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Transfer and Expression of a Multiple Antibiotic Resistance Plasmid in Marine Bacteria

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Abstract. Conjugal transfer of a multiresistance plasmid from *Pseudomonas fluorescens* to halophilic and halotolerant bacteria was studied under in vitro and in situ conditions. Mating conducted in broth as well as on plates yielded a plasmid transfer frequency of as high as 10^{-3}. Among these two, plate mating facilitated conjugal transfer of plasmid, because the cell-to-cell contact is more in plate mating. When *P. fluorescens* was incubated in seawater, the organism progressively lost its colony forming activity within 15 days. Microscopic examination revealed the presence of very short rods, indicating that the cells have become viable but nonculturable (VNC). Mating conducted in natural seawater without any added nutrients revealed that the conjugal transfer is influenced by the physical state of the donor and the recipients as well as the availability of nutrients. But a plasmid transfer frequency of 10^{-7} was obtained even after the donor cells have become VNC suggesting that the nonculturable state and nutrient deprived condition may not limit plasmid transfer. The results suggest that the terrestrial bacteria entering into the seawaters with antibiotic resistance plasmids may be responsible for the prevalence of resistance genes in the marine environment.

The use of antimicrobial agents in both human and veterinary medicine exerts a strong selective pressure among bacteria, leading to the emergence and dissemination of antibiotic resistance genes [13, 18, 36]. Generally, bacteria with the highest level of resistance are isolated from environments such as hospitals, sewage, effluents, and wastewater which are contaminated with antimicrobial agents [8, 11, 21, 31]. However, resistant bacteria have also been isolated from nonselective environments. For instance, Kobori et al. [16] reported the incidence of antibiotic resistance among bacteria isolated from Antartica, where there is very little human activity. Thus, it remains a mystery for the microbial ecologists to understand the evolution of antibiotic resistance in natural environments, especially in a marine ecosystem, which is essentially free of selective pressure due to anthropogenic materials.

Horizontal transfer of plasmid-encoded genes is the primary reason for the dissemination of resistance genes in the environment [29]. Plasmid transfer between bacteria occurs in a variety of natural habitats, e.g., wastewater [19], sewage [8, 11], riverwater [2, 12], lakewater [25], sediments [30], and soil [24, 28, 34]. Plasmid transfer has been shown to occur even between ecologically and evolutionarily disparate organisms [31,33], and intrageneric and intergeneric transfer has been demonstrated under a variety of laboratory conditions [20]. However, the number of studies on gene transfer in natural environments is limited.

Large volume of sewage and effluents containing antibiotic resistance bacteria is discharged into marine ecosystem. Besides, the rain waters and flood displace several antibiotic-resistant terrestrial bacteria into the marine waters. The fate of these resistant bacteria is not clearly understood. When fresh-water or terrestrial bacteria enter seawater, they undergo stress owing to salinity and starvation. As a result, the cells enter into a dormant viable but nonculturable (VNC) state [23]. During VNC state, the cells lose their ability to form colonies on culture media, but they maintain their metabolic potential, and the biological function is not totally switched off or impaired seriously [9]. However, it is not known whether plasmid transfer could occur after the cells have entered the VNC state.

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The aims of the study reported here were to determine whether a plasmid could transfer from a non-halophilic bacterium (*Pseudomonas fluorescens*) to marine bacteria; whether the plasmid transfer could occur even after the donor has become VNC; and whether salinity affects the conjugal frequency. To achieve these aims, we studied the plasmid transfer under both in vitro and in situ conditions.

Materials and Methods

Bacterial strains and plasmid. *Pseudomonas fluorescens* harboring a 140-kb multiple antibiotic and heavy metal resistance plasmid, pSCL, was used as the donor. The characteristics of the donor as well as its plasmid were described previously [4]. Among the four marine isolates used as recipients, *Micrococcus halobius* ATCC21727 and *Flavobacterium antarticus* MTCC676 were received from the Institute of Microbial Technology, Chandigarh, India, and the other two were isolated from seawaters collected from the Bay of Bengal. On the basis of the biochemical characteristics, they were identified as *Bacillus brevis* and *Bacillus pumilus*.

Culture conditions. *P. fluorescens* was grown in Luria Bertani (LB) medium (peptone 1%, yeast extract 0.5%, NaCl 0.5%), and the marine isolates were regularly grown in Upper Bay Yeast extract (UBY) medium [5] (NaCl 0.5 g, KCl 0.016 g, Peptone 0.1 g, yeast extract 0.1 g, agar 1.5 g, aged seawater 75 ml, distilled water 25 ml).

