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Capacity of Aquatic Bacteria To Act as Recipients of Plasmid DNA

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A total of 68 gram-negative freshwater bacterial isolates were screened for their ability to receive and express plasmids from *Pseudomonas aeruginosa* donors. The plate mating technique identified 26 of the isolates as recipient active for the self-transmissible wide-host-range plasmid R68; 10 were recipient active by R68 mobilization for the wide-host-range plasmid cloning vector R1162. Frequencies of transfer were compared by using three conjugal transfer procedures: broth, plate, and filter mating. For every recipient tested, a solid environment was superior to a liquid environment for transfer. The broth mating technique failed to demonstrate R68 transfer in 63% of the recipient-active isolates. Filter mating, in general, yielded the highest transfer frequencies. The more-rapid plate mating procedure, however, was just as sensitive for testing the capacity of natural isolates to participate in conjugal plasmid transfer.

One of the concerns regarding the release of genetically engineered microorganisms to the environment is that introduced strains may transfer their novel DNA sequences to the indigenous population. To assess the potential risk associated with the release of novel organisms to the environment, it is necessary to determine the capacity of the indigenous community to receive DNA from introduced strains. Plasmids are commonly used as vehicles for recombinant DNA, and their transfer by conjugation may be a significant mode of gene transfer in natural habitats (10). Thus, plasmid-mediated gene transfer was the focus of this investigation.

While there are numerous reports on the ability of laboratory-developed strains to act as donors and recipients in plasmid transfer experiments under laboratory conditions, there are relatively few studies that use environmental isolates. Schilf and Klingmuller (8) demonstrated that bacterial isolates from agricultural soil and water possessed the capacity to act as recipients for plasmid transfer under both laboratory and simulated environmental conditions. Walter et al. (11), working with *Escherichia coli* and *Pseudomonas cepacia* donors harboring self-transmissible plasmids from three incompatibility groups, found that no single conjugation mating technique was completely reliable in detecting exconjugants.

The conjugal transfer of plasmid R68 (IncP; 4) and its mobilization of the transfer-deficient plasmid R1162 (IncP-4; 2) were chosen as a model for gene transfer. Both plasmids are wide host range. Small non-self-transmissible plasmids like R1162 are the most commonly used vehicles for carrying recombinant DNA; thus, we also examined the capacity of freshwater bacteria to receive this cloning vector by triparental mating with R68 as the helper plasmid. This mode of transfer may be environmentally significant, because indigenous bacterial strains capable of mobilizing non-self-transmissible plasmids have been isolated from wastewater (7).

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas aeruginosa* donor strains RM2046, a leucine auxotroph harboring R68 (Cb^r Km^r Tc^r), and RM2021, a prototroph harboring R1162 (Sm^r Su^r), were obtained from R. V. Miller, Loyola University Medical Center, Maywood, Ill. Most recipient strains (Table 1) were randomly isolated from colonies arising on plate count agar (Difco Laboratories, Detroit, Mich.) plates inoculated with dilutions of sediment samples obtained from Onandaga and White lakes (1). These recipients were identified by the procedure of Ward et al. (12). Isolates belonging to the genus *Acinetobacter* were confirmed by the method of Juni (6). *Pseudomonas putida* CYM318, a spontaneous nalidixic acid- and rifampin-resistant mutant, was derived from CYM3 (P. J. Chapman, University of Minnesota, St. Paul). *Beggiatoa alba* B18LD was obtained from T. Schmidt, University of Wisconsin, Milwaukee Center for Great Lakes Studies.

Media and growth conditions. *B. alba* was grown as described by Strohl et al. (9). All other organisms were grown in plate count broth (Difco) with shaking at 200 rpm at 30°C in a 125-ml Erlenmeyer flask containing 10 ml of broth.

All antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Unless indicated otherwise, antibiotics were added as required at the following concentrations (micrograms per milliliter): kanamycin, 25; nalidixic acid, 500; rifampin, 150; and tetracycline, 25.

