MilliQ).

Organisms were recovered on either TSS or LB15 plates. TSS consisted of (grams per liter): tryptone soy broth (Oxoid), 30; and NaCl, 20. LB15 consisted of (grams per liter): Luria Broth (22) with extra salt, i.e., yeast extract, 5; tryptone, 10; and NaCl, 15. One and 0.33 ml of separately autoclaved 1 M solutions of $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were added to TSS and LB15. Solidified media contained 1.5% (wt/vol) agar. All media were sterilized by autoclaving at 120°C for 20 min unless otherwise stated. Stock cultures were maintained at −70°C; *E. coli* was suspended in 1 ml of LB5 (as for LB15 but containing only 5 g of NaCl per liter) plus 1 ml of 80% glycerol, and *Vibrio* strains were suspended in 1 ml of TSS plus 1 ml of 80% glycerol.

Where appropriate, filter-sterilized antibiotics were added to the media at the following concentrations (micrograms per milliliter): kanamycin, 200; rifampin, 100; streptomycin, 100; and tetracycline, 10.

Starvation regimen. Cells were grown overnight in VNSS at 28 to 30°C for *Vibrio* strains and at 37°C for *E. coli*, subcultured the following day, and grown to an optical density at 600 nm of 0.3 to 0.4, which was the early- to mid-log phase. Cells were washed twice in NSS by centrifugation and resuspension and resuspended in a final volume of 6 to 8 ml of NSS, at about 10^8 cells per ml, in a 50-ml screw-capped Falcon tube. This represented T_0 or zero time of starvation. The tubes were incubated, lying on their sides with the caps tightly closed, at 26 to 28°C. CFU were determined at T_0 and at various times during the starvation regimen.

Bacterial enumeration. Viable cells were determined as CFU on appropriate selective (plus antibiotics) and nonselective plates by plating 100 μl of appropriate dilutions of cell suspensions. Plates were incubated at 28 to 30°C. Colony counts were made in duplicate, and the means are given in the data. For the gravity-feed experiments, total cell numbers were counted with a Zeiss phase-contrast microscope. In the filter-float experiments, the total number of cells was obtained using a model Z_{B1} Coulter Counter with a 30-μm orifice tube. Total count determinations were also made in duplicate.

Conjugation. (i) **Gravity-feed method.** The apparatus consisted of a 20-ml glass filter apparatus (Sartorius, Göttingen, Germany) sterilized by autoclaving the separate parts. A coarse glass filter (Whatman, Maidstone, United Kingdom) was placed over the sintered glass. One milliliter each of appropriate dilutions from the starvation suspensions of donors and recipients were mixed and aseptically drawn onto a 0.2-μm-pore-size filter (Nuclepore Corp., Pleasanton, Calif.), which was placed on top of the glass filter. The entire assembly was then clamped together aseptically. The surface area of the filter was 3.8 cm^2. A reservoir containing 1 liter of sterile NSS was held above the filter apparatus and connected to it by a sterile tube of 3-mm diameter. Flow was begun by pumping the NSS from the reservoir into the filter apparatus. When about 10 ml had been pumped in, the tubing was released from the peristaltic pump and the NSS continued to drain into the filter apparatus by gravity feed. Between 200 and 400 ml of NSS passed through the filter during the conjugation period of each experiment. Cells were left to conjugate for 24 h in the filter apparatus, which was at room temperature (24 to 26°C).

Controls consisted of 1-ml aliquots of donor or recipient, taken from the dilutions made for the conjugation experiment, separately drawn onto filters which were then immediately resuspended separately in 0.5 ml of NSS. Controls were also made from undiluted starvation suspensions. CFU were determined and 100- or 50-μl aliquots of each were spread onto streptomycin-kanamycin or streptomycin-tetracycline selection plates, both separately and mixed. All experiments were conducted in duplicate, and representative data are shown, unless stated otherwise.