Susceptibility testing and plasmid isolation. The susceptibility of marine bacteria to various antibiotics (ampicillin, chloramphenicol, gentamicin, kanamycin, and tetracycline) was determined by plating them on UBY agar containing increasing concentrations of antibiotics. The minimum concentration required to arrest the growth completely was considered as the MIC. Plasmid DNA from *P. fluorescens* was isolated as described previously [4], and the marine isolates were examined for the presence of plasmids by the method of Kado and Liu [15]. Plasmid DNA was resolved in horizontal agarose gel (0.7% wt/vol) electrophoresis.

Conjugation experiments

In vitro conjugation. (1) *Broth mating.* The cell density of the overnight cultures of donor and recipient bacteria was adjusted to an O.D. of 1.0 at 600 nm. 100 µl of culture from the donor as well as from the recipient were aliquoted into a sterile test tube to which 800 µl of sterile LB broth was added, and it was incubated at 37°C on a rotary shaker. At regular intervals, aliquots of this suspension were serially diluted and plated on UBY medium amended with tetracycline (25 µg/ml). The plates were incubated at 37°C for 24 h, and the putative transconjugants were replica plated onto UBY agar plates amended with other antibiotics (gentamicin 25 µg/ml; kanamycin 25 µg/ml; chloramphenicol 25 µg/ml).

(2) *Plate mating.* Plate mating was performed with overnight cultures of donor and recipients. The density of the cells was adjusted to $O.D._{600} = 1.0$, and 100 µl of donor and recipient culture was spotted on LB agar plates. The plates were incubated at 37°C. At appropriate time intervals, the dried bacterial spots were moistened with sterile phosphate buffer (0.1 M, pH 7) and gently scraped with a sterile glass rod. The cell suspension was serially diluted and plated on UBY agar plates amended with tetracycline (25 µg/ml). The colonies that appeared on these plates were further replica plated on other antibiotics as described above.

Table 1. Frequency of plasmid transfer from *P. fluorescens* to marine bacteria during broth mating

Time of incubation (h)	*P. fluorescens* vs *B. brevis*	*P. fluorescens* vs *B. pumilus*	*P. fluorescens* vs *M. halobius*	*P. fluorescens* vs *F. antarticus*
1	2.8×10^{-4}	2.4×10^{-4}	3×10^{-4}	2×10^{-4}
2	7.2×10^{-4}	5×10^{-4}	6×10^{-4}	6×10^{-4}
3	8.8×10^{-4}	5.2×10^{-4}	7.5×10^{-4}	7×10^{-4}
4	8.8×10^{-4}	8×10^{-4}	1×10^{-3}	8.7×10^{-4}
5	9.6×10^{-4}	8×10^{-4}	1.7×10^{-3}	1×10^{-3}
6	5.2×10^{-3}	8.8×10^{-4}	3×10^{-3}	1.5×10^{-3}

In situ conjugation. The conjugal transfer of plasmid pSCL from *P. fluorescens* to marine recipients was studied under two different conditions.

(i) The donor and recipient cells were grown overnight in LB and UBY medium respectively and centrifuged. The concentration of the cells was adjusted to 1.0 at 600 nm with marine water, and the cells were incubated at room temperature. On every third day, 1 ml each from the donor and recipients was aliquoted into a sterile test tube and incubated for 6 h on a rotary shaker, after which it was serially diluted and plated on UBY agar media containing antibiotics.

(ii) The conjugal transfer was studied after the donor cells have become VNC (see below). They were mixed with similarly starved cells of the recipients and incubated for 6 h. After suitable dilution, they were plated onto selective media as described above.

Viability of *P. fluorescens* in sea water. Freshly grown cells of *P. fluorescens* were centrifuged and suspended in sterile sea water. They were incubated on a rotary shaker, and the viable count of the cells was determined every day by plating them onto LB media.

Results

Phenotype of donor and marine recipients. *P. fluorescens*, which served as the donor, was resistant to almost all antibiotics [4] and was unable to grow on UBY agar medium. Therefore, these characters were taken as markers. Among the marine recipients, *F. antarticus* was sensitive to all the antibiotics, and *M. halobius*, *B. brevis*, and *B. pumilus* were moderately resistant to ampicillin (MIC value 25 µg/ml) and sensitive to all other antibiotics. Hence their sensitivities to tetracycline, gentamicin, kanamycin, and chloramphenicol were taken as markers.