Mating procedures. To permit selection against the donor in conjugation experiments, spontaneous rifampin-resistant recipients were used. A nalidixic acid-resistant derivative of RM2046 was generated by subculture in increasing concentrations of nalidixic acid in plate count broth. Nalidixic acid was then used to select against recipients when donors were enumerated in the mating mix. Broth matings were performed by mixing 1.0 ml each of overnight (16- to 18-h) donor (RM2046) and recipient culture at 30°C for 3 h. Appropriate dilutions in G buffer (10 mM Tris hydrochloride–10 mM NaCl–1.0 mM $MgCl_2$–1.0 mM $CaCl_2$ [pH 7.8]) were plated on plate count agar with rifampin and either kanamycin or tetracycline to enumerate exconjugants. Controls, donor or recipient alone, were also plated on the appropriate selective medium. Viable counts of the donor cells were done by plating on plate count agar and nalidixic acid with either kanamycin or tetracycline.

Plate matings were performed with overnight (18-h) cultures of donor and recipient cells mixed in a 1:1 ratio. A 1:1:1 donor/helper/recipient ratio was used for triparental matings. Three 20- or 40-μl samples of the mixtures were spotted on plate count agar plates. Plates were incubated for 3 to 5 h at 30°C to allow genetic exchange. The dried bacterial spots

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TABLE 1. Potential of aquatic bacteria to serve as recipients for plasmid transfer

Organism or type	No. tested	No. of recipients (% of total) by:	
		Conjugal transfer[a]	Mobilization[b]
Pseudomonas sp.	30	12 (40)	4 (13)
Acinetobacter sp.	7	2 (29)	0
Alcaligenes sp.	3	3 (100)	0
Beggiatoa alba	1	0	0
Chromobacterium violaceum	1	1 (100)	1 (100)
Flavobacterium sp.	2	0	0
Achromobacter sp.	1	1 (100)	ND[c]
Moraxella-like sp.	4	1 (25)	1 (25)
Unknown gram-negative rods	19	6 (32)	4 (21)
Total	68	26 (38)	10 (15)

[a] Frequency of $\geq 1.5 \times 10^{-8}$ exconjugants per donor.
[b] Frequency of $\geq 2.0 \times 10^{-8}$ exconjugants per donor.
[c] Unable to determine because of resistance of the recipient to streptomycin.

were distributed evenly over the agar surface by adding 0.2 ml of G buffer and spreading with a bent glass rod. Plates were then overlaid with antibiotic-containing soft agar (0.5 to 0.8% agar in G buffer with rifampin and either kanamycin or tetracycline for R68 exconjugants and rifampin plus streptomycin for R1162 exconjugants) and were incubated at 30°C for 48 h.

Filter matings were performed by mixing 0.15 ml each of the recipient and donor cultures and filtering them through 2.5-cm-diameter, 0.22-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). The filter was aseptically placed on a plate count agar plate and incubated at 30°C. After 3 h, the filter was lifted from the plate and immersed in 2 ml of G buffer. The cells were washed from the filter by vortexing in buffer for 0.5 min, and appropriate dilutions were plated on media selective for donors and exconjugants. Plates were incubated at 30°C. Colonies were counted at 48 h.

For all mating procedures, the frequency of transfer was calculated as the number of exconjugants per donor cell. Plasmid DNA in the donor, selected exconjugants, and other isolates was confirmed by visualization on agarose gels by the procedure of Birnboim and Doly (3).

RESULTS

Of the 68 freshwater isolates screened for their capacity to participate in conjugal plasmid transfer, 26 (38%) were found to be recipient active for R68 and 10 (15%) were found to be recipient active for R1162 by R68 mobilization (Table 1). Additional recipient-minus isolates were not obtained at low kanamycin concentrations (2 to 10 μg/ml). The presence of plasmids R68 and R1162 was confirmed by agarose gel electrophoresis of DNA preparations from selected isolates (data not shown).

The rapid plate mating procedure was evaluated as a method for screening the capacity of bacteria for conjugal plasmid transfer by rescreening all R68 recipient-minus isolates for R68 transfer by the filter mating procedure. Of the 42 isolates examined, no additional recipients were identified by this procedure.

Transfer frequencies were compared by three techniques: broth, plate, and filter mating (Table 2). R68 transfer was not detected by the broth mating procedure in 15 of the 24 previously identified recipients. A solid surface (plate mating or filter mating) yielded the highest transfer frequencies. Filter mating frequencies were at least 10-fold greater than plate mating frequencies for 11 of the 26 recipient-active isolates. In contrast, plate mating frequencies were at least 10-fold greater than filter mating frequencies in only three of the recipient-active isolates. For the remaining 12 recipient-active isolates, the plate and filter mating frequencies were within a factor of 10 of each other.