(ii) Filter-float method. Fifty microliters each of donors and recipients from the undiluted starvation cell suspensions (at various times after $T_{1\ day}$ starvation) were mixed and aseptically drawn onto 0.1-μm-pore-size Nuclepore filters and floated on 20 ml of NSS in a sterile petri dish, which was covered by its lid. Approximately 5×10^6 to 1×10^7 cells each of the starved donor and recipient were mixed for each experiment. Incubation was for 24 h at 26 to 28°C. Controls were treated in the same way as mating mixes. All experiments were done in duplicate, and, unless otherwise stated, representative data are shown. For the S141 × S142(RP1) pair, the entire experiment for cells starved for 24 h, then up to 6 days of starvation, was repeated in duplicate four and three times, respectively.

For both conjugation methods, the cells on the filter were treated after 24 h as follows. The filter was vortexed for 1 to 2 min in 1 ml of NSS, and 100 μl of serial dilutions of the suspensions was plated on the appropriate antibiotic supplemented plates. Donors [either S142(RP1) or *E. coli* 803(RP1)] were recovered on kanamycin, which counterselected the recipient, recipients (S141) were recovered on streptomycin, which counterselected the donor, and transconjugants were selected on streptomycin-kanamycin or streptomycin-tetracycline plates, which allowed growth of recipient cells that had acquired the RP1 plasmid. Transconjugants (10 to 15 colonies) recovered on streptomycin-kanamycin or streptomycin-tetracycline were replica plated to streptomycin-tetracycline or streptomycin-kanamycin, respectively, to determine whether simultaneous acquisition of a second antibiotic resistance had occurred, as well as to rifampin plates, to ensure that they were not spontaneous Sm^r donor mutants. The simultaneous acquisition of Km^r and Tc^r has been used to indicate receipt and maintenance of RP1 (27). No Sm^r donor mutants were ever recovered. Plates were incubated at 28 to 30°C. At the concentrations of antibiotics used, spontaneous mutation of donors to Sm^r or of recipients to either Km^r or Tc^r was less than 10^{-9}.

Controls. To determine whether cells were conjugating while they were recovering on the rich medium antibiotic selection plates, we plated 100 μl each of starved donors and recipients from the controls together on the selection plates. These plates showed no recovery of transconjugants, except on two occasions on which the numbers recovered were 50-fold and 1,000-fold lower than the number of transcon-

jugants recovered from the mating mixes. These colonies were subtracted from the final transconjugant CFU presented in the data. When 100 μl of recipient or donor controls was plated separately onto either streptomycin-tetracycline or streptomycin-kanamycin plates, no colonies were recovered.

To determine whether plasmid transfer might have resulted from either transformation or transduction, an aliquot of donor cells was filtered through a 0.22-μm-pore-size membrane filter and 50 μl of the filtrate was mixed with 50 μl of recipient cells, drawn onto a 0.1-μm-pore-size Nuclepore filter, floated on NSS, and treated as described above. Transconjugants were not recovered.

In a separate experiment to test for transformation only, donor and recipient cells were drawn onto a filter that had been soaked in DNase I (200 μg/ml; Boehringer GmbH, Mannheim, Germany) for 2 h at 4°C and which was then placed on NSS solidified with agar containing 10 μg of DNase I per ml. After 24 h, the filter was treated as described above. Transconjugants were recovered after this treatment.

TOC determinations. In this study, deionized water supplemented with salts (i.e., the NSS solution) was used for the starvation regimen and conjugation experiments. Organic carbon was not added. However, because the salts may have contained trace amounts of contaminants containing organic carbon, or plasticizers may have leached from the plastic Falcon tube, and because the conjugation experiments were exposed to laboratory air that may have contained volatile organic compounds, the total organic carbon (TOC) of six samples both from the laboratory in Sweden as well as that in Australia was measured. These included the deionized water, sterile NSS solutions, and the NSS after conjugation by the gravity-feed and the filter-float methods. The TOC was determined by the combustion-infra red method, as described by the American Public Health Association, method number 5310B (1). The TOC was found to be between 4 to 14 mg/liter with no apparent trend regarding source of the water or added salts.