Plasmid transfer during broth mating. The broth mating between *P. fluorescens* and the marine isolates was studied at various time intervals at room temperature (30°C), and the frequency of transfer is summarized in Table 1. Although the frequency increased with the increase of incubation time, after 6 h there was no increase, and when mating was allowed to proceed overnight there was not any marked increase in the number of transconjugants obtained. Among the four marine isolates, *M. halobius* showed the highest conjugation frequency. The transconjugants were able to grow in

Table 2. Frequency of plasmid transfer from *P. fluorescens* to marine bacteria during plate mating

Time of incubation (h)	*P. fluorescens* vs *B. brevis*	*P. fluorescens* vs *B. pumilus*	*P. fluorescens* vs *M. halobius*	*P. fluorescens* vs *F. antarticus*
1	6.4×10^{-4}	6×10^{-4}	5.2×10^{-4}	3.4×10^{-4}
2	8×10^{-4}	6.5×10^{-4}	7×10^{-4}	6×10^{-4}
3	1.2×10^{-3}	9.2×10^{-4}	1.1×10^{-3}	7.2×10^{-4}
4	1.2×10^{-3}	1.1×10^{-3}	1.3×10^{-3}	1×10^{-3}
5	2.5×10^{-3}	1.5×10^{-3}	1.8×10^{-3}	1.2×10^{-3}
6	5.6×10^{-3}	2.8×10^{-3}	2.2×10^{-3}	1.8×10^{-3}

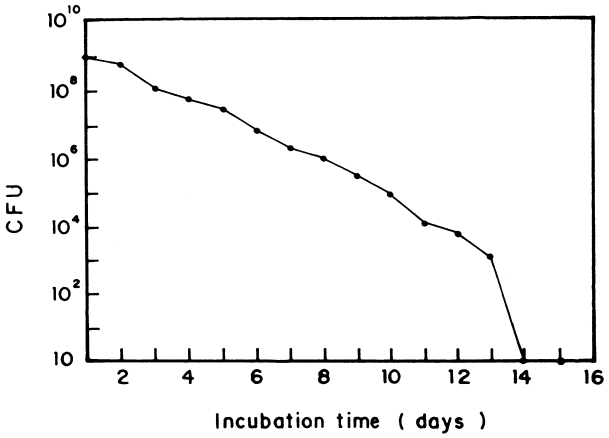

Fig. 1. Viability of *P. fluorescens* in marine water.

UBY medium containing antibiotics, and they harbored the plasmid, pSCL.

Plasmid transfer during plate mating. When compared with broth mating, the frequency of conjugal transfer of plasmid DNA was high in plate mating, as evident from Table 2. Interestingly, mating with all four marine recipients recorded almost similar plasmid transfer frequency. As observed in broth mating, the frequency of transfer did not increase after 6 h incubation time.

Viability of *P. fluorescens* in sea water. The initial concentration of bacteria was ca. 1×10^9 cells per ml, and a progressive loss of colony-forming ability was observed when *P. fluorescens* was incubated in marine water without any added nutrients (Fig. 1). After 2-week incubation time, only a few colonies were obtained. From the 15th day onwards there were no colonies at all indicating that the cells have lost their colony-forming ability. However, the microscopic observation showed the presence of cells. But the cells that are originally rods have become very short rods.

Plasmid transfer under in situ conditions. The intergeneric transfer of plasmid took place from *P. fluorescens* to marine bacteria under in situ condition, but at lower frequencies. On day 1, the conjugation frequency was as high as 1×10^{-3}, after which there was decline as the incubation time progressed (Table 3). However, after the 15th day, when the donor cells reached the VNC state, the number of transconjugants obtained reached a steady state with a frequency of around 10^{-7}.

Table 3. Frequency of plasmid transfer from *P. fluorescens* to marine bacteria in natural seawater

Days	*P. fluorescens* vs *B. brevis*	*P. fluorescens* vs *B. pumilus*	*P. fluorescens* vs *M. halobius*	*P. fluorescens* vs *F. antarticus*
1	3.1×10^{-4}	9.1×10^{-4}	7.2×10^{-4}	8.1×10^{-4}
3	3.2×10^{-4}	5.9×10^{-4}	2.2×10^{-4}	6.7×10^{-4}
6	6.9×10^{-5}	6.2×10^{-5}	4.1×10^{-5}	5.5×10^{-5}
9	3.7×10^{-5}	2.9×10^{-5}	2.2×10^{-5}	2.0×10^{-5}
12	1.1×10^{-5}	9.2×10^{-6}	9.0×10^{-6}	9.2×10^{-6}
15	9.8×10^{-5}	7.9×10^{-6}	8.7×10^{-6}	8.7×10^{-6}
18	9.2×10^{-6}	8.5×10^{-7}	8.2×10^{-6}	7.7×10^{-6}

