Plasmid Transfer between Strains of *Pseudomonas aeruginosa* on Membrane Filters Attached to River Stones

By MARK J. BALE, JOHN C. FRY* AND MARTIN J. DAY

Department of Applied Biology, University of Wales Institute of Science and Technology,
PO Box 13, Cardiff CF1 3XF, UK

(Received 12 May 1987; revised 8 August 1987)

A naturally occurring mercury-resistance, conjugative plasmid, designated pQM1, was isolated from a bacterial population on the surface of stones from a river using *Pseudomonas aeruginosa* as a recipient. This was a narrow-host-range plasmid [IncP-13; 165 MDa; Tra⁺, Hg^r, fluorescein mercuric acetate^r, merbromin^r, Phi(E79), UV^r] confined to some *Pseudomonas* spp. It was used to demonstrate transfer between bacteria on stones in laboratory microcosm experiments and *in situ*. Transfer occurred (3.3×10^{-1} to 6.8×10^{-9} per recipient) at all the temperatures used (6–20 °C), although frequencies were lower in the cold. Nutrient status also affected transfer frequency, rich conditions promoting transfer. The presence of competing bacteria in the natural epilithon lowered transfer frequencies, but when unscrubbed stones were heat treated, transfer was enhanced, perhaps because of nutrient release from the heated epilithon.

INTRODUCTION

Attempts have been made to show that plasmid transfer occurs in a variety of natural habitats. Many studies have demonstrated gene transfer in the mammalian gut and most have used plasmid-encoded antibiotic resistances in *Escherichia coli*. Transfer is most frequent during starvation, during antibiotic treatment, in very young and in gnotobiotic animals but is rarely observed in the normal gut populated by an undisturbed microflora (Freter, 1984). Plasmid transfer has also been shown to occur on plant surfaces between *Klebsiella* spp. on whole radish plants and between phytopathogenic *Pseudomonas* spp. on bean leaves (Lacey & Leary, 1975; Talbot *et al.*, 1980). In aquatic environments gene transfer has been demonstrated in containers but not when the bacteria are unenclosed. Transfer of plasmid-borne antibiotic resistance has been shown in membrane diffusion chambers suspended in waste water treatment plants (Altherr & Kasweck, 1982; Mach & Grimes, 1982) and dialysis sacs in freshwater (Grabow *et al.*, 1975; Gowland & Slater, 1984). These experiments were all done with pure cultures of enterobacteria and in only one instance were the competing indigenous microflora present (Grabow *et al.*, 1975). Unenclosed experiments are important if plasmid transfer in aquatic habitats is to be unequivocally demonstrated.

Thus the aim of the work described here was to isolate a natural plasmid and to study its transfer in laboratory microcosms and in unenclosed *in situ* experiments. We chose the epilithon because it has a dense population of closely spaced bacteria (Lock *et al.*, 1984) which are fixed and hence more likely to transfer plasmids by conjugation than other communities of aquatic bacteria. *Pseudomonas* spp. were used because these are very commonly occurring aquatic bacteria (Holder-Franklin *et al.*, 1978; Nuttall, 1982; Jones *et al.*, 1986) which often contain plasmids (Burton *et al.*, 1982).

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are shown in Table 1. The plasmids pMG1 and pMG25 were used in *P. aeruginosa* PU21 and PAO303 respectively. Standard plate count agar (PCA; Oxoid CM463) was used for routinely growing all the strains. Antibiotics were from Sigma and were

Table 1. *Bacterial strains used in this study*

Species	Strain	Genotype/phenotype	Reference/source*
<i>Pseudomonas aeruginosa</i>	PAO303	<i>arg-18 chl-2</i> FP ⁻	Isaac & Holloway (1972)
	PAO2002	<i>argH32 str-39 chl-2 rec-1</i>	Chandler & Krishnapillai (1974)
	PAO2002R	Prototrophic mutant	This study
	PU21	<i>ilvB112 leu-1 str-1</i> Rif ^r	Jacoby (1974)
<i>P. putida</i>	PaW85	Prototrophic pWW0 ⁻	Bayley <i>et al.</i> (1977)
	PP3	Prototrophic	Senior <i>et al.</i> (1976)
<i>P. fluorescens</i>	PMG1	Prototrophic	M. Mergeay, CEN/SCK, Mol, Belgium
	10038	Prototrophic	NCTC 10038
	AR41	Prototrophic	Epilithic isolate
<i>P. maltophilia</i>	10499	Methionine auxotroph	NCTC 10499
	AR27	Methionine auxotroph	Epilithic isolate
	10661	Prototrophic	NCTC 10661
<i>P. cepacia</i>	10689	Prototrophic	NCTC 10689
<i>P. fragi</i>			
<i>Escherichia coli</i>	K12	Prototrophic	Bachmann (1972)
<i>Proteus mirabilis</i>	PM61	Prototrophic	D. Stickler, UWIST, Cardiff, UK
<i>Klebsiella pneumoniae</i>	8172	Prototrophic	NCTC 8172, as <i>K. aerogenes</i>
<i>Rhizobium leguminosarum</i>	T83K3	—	Ruiz-Sainz <i>et al.</i> (1984)