Statistical analysis. Counts were transformed by $\log_{10}^{x}$. The means ($\bar{x}$), standard deviations (SD), and standard errors (SE) of recovered transconjugant numbers were calculated for the following sets of experiments: *E. coli* 803(RP1) × S141 starved for 24 h and mixed for 24 h in the gravity-feed apparatus (see Table 1); S142(RP1) × S141 starved for 24 h and mixed for 24 h by the filter-float method (see Table 3). Other values were then compared with these means. Significant differences are quoted for $P < 0.05$.

RESULTS

Gravity-feed apparatus. Four separate experiments revealed that plasmid transfer occurred between cells of *E. coli* 803(RP1) and S141 starved separately for 24 h and held together under oligotrophic conditions for a further 24 h (Table 1). No transconjugants were detected when the cells were starved for 5 days prior to mixing, even though recoverability of both donors and recipients was 2×10^6 CFU/ml.

Plasmid transfer between exclusively marine strains was shown when transconjugants were recovered following starvation of both donor [S142(RP1)] and recipient (S141) cells for 15 days before being mixed and from cell mixtures containing recoverable CFU as low as 1.1×10^5 donors per ml and 6.0×10^4 recipients per ml (Table 2). Recovery of transconjugants on streptomycin-kanamycin plates was better than on streptomycin-tetracycline plates, indicating that the type of antibiotic used for selection affected the recovery (Table 2). Replica plating confirmed that transconjugants recovered on streptomycin-kanamycin or streptomycin-tetracycline plates had simultaneously acquired tetracycline or kanamycin resistance, respectively, and were not Sm^r donor mutants. After 24 h of mixing, donors and recipients always showed a recovery of CFU within an order of magnitude of initial numbers placed on the filters. No cell multiplication on the filters occurred, as no differences in total cell numbers, determined microscopically, were observed in samples taken before or after 24 h of mixing (data not shown).

TABLE 1. Conjugation between *E. coli* 803(RP1) and *Vibrio* strain S141 starved for 24 h and subsequently mixed for 24 h, using the gravity-feed apparatus

Vibrio strain S141 (CFU/ml)	*E. coli* 803(RP1) (CFU/ml)	Transconjugants recovered on streptomycin-tetracycline (CFU/ml)[a]
2.0×10^7	1.4×10^6	2.5×10^4
2.9×10^7	3.8×10^6	4.7×10^4
4.2×10^7	1.6×10^5	4.6×10^3
1.5×10^6	3.0×10^5	1.1×10^3

[a] $n = 4$; $\bar{x}$ ($\log_{10}^{x}$) = 3.94; SD = 0.74; SE = 0.37.

Filter-float method. Mixing 803(RP1) × S141 and S142(RP1) × S141 on filters floating on NSS liquid gave results (Table 3) similar to those found above (Tables 1 and 2), in that both 803(RP1) and S142(RP1), when starved for 3 and 14 days, respectively, prior to mixing produced transconjugants on mating for 24 h with S141 prestarved for similar periods. After S14 donor or recipient cells had been prestarved for 6 days or more, the numbers of CFU recovered decreased with increasing prestarvation times. By 20 days, when transconjugants were not recovered, only about 10^4 and 10^3 CFU of donors and recipients, respectively, per ml were recovered; this represented a loss in recoverability of 3 to 4 orders of magnitude. All Km^r transconjugants showed simultaneous acquisition of tetracycline resistance and sensitivity to rifampin after recovery on streptomycin-kanamycin plates followed by replica plating on streptomycin-tetracycline and rifampin plates. As found above, cell multiplication on the filters did not occur. Controls for gene

TABLE 2. Conjugation between *Vibrio* strains S142(RP1) and S141 starved for different lengths of time and subsequently mixed for 24 h, using the gravity-feed apparatus