Table 4. Frequency of plasmid transfer from the VNC cells of *P. fluorescens* to marine bacteria

Days	*P. fluorescens* vs *B. brevis*	*P. fluorescens* vs *B. pumilus*	*P. fluorescens* vs *M. halobius*	*P. fluorescens* vs *F. antarticus*
1	7.0×10^{-5}	7.9×10^{-5}	6.8×10^{-5}	4.9×10^{-5}
3	3.0×10^{-5}	4.1×10^{-5}	1.2×10^{-5}	1.2×10^{-5}
6	9.8×10^{-6}	1.2×10^{-5}	9.0×10^{-6}	8.7×10^{-6}
9	7.5×10^{-6}	8.0×10^{-6}	6.4×10^{-6}	6.2×10^{-6}
12	1.2×10^{-6}	1.2×10^{-6}	1.0×10^{-6}	1.1×10^{-6}
15	7.2×10^{-7}	8.4×10^{-7}	6.7×10^{-7}	7.9×10^{-7}
18	4.2×10^{-7}	4.9×10^{-7}	4.2×10^{-7}	4.4×10^{-7}
21	2.7×10^{-7}	2.7×10^{-7}	2.0×10^{-7}	1.9×10^{-7}
24	2.0×10^{-7}	2.0×10^{-7}	1.8×10^{-7}	1.3×10^{-7}
27	1.7×10^{-7}	1.8×10^{-7}	1.5×10^{-7}	1.2×10^{-7}
30	1.0×10^{-7}	1.2×10^{-7}	1.2×10^{-7}	1.0×10^{-7}

Conjugation experiments conducted with the VNC cells showed interesting results. In the initial days of VNC state (14th and 15th days), the conjugal frequency was comparatively more (Table 4). Afterwards there was a slight decrease in the frequency, and interestingly, in mating experiments conducted 15 days after the donor cells had entered into VNC state, a steady number of transconjugants was obtained with a conjugation frequency of around 10^{-7}. Even after prolonged storage, the VNC cells were still found to engage in conjugation, albeit at a low frequency. Control experiments were also carried out in which the VNC cells were periodically plated on LB agar plates to see whether the VNC cells had reverted to vegetative cells, and it was found that as long as the VNC cells were maintained in salinity and starvation stress, they never became culturable.

Stability of transconjugants. To investigate the stability of plasmid in the marine hosts under nonstress condition, we subcultured the transconjugants in UBY broth several times and screened them for the presence of plasmid DNA. It was found that, after a few generations, the transconjugants lost the plasmid DNA. However, when the transconjugants were grown in UBY broth amended with antibiotics, the plasmid was stably maintained.

Discussion

Currently, there is great interest in gene transfer in natural habitats. Although there are reports of plasmid transfer in aquatic environment, such as waste waters, sewage, and rivers, there is no systematic study on the transfer of antibiotic resistance plasmids from terrestrial to marine bacteria. As large numbers of bacteria are discharged into marine waters, the details about the feasibility of transfer of R plasmids from these organisms to marine bacteria will give a clue to the cause of prevalence of antibiotic resistance plasmids in the marine environment. In view of this background, the present study was initiated to find out whether conjugal transfer of plasmid occurs between terrestrial and marine bacteria. Besides, this study represents the first report of plasmid transfer from terrestrial donor to the marine recipients after the donor has entered into the VNC state.

Among the four marine recipients, *M. halobius* and *F. antarticus* are halophiles, and the other two, viz., *B. brevis* and *B. pumilus*, have been isolated from the coastal waters of the Bay of Bengal and hence they may be termed ''halotolerant'' [26]. Conjugation experiments were performed both in vitro and in situ in order to determine whether the presence of nutrients affects the frequency of conjugal transfer. Quite expectedly, the transfer was higher under laboratory conditions than that conducted in natural seawater. In marine oligotrophic conditions, the coastal areas are comparatively rich in nutrients owing to the inflow of sewage and effluents, and their salinity is also less. Hence it may be expected that plasmid transfer will be high in these regions, as is evident from the higher number of transconjugants obtained in the nutrient-rich laboratory conditions. Plate mating yielded comparatively more transconjugants than broth mating. This may be because the cell-to-cell contact is greater in plate mating than in broth mating. A similar conclusion was reached by Genthner et al. [10], who reported that a solid environment was superior to a liquid environment for plasmid transfer.