*NCTC, National Type Culture Collection.

added as required after autoclaving as a solution of the salt. Rifampicin (100 mg l⁻¹) was dissolved in sterile distilled water containing 0.1 ml 5 M-NaOH; mercuric chloride (27.2 mg l⁻¹) was added as a 27.2 g l⁻¹ aqueous stock solution. *Pseudomonas* selective medium (PSM; Oxoid CM559) was used with a ceftrimide/nalidixic acid supplement (Oxoid SR102); PSM + 2 g streptomycin l⁻¹ was also used. Fluorescent *pseudomonads* were counted on Kings B medium after replica plating from an ampicillin/cycloheximide/chloramphenicol selective medium (Simon & Ridge, 1974). Minimal salts medium (B22) contained (g l⁻¹): K₂HPO₄, 12.5; KH₂PO₄, 3.89; MgSO₄ · 7H₂O, 0.19; (NH₄)₂SO₄, 1.09 and Bacto-agar (Difco), 15. Sterile glucose solution (50%; w/v) was added (1 g l⁻¹) to molten B22 (50 °C) to give minimal salts + glucose (MG). Amino acids (100 mg l⁻¹) were added when needed as filter-sterilized solutions. Unless otherwise stated liquid cultures were grown in 10 ml nutrient broth (Oxoid CM3) at 30 °C for 18 h in an orbital incubator and all viable counts were at 30 °C for 24 or 48 h.

Laboratory mating experiments on agar. Samples of donor (0.2 ml) and recipient (1.8 ml) were mixed in sterile universal bottles. The mating mixture was immediately filtered through a 0.22 µm pore size, 25 mm diameter cellulose acetate membrane filter. The filters were placed face up on a PCA plate using sterile filter forceps and incubated (24 h) at the required temperature. The filter was then transferred to 10 ml one-tenth-strength nutrient broth (NB/10) and vortex mixed for 2 min. The sample was serially diluted in NB/10 and viable counts were done by a drop-counting method, using three 20 µl drops per dilution. In addition, 100 µl spread plates of the highest dilutions were used to count transconjugants. Viable counts were made of donors and recipients on the appropriate selective media, MG for prototrophs, MG + methionine for *P. maltophilia* and MG + aginine for PAO303. Rifampicin-resistant mutants were counted on PCA + rifampicin. Transconjugants were counted using the recipient selective agar with HgCl₂ (27.2 mg l⁻¹).

To determine the plate-to-broth mating ratio (Bradley, 1983), matings were begun as described above but the broth matings were not filtered. The broth matings and a control filter mating were incubated for 5 h before being counted as described above.

Isolation of the natural plasmid. Typical flat, smooth stones (pennant sandstone; about 10 × 15 × 2 cm) were taken from the River Taff, an organically polluted river in South Wales. The bacteria in the epilithon were removed by scrubbing with a sterile nylon toothbrush in 100 ml sterile distilled water (Burton *et al.*, 1982). Matings were done by mixing 7.8 × 10⁷ epilithic bacteria, estimated by acridine orange direct count (Burton *et al.*, 1982), with 1.9 × 10⁸ PAO2002R, estimated by viable count on PCA, and depositing the mixture onto a 0.22 µm cellulose acetate membrane filter (25 mm diameter) which was incubated (20 °C, 24 h) on PCA. The filter was removed and vortex mixed in 10 ml NB/10, and presumptive recipients were grown on PSM + streptomycin + HgCl₂ (27.2 mg l⁻¹). The identity of 100 of the recipients was confirmed by growth at 42 °C on acetamide, histidine (Day *et al.*, 1975), PSM and PSM + streptomycin. Additional confirmation was provided by biochemical tests (API20B). Confirmed recipients were screened for plasmids by agarose gel electrophoresis (Rochelle *et al.*, 1986), using

Rhizobium leguminosarum T83K3 (Ruiz-Sainz *et al.*, 1984) as an additional molecular mass standard because of the several large plasmids it contained (100–310 MDa). The presence of conjugative plasmids was shown by transfer into PU21 using the filter mating method. The one conjugative plasmid isolated by this technique was designated pQM1.

Tests for pQM1-encoded characteristics: mercury resistance. Organomercurials and salts were dissolved in sterile distilled water and added aseptically to PCA to give the required final concentrations, which were just above the minimum inhibitory concentration for plasmid-free bacteria. These were as follows (mg l^{-1}): Hg^+ (as $\text{Hg}_2(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$; 56.1), Hg^{2+} (as HgCl_2 ; 27.2), phenylmercuric acetate (168), fluorescein mercuric acetate (85.0), merbromin (375) and thimerosal (40.5). The media were inoculated, with a multipoint inoculator, from a broth culture of PAO2002R(pQM1) and PU21(pQM1).