Starvation time (days)	S141 (CFU/ml)	S142(RP1) (CFU/ml)	Transconjugants (CFU/ml) recovered on:	
			Streptomycin-tetracycline	Streptomycin-kanamycin
1	6.7×10^6	3.0×10^6	1.0×10^4	4.8×10^4
2	7.0×10^5	6.3×10^5	3.5×10^2	2.5×10^4
3	2.2×10^5	1.5×10^5	ND[a]	3.9×10^2
6	6.5×10^3	7.0×10^3	ND	ND
6	6.6×10^3	1.4×10^4	ND	ND
6	6.0×10^4	1.1×10^5	ND	2.2×10^2
15	1.2×10^5	5.0×10^5	—[b]	8.0×10^2
15	3.2×10^4	4.0×10^5	—	9.0×10^2

[a] ND, not detected.
[b] —, not done.

TABLE 3. Conjugation between *E. coli* 803(RP1) and *Vibrio* strain S141 and between *Vibrio* strains S142(RP1) and S141 starved for different lengths of time and subsequently mixed for 24 h, using the filter-float method

Starvation time (days)	*Vibrio* strain S141 (CFU/ml)	*E. coli* 803(RP1) (CFU/ml)	*Vibrio* strain S142(RP1) (CFU/ml)	Transconjugants recovered on streptomycin-kanamycin (CFU/ml)
1	4.5×10^6	4.8×10^6	—[a]	1.2×10^3
2	5.3×10^6	7.1×10^5	—	2.6×10^3
3	1.6×10^6	1.1×10^6	—	4.4×10^3
8	1.2×10^4	1.1×10^6	—	ND[b]
1	6.6×10^6	—	8.2×10^6	2.6×10^3[c]
2	7.4×10^5	—	6.7×10^6	7.7×10^2
3	2.5×10^6	—	1.1×10^6	3.3×10^3
6	2.3×10^4	—	6.0×10^5	3.3×10^2
14	3.7×10^3	—	4.1×10^4	2.0×10^1
20	1.1×10^3	—	9.2×10^3	ND

[a] —, not done.
[b] ND, not detected.
[c] Geometric mean of $n = 8$; $\bar{x}$ ($\log_{10}^x$) = 3.41; SD = 0.81; SE = 0.29.

transfer by either transduction or transformation were negative.

Effect of varying parameters on recovery of transconjugants. In all the following experiments, S142(RP1) was used as the donor and S141 as the recipient. The filter-float method was used, except where stated otherwise.

(i) Conjugation time. When donor and recipient cells were starved for 24 h prior to mixing and then allowed to conjugate for periods of 2, 5, or 24 h, the numbers of transconjugants recovered on streptomycin-kanamycin plates were 4×10^3 (2 h), 2×10^4 (5 h), and 3×10^4 (24 h) CFU/ml. These transconjugant numbers did not differ significantly from those found for cells starved for 24 h and mixed for 24 h ($P > 0.05$, Tables 1 and 3). For each mating time, the numbers of donors and recipients placed on, and recovered from, the filter ranged from 6×10^6 to 1×10^7 CFU/ml.

(ii) Conjugation temperature. When donor and recipient cells were starved for 24 h and allowed to conjugate at 4 and 26°C for 24 h, the numbers of transconjugants recovered were 2×10^3 and 3×10^4 CFU/ml, respectively. These transconjugant numbers were not significantly different from those for cells starved for 24 h and mixed for 24 h at 26 to 28°C ($P > 0.05$, Tables 1 and 3). At each temperature, the numbers of donors and recipients placed on, and recovered from, the filter ranged between 4×10^6 and 1×10^7 CFU/ml. When donors and recipients, grown to the mid-log phase, were mixed and placed at 4 and 26°C on filters floated on rich TSS broth, instead of on NSS, transconjugant numbers recovered at 4°C (10^3 CFU/ml) were not significantly different from those mated on NSS ($P > 0.05$, Table 3), whereas numbers recovered from the 26°C mating were 6 orders of magnitude higher (10^9 CFU/ml). After accounting for differences in cell numbers, this represented about a 10,000-fold-higher plasmid transfer frequency (as transconjugants/donors).