DISCUSSION

Walter et al. (11) reported that, although their membrane filtration method produced transconjugants most consistently, no single mating technique was completely reliable in detecting transconjugants. The data we present, however, suggest that a simple and rapid spot mating technique, which allowed for phenotypic expression before challenge with selective antibiotics, proved completely reliable for testing the capacity of natural isolates to participate in conjugal plasmid transfer.

TABLE 2. Comparison of three methods of assessing conjugal plasmid transfer to aquatic bacteria

Strain	Frequency[a] of R68 transfer by:		
	Broth mating	Plate mating	Filter mating
Pseudomonas			
CYM318	1.9×10^{-5}	7.5×10^{-4}	9.3×10^{-3}
EFL1301	ND[b]	2.0×10^{-4}	2.0×10^{-4}
EFL6001	6.2×10^{-9}	3.9×10^{-6}	4.7×10^{-6}
PS1701	2.5×10^{-8}	2.0×10^{-8}	1.2×10^{-4}
PS2601	ND	3.8×10^{-6}	1.1×10^{-3}
PS3101	ND	1.4×10^{-6}	1.0×10^{-6}
PS5301	ND	3.0×10^{-4}	2.2×10^{-5}
PS7701	ND	3.2×10^{-5}	4.7×10^{-5}
PS8901	2.0×10^{-8}	3.0×10^{-3}	2.5×10^{-2}
9MC3001	6.9×10^{-8}	1.1×10^{-5}	5.7×10^{-5}
WTL7101	ND	2.4×10^{-5}	3.4×10^{-5}
CL118	ND	2.0×10^{-6}	1.2×10^{-6}
Acinetobacter			
EFL3701	ND	5.0×10^{-5}	1.0×10^{-5}
9MC6001	ND	1.3×10^{-5}	8.7×10^{-5}
Achromobacter			
PS1101	5.1×10^{-7}	7.2×10^{-5}	6.3×10^{-4}
Alcaligenes			
PS3201	ND	9.0×10^{-7}	2.3×10^{-5}
PS6201	2.3×10^{-7}	3.1×10^{-5}	1.8×10^{-3}
EFL5701	ND	1.5×10^{-8}	2.1×10^{-6}
Moraxella-like sp.			
PS1501	2.0×10^{-8}	9.0×10^{-6}	1.5×10^{-4}
Chromobacterium			
CV101	7.3×10^{-8}	1.6×10^{-7}	4.1×10^{-4}
Unknown species			
PS4601	ND	6.9×10^{-4}	1.2×10^{-5}
PS7801	ND	5.3×10^{-4}	1.6×10^{-3}
PS8001	ND	2.1×10^{-4}	1.3×10^{-6}
WTL4101	7.0×10^{-7}	7.4×10^{-6}	2.9×10^{-4}
WTL8401	1.8×10^{-5}	7.7×10^{-4}	2.9×10^{-2}
WTL9401	ND	1.6×10^{-6}	1.3×10^{-4}

[a] Number of exconjugants per donor cell.
[b] ND, None detected; frequency less than 1.0×10^{-9}.

In our study, filter mating frequencies were at least 10-fold greater than plate mating frequencies for 11 of 26 recipient-active isolates. In contrast, only three recipient-active isolates gave plate mating frequencies greater than the filter mating frequencies, and the plate mating technique most likely generated disproportionately lower frequencies because the recipient and the faster-growing donor were enumerated before the mating mixture was spotted on the plates.

The relatively large percentage of aquatic isolates that were recipient active for R68 transfer (38%) and R1162 mobilization (15%) and the discovery of indigenous mobilizers in wastewater by McPherson and Gealt (7) suggest that a novel gene located on a transmissible genetic element might spread in an aquatic environment. This potential exists whether the gene is located on a conjugative or a nonconjugative plasmid. In addition, plasmid R68 possesses chromosomal mobilizing ability (5). Therefore, its transfer suggests a potential for the spread of chromosomal genes to the identified recipients. A preliminary study conducted by Schilf and Klingmuller (8) showed than 17.3% of their aquatic bacterial isolates were also recipient active for wide-host-range plasmids RP4 and pRD1. We found that 38% of our aquatic bacterial isolates were recipient active for the wide-host-range plasmid R68. The differences between the data of Schilf and Klingmuller (8) and the results reported here can be attributed to their use of (i) a different procedure to identify recipient-active isolates, (ii) a different donor (*E. coli*), (iii) different plasmids, and (iv) different recipients, mainly members of the family *Enterobacteriaceae*.

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