Phage plating characteristics. To test for plasmid-encoded restriction systems and/or a phage inhibition phenotype, phage E79 were enumerated using the overlay method (Willett, 1984). Lysates (about 1×10^{12} p.f.u. ml^{-1}) were prepared from PU21 and PU21(pQM1) and phage were enumerated on both hosts.

UV resistance. The UV-resistance phenotype was determined (Jacoby, 1974) by irradiation with a UV transilluminator (1.3 mW cm^{-2} at 302 nm) using a dilution series of PU21, with and without pQM1 or pMG1, spread on PCA. After irradiation plates were incubated in the dark to determine resistance.

Microcosm experiments. In all these and subsequent experiments matings were done using PAO2002R(pQM1) as donor (0.2 ml) and PU21 as recipient (1.8 ml), which were mixed and filtered as described for laboratory matings. The membrane was placed face down on the surface of a sterile scrubbed stone fragment (about $3 \times 5 \times 1.5 \text{ cm}$), covered with a 47 mm glass fibre filter and secured with rubber bands. The stone was then placed in a sterile plastic beaker and covered with 300 ml sterile PCA broth (yeast extract, 2.5 g l^{-1} ; Bacto-casitone, 5 g l^{-1} ; glucose, 1 g l^{-1}), B22 salts medium or River Taff water (TW) collected in sterile glass bottles 1 h before use. The beaker was then sealed with sterile polythene and incubated (6°C or 20°C , 24 h) in the dark without aeration. To sample the microcosms, the membrane filter was placed into 10 ml NB/10 and vortex mixed for 2 min. The stone was placed in a sterile polythene stomacher bag with NB/10 (10 ml) and the surface where the filter had lain was scrubbed with a sterile toothbrush for 30 s. The sample was then placed in a sterile bottle and vortex mixed for 30 s. The liquid phase was sampled by scrubbing the inside surface of the beaker, agitating the rubber bands and the glass fibre filter and then removing a 1 ml sample. All samples were stored at 4°C for a maximum of 3 h before use. Each sample was counted as described for agar matings, using $\text{MG} + 500 \mu\text{g streptomycin l}^{-1}$ for the donors, PCA + rifampicin for the recipients and PCA + rifampicin + HgCl_2 for the transconjugants. Transconjugants from TW experiments at 6°C were counted using membrane filter counts: 1, 10 or 100 ml of sample was filtered through a $0.45 \mu\text{m}$ sterile cellulose nitrate membrane filter (47 mm diameter) and incubated on PCA + rifampicin + HgCl_2 .

Presumptive transconjugants were confirmed by testing for the PU21 phenotype (Table 1) using PCA + rifampicin + HgCl_2 , MG, MG + isoleucine + leucine + valine and PCA + streptomycin (2 g l^{-1}). Colonies showing suitable phenotypes after growth were then tested for the presence of pQM1.

In situ methodology. Experiments with scrubbed stones were the same as described for microcosms with the following exceptions. Stones were placed directly in a beaker or on a wire mesh in a beaker, covered with fresh TW and then carried to the field site for incubation. Stones on wire mesh were removed from the beaker before being put in the Taff Feeder Canal. Transport took a maximum of 15 min. In the Taff Feeder Canal beakers and wire mesh supports were incubated in midwater and suspended by a nylon fishing line. In the River Taff stones were enclosed in nylon mesh (size $2 \times 2 \text{ cm}$) formed into a bag and tied to a stake in the river-bed. Temperatures given are averages of the minimum and maximum recorded. Recovered stones were transported in sterile plastic bags.

Unscrubbed stones were freshly collected from the River Taff and were either untreated (maintained in sterile plastic bags, 15°C), autoclaved (121°C , 15 min) and cooled rapidly or pasteurized in a sterile plastic bag by immersion in a water bath (65°C , 10 min).

Statistical methods. One-way or crossed analysis of variance followed by Duncan's test was used to compare differences between means using the SPSS-X computer package (Hull & Nie, 1981). All counts and frequencies were transformed by $\log_{10}x$ and percentages by $\arcsin(x/100)^{1/2}$, which ensured normality of distribution and homogeneity of variances. Minimum significant differences were calculated by the Tukey-Kramer method (Sokal & Rohlf, 1981). All significant differences are quoted for $P < 0.05$ unless otherwise stated.

RESULTS AND DISCUSSION

Plasmid isolation and characteristics

From the initial 100 presumptive recipients isolated after mating *P. aeruginosa* PAO2002R with epilithic bacteria only two were confirmed, and these were screened for plasmids by agarose gel electrophoresis. One recipient contained a large plasmid (named pQM1) and this was used in all subsequent experiments. The presence of this plasmid was confirmed by mating with