(iii) Starvation time. All times given below refer to starvation treatments prior to the conjugation experiments. Transconjugants (2×10^1 to 3×10^3 CFU/ml) were obtained when donors were starved for 1 day and mixed with donors starved for 72 days. In a similar gravity-feed experiment, in which the recipients were starved for up to 100 days and the donors for 1 or 9 days, the numbers of transconjugants produced from mixes ranged from 1×10^3 to 3×10^4 CFU/ml. For each mating pair, the number of recovered CFU of the strain starved for 1 day prior to mating was about the same as the number of cells placed on the filter, whereas the strain starved for the longer time showed a recovered CFU lower, by 1 (9 days) to 3 (100 days) orders of magnitude, than the number of cells placed on the filter.

DISCUSSION

This study represents the first report of plasmid transfer between donor and recipient strains starved before being mixed to allow conjugation. The plasmid transfer was demonstrated at a surface, under ideal model marine oligotrophic conditions, between nutrient-deprived donor [either *E. coli*(RP1) or the marine bacterium S142(RP1)] and recipient (marine bacterium S141) strains. Numbers of donors and recipients below a critical level produced no transconjugants. The lowest concentration at which conjugation was detected in the gravity-feed apparatus was 1.1×10^5 CFU of donors per ml and 6.0×10^4 CFU of recipients per ml, which corresponded to 2.9×10^4 and 1.6×10^4 CFU/cm^2, respectively. When the cell density on the filters was decreased to about 10^3 CFU/cm^2, no transconjugants were detected. Rochelle et al. (30) reported that an initial cell density of about 10^4 CFU/cm^2 was critical for detection of conjugation between growing donors and recipients, even though cell numbers following growth were about 10^6 CFU/cm^2. It is only under conditions in which cell multiplication does not occur that the minimum numbers of donors and recipients needed to detect conjugation can be estimated. Such a critical cell density on the filter may reflect the point below which the cells are too far apart for the RP1-encoded sex pili to make contact with recipients.

It is uncertain how long the marine *Vibrio* strain S14 pair can be starved before recovery of transconjugants becomes undetectable. The results demonstrating transconjugant recovery from pairs in which one parent had been starved for up to 100 days when the other had been starved for 1 or 9 days were unexpected. Although transconjugants were not recovered from the mating pair in which both donor and recipient had been prestarved for 20 days, this was possibly an artifact of the filter-float method, resulting from low recoveries of the donor and recipient from the filter after this time. It is possible that the additional stress of the surface of the filter exposed to the air-water interface may have resulted in the cells being too debilitated for efficient recovery on rich media containing antibiotics. This phenomenon of antibiotic pressure affecting the initial recovery of stressed bacteria has been reported previously (5, 6, 28). In contrast, recovered CFU were within an order of magnitude of the initial numbers for prestarvation times of up to 15 days in the gravity-feed apparatus, where NSS was constantly flowing past the cells on the filter. The filter-float method proved adequate for use with cells prestarved for short times and was simpler to use than the gravity-feed method.

At this stage, it is not clear why transconjugants from the *E. coli* 803(RP1) and S141 pair were not detected after a few days of prestarvation, whereas those from the S142(RP1) and S141 pair prestarved for longer times were recovered. The efficiency of plasmid transfer between bacterial strains depends on the plasmid, the donor strain, and the recipient strain (8, 15, 30). The marine *Vibrio* strain S14 should be better adapted to a marine oligotrophic environment than an enteric bacterium, such as *E. coli*, which may rapidly lose its ability to transfer plasmids under these conditions. Once a plasmid has been transferred from an enteric to a native

marine bacterium, however, its dissemination to other marine microbial community members could occur over a much longer time.