At present, very little is known about the effect of introduced organisms on the marine environment. Moreover, only a few reports have dealt with the viability of terrestrial bacteria in high salinity. In the present study, we found that *P. fluorescens* progressively lost its colony-forming ability within 2 weeks when maintained in seawater without any nutrients. Studies by Byrd and Colwell [3] demonstrated that when *E. coli* cells harboring pBR322 and pUC18 were placed in sterile artificial seawater, the direct cell count did not change, but the cells became nonculturable. While entering into the VNC state, the cells undergo both physiological and metabolic changes and become reduced in size with fewer ribosomes. Their membrane fatty acids also change with the appearance of several short-chain fatty acids [17]. However, the VNC cells of *Shigella dysenteriae* Type 1 were reported to retain several virulence factors, and no change was observed in the DNA content of these cells during VNC state [3, 9]. Besides, it has recently been shown that the VNC cells of *E. coli* strains maintained the ability to express plasmid-encoded antibiotic resistance phenotype [2]. While Linder and Oliver [17] reported that *Vibrio vulnificus* cells lost virulence after they attained the VNC state, Colwell et al. [6] found that the non-culturable cells of attenuated *Vibrio cholerae* 01 reverted to a cultivable state and regained the capacity to colonize while passing through the intestinal tracts of healthy, human volunteers. Therefore, it will be epidemiologically significant to know whether the VNC cells can engage in horizontal gene transfer.

The results show that the frequency of plasmid transfer was comparatively high when the donor was in the culturable state. Quite interestingly, the terrestrial bacteria were able to act as donors even after they had entered the VNC state. However, the VNC state is not achieved quite abruptly. There is a cascade of genetic switches that bring about the VNC state [22]. Therefore, even if it appears that the cells have become nonculturable, they may still have the ability to engage in the conjugation process. The results of the present study confirm this fact. In the initial days of the VNC state, the frequency was high, and it declined thereafter. However, the VNC cells did not become totally refractile to conjugation even after several days of their dormant state, and transconjugants could be obtained whenever they were mated with the marine bacteria.

While the availability of nutrients also plays a decisive role in the incidence of plasmid transfer [14], it appears that the transfer may occur even under nutrient-deprived conditions [27, 32]. In general, the efficiency of plasmid transfer between bacteria depends on the plasmid, the donor strain, and the recipient strain [1, 7].

Under normal physiological conditions, the limiting step in mating pair formation is the production of pili by the donor [35]. However, from the above results, it appears that the VNC state does not preclude the formation of sex pili. At present very little is known about the

physiology of the VNC cells, and further studies are required to understand whether the ability of VNC cells to engage in conjugation is confined only to a limited bacterial genera or is a widespread phenomenon. If the latter is found to be true, then it may explain how the resistance genes are disseminated in the marine environment.