PU21, which gave mercury-resistant transconjugants at high frequency (1.2×10^{-1} per recipient), all of which contained pQM1. Procedures using two other strains of *P. aeruginosa* (PAO1 and PU21) and several other selective agents (*a*, streptomycin, tetracycline, Pb^{2+} , Zn^{2+} , Co^{2+} , Cr^{6+} , Ag^{+} ; *b*, gentamicin, kanamycin, potassium tellurite) were unsuccessful. It was not possible to differentiate resistant colonies against the background growth for group (*a*), and plasmid transfer was not detected with group (*b*), or to the other strains of *P. aeruginosa*. The isolation procedure used here is novel because a plasmid was transferred in a single step, without the use of overt selective pressure during mating, from a natural mixture of epilithic bacteria to a plasmid-free recipient. This contrasts with conventional methods in which conjugative plasmids are invariably isolated by screening bacteria on selective plates, with or without pre-enrichment, followed by transfer to another strain. Mercury resistance was chosen as the selective phenotype because it is often plasmid encoded (Kelly & Reanney, 1984) and gave good discrimination between sensitive and resistant phenotypes on our selective media. The isolation was carried out at 20 °C to bias selection for a plasmid capable of transferring at environmentally relevant temperatures. It is likely that this method will preferentially select plasmids which transfer at high frequency and which are immune to the restriction systems of the recipient (Holloway, 1969).

Before plasmid transfer was investigated further, a range of potential phenotypes commonly encoded by plasmids was examined. These tests showed that pQM1 carried genes conferring resistance to Hg^{+} , Hg^{2+} , fluorescein mercuric acetate and merbromin, which indicated a typical narrow-spectrum phenotype (Clark *et al.*, 1977). The comparison of pQM1 with pMG1, a UV-resistance plasmid (Jacoby, 1974), showed that pQM1 also conferred UV-resistance. Stony rivers are nearly always shallow and swift, thus sunlight can easily penetrate to the epilithon on the river bed. Bacteria carrying UV-resistance genes, like those on pQM1, will be more tolerant of the high illumination than *E. coli* (Gameson & Saxon, 1967). It is possible that the UV^r phenotype provides a selective pressure for pQM1 in the epilithon as the levels of mercury in the River Taff are low (Welsh Water Authority, personal communication).

The efficiency of plating (e.o.p.) of phage E79 was influenced by the presence of pQM1. Lysates of E79 (1.4×10^{11} p.f.u. ml⁻¹) prepared on PU21 and plated on PU21(pQM1) showed an e.o.p. of 9.3×10^{-3} . This reduction in titre could not have been due to a plasmid-encoded restriction/modification system (Jacoby & Sutton, 1977) because another lysate of E79 (1.1×10^{12} p.f.u. ml⁻¹) grown and plated on PU21(pQM1) also showed a substantial reduction in the e.o.p. (5.2×10^{-3}). This Phi(E79) phage inhibition phenotype was similar to that seen for G101 and the derepressed IncP-10 plasmid, R91-5 (Chandler & Krishnapillai, 1977).

The host range of pQM1 was examined by filter matings using PU21 donors. No transfer was detected to *E. coli* K12, *Proteus mirabilis* PM61, *Klebsiella pneumoniae* NCTC 8172 and *P. fragi* NCTC 10689 (lowest detectable frequency $\leq 3.6 \times 10^{-7}$ per recipient). pQM1 did transfer to *P. aeruginosa* PAO2002R (5.5×10^{-1}), *P. putida* PaW85 (4.3×10^{-3}) and PP3 (2.0×10^{-2}), *P. fluorescens* PMG1 (4.6×10^{-4}), NCTC 10038 (9.0×10^{-5}) and AR41 (4.5×10^{-3}), *P. maltophilia* AR27 (3.6×10^{-6}) and NCTC 10499 (4.0×10^{-8}) and *P. cepacia* NCTC 10661 (2.7×10^{-7}). Thus pQM1 is a narrow-host-range *Pseudomonas* plasmid. The ratio of transfer frequency from membrane and broth matings was 1.87 and suggests that pQM1 encodes for thick, flexible pili (Bradley, 1983). Thus pQM1 transfers almost as well in broths as on surfaces, unlike many plasmids (e.g. RP1, R2 and R91; Bradley, 1983). The size of pQM1 was estimated by gel electrophoresis at 165 ± 12 MDa ($n = 9$). The incompatibility group of pQM1 was determined using IncP-1, P-4, P-5, P-6, P-7, P-9, P-10, P-11 and P-13 plasmids. Of these, pQM1 was only incompatible with the IncP-13 group plasmid (pMG25; Jacoby, 1980) and it was thus assigned to the IncP-13 group.

Transfer experiments on sterile scrubbed stones

Laboratory microcosm experiments. Studies of plasmid transfer with enclosed experiments in aquatic habitats have found transfer frequencies to be lower than those observed in laboratory experiments (Grabow *et al.*, 1975; Altherr & Kasweck, 1982; Mach & Grimes, 1982). Consequently, it was thought necessary to perform preliminary laboratory microcosm

Table 2. *Effect of temperature and suspending medium on transfer frequencies of pQM1 between P. aeruginosa strains on sterile scrubbed stones incubated in laboratory microcosms*

Mating experiments using PAO2002R(pQM1) donors and PU21 recipients were done on filters on PCA plates or on scrubbed stones in three different suspending media in simple microcosms for 24 h. Details of sampling and counting procedures are given in Methods. Control matings on PCA agar plates gave pQM1 transfer frequencies of 2.8×10^{-1} at 20 °C and 1.6×10^{-5} at 6 °C. Minimum significant difference ($\log_{10}x$) = 0.56; average coefficient of variation between replicate experiments = 8.7%.