Transconjugant recovery has been expressed as CFU per milliliter rather than in terms of transfer frequency since, as pointed out by Fernandez-Astorga et al. (8), increasing transfer frequencies may be an artifact when recoverability of one or both of the parent strains is declining, as found in this study. Recovery of transconjugant CFU was affected by the antibiotic employed in the media, as evidenced by the different CFU obtained on plates containing either tetracycline or kanamycin for plasmid selection. After recovery, however, Km^r transconjugants were able to grow on tetracycline plates. The mechanisms of resistance to tetracycline and kanamycin encoded by plasmid RP1 are known to be different (38), and it is clear that resistance to kanamycin could be maintained in long-term starved S14 cells but not resistance to tetracycline. O'Morchoe et al. (26) noted a similar phenomenon for transfer of R68.45 among freshwater bacteria, in which carbenicillin selection resulted in a several hundredfold greater recovery than when tetracycline or G-418 was used. In addition, Griffiths et al. (12) found that long-term starved cells of *E. coli* containing the R plasmid pSa were not recoverable on antibiotic-containing media, but were able to express the plasmid-encoded antibiotic resistance following resuscitation on a nonselective medium.

TOC concentrations in the NSS used in the present study varied between 4 and 14 mg of C per liter. Even if some of this organic carbon was available to the bacteria, total cell counts indicated that it was insufficient to allow cell multiplication. The level of assimilable organic carbon is the most meaningful indicator of bacterial activity and growth in aquatic systems, but the assimilable organic carbon is generally only a small proportion (0.1 to 9%) of the TOC (16). However, even assuming that all the TOC in our system was available to the bacteria, then this amount would not have been sufficient to stimulate conjugative plasmid transfer. Fernandez-Astorga et al. (8) found that an organic carbon concentration greater than 1,000 mg of TOC per liter was necessary to have an appreciable stimulatory effect on conjugation. It is feasible that some nutrients, originating as contaminants of the inorganic salts or from cell lysis, may have concentrated at the filter-water interface and increased cellular metabolic activity. This situation would be similar to that pertaining in natural biofilms, where nutrients concentrate from the bulk water phase and where lytic products from cells within the biofilm are metabolized by adjacent bacteria (19). Plasmid transfer has been demonstrated among freshwater biofilm bacteria in situ and in laboratory microcosms where the organically polluted river water may have provided relatively high nutrient levels (10). Hermansson et al. (14) reported higher frequencies of antibiotic- and heavy metal-resistant isolates at the air-water interface compared with the bulk water phase in coastal marine samples, with 55% of the resistant bacteria carrying plasmids. It was suggested that surfaces in general, with their higher bacterial population densities, are sites where plasmid transfer is most likely to occur.

A report of plasmid RP1 transfer among *P. aeruginosa* strains on agar plates with no added nutrients (30) may not represent transfer between starving bacteria because of the likelihood of contaminating nutrients in the agar (8, 20). Two recent studies have demonstrated plasmid transfer in unsupplemented freshwater microcosms (8, 15), but in these studies the bacteria were not starved before mixing. Plasmid transfer has been reported in agricultural soils containing 2 to 5% organic matter (36), which is considered to be high (41). Such reports, however, are difficult to assess in terms of starvation of the mating organisms, because of an unavoidable lack of suitable controls and the fact that autoclaving or irradiating soil as well as rewetting of dry soils is well known to result in a burst of microbial activity as a result of the release of available organic matter (18). None of the above studies involved plasmid transfer between strains that were starved both before and during the conjugation period.

Both time and temperature differences during the conjugation process had no effect on the yield of transconjugants in the present starvation studies. These results are in agreement with those reported by Fernandez-Astorga et al. (8) for plasmid transfer between starved *E. coli* strains. When donor and recipient cells are growing, on the other hand, RP1 transfer is most efficient at temperatures optimal for cell growth and is low at 4°C, and transfer frequencies increase with increasing conjugation times (8, 10, 11, 17, 27, 30, 42). It may be that different mechanisms exist for plasmid transfer under nongrowth conditions, such as under nutrient deprivation and/or low temperatures.

Generally, nutrients are scarce in marine environments (3, 29), and most bacteria exist at temperatures below those optimal for exponential growth. Plasmid transfer under oligotrophic conditions may be an ecologically significant process resulting in gene transfer in natural marine environments. The present studies are being extended to investigate plasmid transfer among marine bacteria under more natural environmental conditions.

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