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Literature Cited

1. Arana I, Justo JI, Pocino M, Barcina I (1997) Influence of a survival process in a freshwater system upon plasmid transfer between *Escherichia coli* strains. Microb Ecol 33:41–49
2. Bale MJ, Day MJ, Fry JC (1988) Novel method for studying plasmid transfer in undisturbed river epilithon. Appl Environ Microbiol 54:2756–2758
3. Byrd JJ, Colwell RR (1990) Maintenance of plasmids pBR322 and pUC8 in nonculturable *Escherichia coli* in the marine environment. Appl Environ Microbiol 56:2104–2107
4. Chandrasekaran S, Lalithakumari D (1998) Plasmid assisted morpholine degradation by *Pseudomonas fluorescens* CAS102. World J Microbiol & Biotechnol 14:7–10
5. Colwell RR, Sayler GS, Nelson JD, Justice A (1976) Microbial mobilization of mercury in aquatic environment. In: Nriagu (ed) Environmental biogeochemistry, vol. 2. Ann Arbor, MI: Ann Arbor Sciences, pp 473–481
6. Colwell RR, Brayton P, Herrington P, Tall B, Huq A, Levine MM (1996) Viable but non-culturable *Vibrio cholerae* 01 revert to a cultivable state in the human intestine. World J Microbiol & Biotechnol 12:28–31
7. Fernandez-Astorga A, Muela A, Cisterna R, Iriberri J, Barcina R (1992) Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Appl Environ Microbiol 58:392–398
8. Fontaine TD, Hoadley AW (1976) Transferable drug resistance associated with coliforms isolated from hospital and domestic sewage. Health Lab Sci 13:238–245
9. Garcia-Lara J, Martinez J, Vilambu M, Vives-Rego J (1993) Effect of previous growth conditions on the starvation-survival of *Escherichia coli* in seawater. J Gen Microbiol 139:1425–1431
10. Genthner FJ, Chatterjee P, Barkay T, Bourquin ALW (1988) Capacity of aquatic bacteria to act as recipients of plasmid DNA. Appl Environ Microbiol 54:115–117
11. Grabow WOK, Prozesky OW (1973) Drug resistance of coliform bacteria in hospital and city sewage. Antimicrob Agents Chemother 3:175–180
12. Grabow WOK, Prozesky OW, Burger JS (1975) Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. Water Res 9:777–782
13. Hinton M, Kaukas A, Lipton AH (1986) The ecology of drug resistance in enteric bacteria. J Appl Bacteriol 61 (Suppl):77–92
14. Jones GW, Baines L, Genthner FJ (1991) Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microb Ecol 22:15–25
15. Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145:1365–1373
16. Kobori H, Sullivan CW, Shizuy H (1984) Bacterial plasmids in antartic natural microbial assemblages. Appl Environ Microbiol 48:515–518
17. Linder K, Oliver JD (1989) Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl Environ Microbiol 55:2837–2842
18. Linton AH, Timoney JF, Hinton M (1981) The ecology of chloramphenicol-resistance in *Salmonella typhimurium* and *Escherichia coli* in calves with endemic salmonella infection. J Appl Bacteriol 50:115–129
19. Mach PA, Grimes DJ (1982) R-Plasmid transfer in a wastewater treatment plant. Appl Environ Microbiol 44:1395–1403
20. Mazolder P, Davies J (1991) Gene transfer between distantly related bacteria. Annu Rev Genet 25:147–171
21. McPherson P, Gealt MA (1986) Isolation of indigenous wastewater bacterial strains capable of mobilizing plasmid pBR325. Appl Environ Microbiol 51:904–909
22. Morita RY (1988) Bioavailability of energy and its relationship to growth and starvation survival in nature. Can J Microbiol 34:436–441
23. Munro PM, Flatau GN, Clement RL, Gauthier MJ (1995) Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella typhimurium* in sea water. Appl Environ Microbiol 61:1853–1858
24. Naik GA, Bhat LN, Chopade BA, Lynch JM (1994) Transfer of broad-host-range antibiotic resistance plasmids in soil microcosms. Curr Microbiol 28:209–215
25. O'Morchoe SB, Ogunseitan O, Sayler GS, Miller RV (1988) Conjugal transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* strains in a freshwater environment. Appl Environ Microbiol 54:1923–1929
26. Oren A, Gurevich P, Azachi M, Henis Y (1992) Microbial degradation of pollutants at high salt concentrations. Biodegradation 3:387–398
27. Rochelle PA, Fry JC, Day MJ (1989) Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. J Gen Microbiol 135:409–424
28. Smit E, Wolters A, van Elsas JD (1995) Genetic stability, conjugal transfer and expression of heterologous DNA inserted into different plasmids and the genome of *Pseudomonas fluorescens* in soil. Rev Microbiol 26:169–179
29. Spratt BG (1994) Resistance to antibiotics mediated by target alterations. Science 264:388–393
30. Stewart KR, Koditschek L (1980) Drug-resistance transfer in *Escherichia coli* in New York Blight sediment. Mar Pollut Bull 11:130–133
31. Toranzo AE, Combarro P, Lemos ML, Barja JL (1984) Plasmid coding for transferable drug resistance in bacteria isolated from cultured rainbow trout. Appl Environ Microbiol 48:872–877
32. Trevors JT, Oddie KM (1986) R Plasmid transfer in soil and water. Can J Microbiol 32:610–613
33. Trevors JT, Barkay T, Borquin AW (1987) Gene transfer among bacteria in soil and aquatic environments: a review. Can J Microbiol 33:191–198
34. van Elsas JD, Nikkel M, van Overbeek LS (1989) Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil bacteria. Curr Microbiol 19:375–381
35. Walmsley RH (1976) Temperature dependence of mating-pair formation in *Escherichia coli*. J Bacteriol 126:222–224
36. Young HK (1993) Antimicrobial resistance spread in aquatic environment. J Antimicrob Agents Chemother 31:627–635