Incubation temp. (°C)	Suspending medium	No. of replicate experiments	Mean transfer frequency per recipient from the		
			Filter	Stone	Liquid phase
20	PCA broth	2	3.8×10^{-2}	$3.3 \times 10^{-1}\dagger$	$1.9 \times 10^{-1}\dagger$
	B22 salts	2	9.3×10^{-3}	1.3×10^{-2}	1.1×10^{-2}
	R. Taff water	1	1.9×10^{-3}	7.5×10^{-3}	1.1×10^{-2}
6	PCA broth	3	3.4×10^{-5}	1.0×10^{-5}	4.4×10^{-6}
	B22 salts	1	1.0×10^{-5}	5.7×10^{-6}	$< 3 \times 10^{-6}\ddagger$
	R. Taff water	2	$1.6 \times 10^{-6}*$	$1.1 \times 10^{-6}*$	$< 5 \times 10^{-9}\ddagger$

* Significantly lower than all other frequencies.

† Significantly higher than all other frequencies.

‡ Transfer not detected.

experiments simulating natural conditions to ensure that the methodology used would work *in situ*. In all subsequent transfer experiments the bacteria [*P. aeruginosa* PAO2002R(pQM1) and PU21] were deposited on sterile membrane filters and incubated face downwards on stones from the River Taff. To mimic nature, the mean number of donors on the membrane filters was $5.3 \times 10^7 \text{ cm}^{-2}$, which was similar to the total bacterial count of the natural epilithon (acridine orange direct count $1.6 \pm 1.5 \times 10^8 \text{ cm}^{-2}$; viable count on PCA $5.1 \times 10^6 \text{ cm}^{-2}$; viable count of fluorescent pseudomonads $1.9 \times 10^3 \text{ cm}^{-2}$). The chance of detecting transfer was maximized by using a recipient-to-donor ratio of 12:1.

These microcosm experiments showed (Table 2) that the transfer frequency of pQM1 was significantly higher ($P < 0.001$) in the complex medium than when the stones were incubated in TW. The results for the mineral salts medium were intermediate. Contact with the stones had no effect on transfer as similar matings on agar were not significantly different from the microcosm results using Duncan's test. With all the suspending media, transfer at 6 °C was at least 1000-fold less than transfer at 20 °C. The direct contact between the membrane filter and the sterile stone clearly resulted in adherence of organisms to the stone surface, as donors, recipients and transconjugants were present on the stones after incubation (Tables 2 and 3). *P. aeruginosa* is motile and had therefore moved from the filters and stones into the liquid phase in all the incubations. After incubation at 20 °C significantly more donor and recipient cells were found in the liquid phase than were originally put on the membrane filters (Table 3), hence growth had occurred. However, at 6 °C the results indicated that cells had become non-recoverable during incubation. The transfer frequency in the liquid phase was very low at 6 °C (Table 2), despite there being more donors and recipients present than on the filter or stone. This implies that movement of the bacteria into the liquid medium occurred before mating took place and so most plasmid transfer occurred on the stone and filter surfaces.

In situ experiments. Transfer experiments were carried out *in situ* that were comparable to the microcosm experiments. Stones with attached membrane filters, inoculated with donors and recipients, were put into open-topped beakers submerged in the Taff Feeder Canal. This canal receives its water from the River Taff, but has a much lower flow rate than the main river and so is ideal for experimental use. Transfer of pQM1 occurred in these beakers at environmental temperatures, but the frequencies were significantly higher at 11–18 °C than at 6–8 °C (Table 4).

Table 3. *Effect of temperature and suspending medium on the distribution of donors and recipients in transfer experiments between P. aeruginosa strains with pQM1 on sterile scrubbed stones incubated in laboratory microcosms*

These results are from the same experiments as reported in Table 2. Details of the media used to count the donors and recipients are given in Methods. The viable counts per filter at 6 °C were: donors, 2.5×10^8 ; recipients, 2.3×10^9 . Those at 20 °C were donors, 1.7×10^8 ; recipients, 5.4×10^9 . Figures with the same letters are significantly different (the comparisons are only for the three samples within each suspension medium). Minimum significant difference ($\log_{10}x$) = 2.03.

Incubation temp. (°C)	Suspending medium	No. of donors expressed as % of no. on filter before incubation			No. of recipients expressed as % of no. on filter before incubation		
		Filter	Stone	Liquid phase	Filter	Stone	Liquid phase
20	PCA broth	0.9 ^a	7.3 ^a	282.8 ^a	0.4 ^g	3.4 ^f	351.8 ^g
	B22 salts	8.3 ^b	57.2 ^c	1128.6 ^{bc}	2.3 ^h	11.7	74.1 ^h
	R. Taff water	0.8 ^d	6.6 ^e	431.4 ^{de}	0.6 ⁱ	4.7	100.0 ⁱ
6	PCA broth	3.0 ^k	7.8	33.1 ^k	4.0 ^l	9.6	29.8 ^l
	B22 salts	5.3	17.3	52.8	6.3	22.2	38.2
	R. Taff water	2.9	16.3	17.8	0.8 ^m	5.3	7.3 ^m

Table 4. *Transfer frequencies of pQM1 between P. aeruginosa strains on sterile scrubbed stones incubated in the Taff Feeder Canal and River Taff*

The mating experiments using PAO2002R(pQM1) donors and PU21 recipients were done on filters held on sterile scrubbed stones supported in a beaker or wire basket and incubated in the Taff Feeder Canal or unsupported in the River Taff for 24 h. Full details are given in Methods. Minimum significant difference ($\log_{10}x$) = 0.34; average coefficient of variation between replicate experiments = 2.27%.

Site and support used for the stones	Average canal or river water temp. over 24 h (°C)	Transfer frequency per recipient from the	
		Filter	Stone
Taff Feeder Canal; plastic beaker	6	$< 1 \times 10^{-8}$ *	6.8×10^{-9}
	7	1.2×10^{-7}	9.8×10^{-9}
	8	9.4×10^{-8}	1.1×10^{-7}
	11	3.0×10^{-6}	1.1×10^{-5}
	12	2.4×10^{-6}	4.2×10^{-6}
	18	5.2×10^{-5}	3.3×10^{-4}
Taff Feeder Canal; wire mesh	8	1.8×10^{-7}	2.0×10^{-7}
	11	2.3×10^{-6}	5.4×10^{-6}
River Taff; unsupported	18	1.1×10^{-3}	2.8×10^{-4}

* Transfer not detected.

Transfer was also observed in experiments in which the stones were incubated on wire-mesh supports, but frequencies were not significantly different from corresponding beaker supports ($P = 0.83$). This indicates that conjugation still occurred when stones were open to the canal's full flow. At 18 °C there were significant differences between the filter transfer frequencies in the River Taff and those in the Feeder Canal (Table 4) but there were no significant differences in the transfer frequencies in the stone sample. This may reflect an increase in aeration beneath the filter in the higher flow conditions of the River Taff.

As the transfer frequencies were much lower in these *in situ* experiments compared with those obtained in the laboratory microcosms (Table 2) it was necessary to perform some control experiments in case false positive results were occurring. The transfer frequency obtained from a single control experiment in Taff Feeder Canal water at 11 °C during transit to and from the field site was 1.84×10^{-8} per recipient. This was at least 100 times less than the result from the corresponding field experiment. The mutation rate to mercury resistance of the recipient (tested during each experiment) was also very low ($< 8.3 \times 10^{-10}$).

The maximum transfer frequency of pQM1 was very high (3.3×10^{-1} per recipient). However, precise comparison with other results is impossible because many different ways of calculating transfer frequencies and performing the experiments are used (Beringer, 1974; Altherr & Kasweck, 1982; Bradley, 1983; Kelly & Reanney, 1984). The high transfer rate of this plasmid made it ideal for use in our ecological experiments because even when the natural aquatic conditions depressed the transfer frequency (minimum observed frequency = 6.8×10^{-9} per recipient), transconjugants were still detectable.

Our results clearly show that plasmid transfer occurs at all temperatures examined (6–20 °C) but is lowest under cold conditions (6–8 °C) and highest at elevated temperatures (11–20 °C). This effect was noticeable in both laboratory microcosms and the *in situ* experiments. The effect of temperature on the transfer frequency of several plasmids has been reported by others. The *E. coli* plasmid R1drd-19 transferred at 17 °C over a 24 h period but not at 15 °C in 48 h (Singleton & Anson, 1981). A mercury-resistance plasmid from a marine pseudomonad transferred to *E. coli* at 25 °C but not at 16 °C (Gauthier *et al.*, 1985) and the plasmid pWK1 transferred between *Erwinia herbicola* strains at 12.5 °C in 24 h (Kelly & Reanney, 1984) but not at 10 °C (Reanney *et al.*, 1982). From this and other evidence it is generally concluded that plasmid transfer does not occur at low temperatures (Reanney *et al.*, 1982). However, two reports have been made of transfer at lower temperatures similar to those we have used. One reported transfer at 10.6 °C, between a range of enterobacteria (Mach & Grimes, 1982) and another (Timoney, 1981) showed a graph, with no experimental details, of IncFII and IncI α plasmids transferring at 4 °C. It therefore seems probable that transfer of plasmids between indigenous aquatic bacteria can occur at environmentally relevant temperatures in the epilithon. However, transfer frequencies will probably be lower in the colder waters of winter than in the warmer summer months.

Temperature has a second and unrelated effect which is shown in Tables 2 and 3. A large proportion of the donors, recipients and transconjugants move away from the filters and stone surface to the surrounding medium. This occurred at both temperatures examined although the effect was far less marked at 6 °C than at 20 °C. This difference implies that migration of plasmid-bearing cells into the river plankton will increase as the temperature rises.

Transfer experiments on unscrubbed stones

Further experiments were done using stones with the intact epilithon to see if conjugation would still occur in the presence of the full, competing, natural microflora (Table 5). Although transfer was not detected at 7 °C it clearly occurred at 11 and 12 °C in both laboratory and *in situ* experiments. The transfer frequencies were significantly lower than those obtained at similar temperatures using sterile scrubbed stones (Table 4). This was presumably due to competition from the natural microflora. When the natural epilithon was killed by heating, the transfer frequencies of pQM1 were significantly greater than those on untreated stones (Table 5) and were almost all significantly higher than those from sterile scrubbed stones (Tables 2 and 4). The enhancement of transfer on pasteurized stones was greater than that on autoclaved stones and this transfer frequency was only about 28 times less than the value obtained in a control laboratory experiment at 12 °C on PCA agar (9.2×10^{-3} per recipient). Consequently, it is probable that heating enhances transfer, by killing the natural competing microflora and by releasing nutrients from the polysaccharide matrix known to be present in the epilithon (Lock *et al.*, 1984). Thus, in addition to temperature, nutrient status appears to be a major factor influencing the rate of pQM1 transfer. These observations are supported by the work of Beringer (1974) who examined RP4 plasmid transfer between two *Rhizobium leguminosarum*

Table 5. *Transfer frequencies of pQM1 between P. aeruginosa strains on unscrubbed stones from the R. Taff incubated in laboratory microcosms and the Taff Feeder Canal*

The matings using PAO2002R(pQM1) donors and PU21 recipients were similar to those using sterile scrubbed stones. The stones were either untreated (Nil), heated at 65 °C for 10 min (Pasteurized) or heated at 121 °C for 15 min (Autoclaved). Full details are given in Methods. The experiment number indicates the results that were obtained on the same date. Minimum significant difference ($\log_{10}x$) = 0.41; average coefficient of variation between replicate experiments = 2.3%.

Experimental site and expt no.	Treatment	Temp. (°C)	Transfer frequency per recipient from the	
			Filter	Stone
Laboratory				
1	Nil	7	$<5 \times 10^{-9}$ *	$<5 \times 10^{-9}$ *
2	Nil	12	4.9×10^{-6}	5.0×10^{-7}
3	Nil	11	1.7×10^{-6}	4.9×10^{-7}
1	Autoclaved	7	2.2×10^{-6}	2.5×10^{-6}
2	Pasteurized	12	3.3×10^{-4}	7.8×10^{-4}
<i>In situ</i>				
3	Nil	11	6.3×10^{-7}	4.0×10^{-7}
3	Autoclaved	11	3.1×10^{-6}	7.2×10^{-7}
3	Pasteurized	11	7.8×10^{-6}	3.1×10^{-5}

* Transfer not detected.

strains. He showed that reductions in the concentrations of nutrient broth or glycine (50–0%) in the mating media lowered the frequency of transfer between 100- and 300-fold.

The overall conclusion from this work is that plasmid transfer is possible between bacteria in the river epilithon. Although this is the first such demonstration, the experiments were performed on membrane filters and so were not exactly like nature. Further work should aim at detecting transfer between organisms actually incorporated into the epilithon. Despite these shortcomings our approach is the only method currently available which can be used as a model system for studying conjugation in aquatic habitats. If pQM1 and the pseudomonad host strains used are typical of indigenous epilithic bacteria, then plasmids will spread rapidly throughout riverine bacterial populations. The overall effect of temperature will be to enhance plasmid transfer throughout rivers in summer and depress it in winter. So it is probable that little transfer occurs during winter in the River Taff when temperatures are below about 6 °C. However, as pQM1 was isolated at 20 °C and transfers more readily at 20 °C than at 6 °C, it is likely that plasmids exist in nature that transfer well in winter but that these need to be isolated at low temperatures. Although the River Taff is organically polluted (Mawle *et al.*, 1985) the sites investigated were not near organically rich effluents. Consequently it is also probable that plasmid transfer will be greatest where available organic substrate concentrations are highest, such as directly below sites of organic enrichment.

We are grateful to the Natural Environment Research Council for the support of one of us (M.J.B.) with a research studentship. We would also like to thank Professor J. E. Beringer, Dr G. A. Jacoby, Dr M. Mergeay, Dr D. J. Stickler and Mr P. Rochelle for supplying bacterial strains and plasmids.

REFERENCES

- ALTHERR, M. R. & KASWECK, K. L. (1982). *In situ* studies with membrane diffusion chambers of antibiotic resistance transfer in *Escherichia coli*. *Applied and Environmental Microbiology* **44**, 838–843.
- BACHMANN, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews* **36**, 525–557.
- BAYLEY, S. A., DUGGLEBY, C. J., WORSEY, M. J., WILLIAMS, P. A., HARDY, K. G. & BRODA, P. (1977). Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. *Molecular and General Genetics* **154**, 203–204.
- BERINGER, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188–198.
- BRADLEY, D. E. (1983). Specification of the conjugative pili and surface mating systems of *Pseudomonas* plasmids. *Journal of General Microbiology* **129**, 2545–2556.
- BURTON, N. F., DAY, M. J. & BULL, A. T. (1982).

- Distribution of bacterial plasmids in clean and polluted sites in a South Wales river. *Applied and Environmental Microbiology* **44**, 1026–1029.
- CHANDLER, P. M. & KRISHNAPILLAI, V. (1974). Isolation and properties of recombination-deficient mutants of *Pseudomonas aeruginosa*. *Mutation Research* **23**, 15–23.
- CHANDLER, P. M. & KRISHNAPILLAI, V. (1977). Characterization of *Pseudomonas aeruginosa* depressed R-plasmids. *Journal of Bacteriology* **130**, 596–603.
- CLARK, D. L., WEISS, A. A. & SILVER, S. (1977). Mercury and organomercurial resistances determined by plasmids in *Pseudomonas*. *Journal of Bacteriology* **132**, 186–196.
- DAY, M. J., POTTS, J. & CLARKE, P. H. (1975). Location of genes for the utilisation of acetamide, histidine and proline on the chromosome of *Pseudomonas aeruginosa*. *Genetical Research* **25**, 71–76.
- FRETER, R. (1984). Factors affecting conjugal plasmid transfer in natural bacterial communities. In *Current Perspectives in Microbial Ecology*, pp. 105–114. Edited by M. J. Klug & C. A. Reddy. Washington, DC: American Society for Microbiology.
- GAMESON, A. L. H. & SAXON, J. R. (1967). Field studies on effect of daylight on mortalities of coliform bacteria. *Water Research* **1**, 279–295.
- GAUTHIER, M. J., CAUVIN, F. & BREITMAYER, J. P. (1985). Influence of salts and temperatures on the transfer of mercury resistance from a marine pseudomonad to *Escherichia coli*. *Applied and Environmental Microbiology* **50**, 38–40.
- GOWLAND, P. C. & SLATER, J. H. (1984). Transfer and stability of drug resistance plasmids in *Escherichia coli* K12. *Microbial Ecology* **10**, 1–13.
- GRABOW, W. O. K., PROZESKY, O. W. & BURGER, J. S. (1975). Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. *Water Research* **9**, 777–782.
- HOLDER-FRANKLIN, M. A., FRANKLIN, M., CASHION, P., CORMIER, C. & WUEST, L. (1978). Population shifts in heterotrophic bacteria in a tributary of the Saint John River as measured by taxometrics. In *Microbial Ecology*, pp. 44–50. Edited by M. W. Loutit & J. A. R. Miles. Berlin: Springer Verlag.
- HOLLOWAY, B. W. (1969). Genetics of *Pseudomonas*. *Bacteriological Reviews* **33**, 419–443.
- HULL, C. H. & NIE, N. H. (1981) *SPSS Update: New Procedures and Facilities for Release 7.9*. New York: McGraw-Hill.
- ISAAC, J. H. & HOLLOWAY, B. W. (1982). Control of arginine biosynthesis in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **73**, 427–438.
- JACOBY, G. A. (1974). Properties of R plasmids determining gentamicin resistance by acetylation in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **6**, 239–252.
- JACOBY, G. A. (1980). Plasmid determined resistance to carbenicillin and gentamicin in *Pseudomonas aeruginosa*. In *Plasmids and Transposons: Environmental Effects and Maintenance Mechanisms*, pp. 83–96. Edited by C. Stutter & K. R. Rozee. London: Academic Press.
- JACOBY, G. A. & SUTTON, L. (1977). Restriction and modification determined by a *Pseudomonas* R plasmid. *Plasmid* **1**, 115–116.
- JONES, J. G., GARDENER, S., SIMON, B. M. & PICKUP, R. W. (1986). Antibiotic resistant bacteria in Windermere and two remote upland tarns in the English Lake District. *Journal of Applied Bacteriology* **60**, 443–453.
- KELLY, W. J. & REANNEY, D. C. (1984). Mercury resistance among soil bacteria: ecology and transferability of genes encoding resistance. *Soil Biology and Biochemistry* **16**, 1–8.
- LACEY, G. H. & LEARY, J. V. (1975). Transfer of antibiotic resistance plasmid RP1 into *Pseudomonas glycinea* and *Pseudomonas phaseolicola* in vitro and in planta. *Journal of General Microbiology* **88**, 49–57.
- LOCK, M. A., WALLACE, R. P., COSTERTON, J. W., VENTULLO, R. M. & CHARLTON, S. E. (1984). River epilithon: toward a structural functional model. *Oikos* **42**, 10–22.
- MACH, P. A. & GRIMES, D. J. (1982). R-plasmid transfer in a waste water treatment plant. *Applied and Environmental Microbiology* **44**, 1395–1403.
- MAWLE, G. W., WINSTONE, A. & BROOKER, M. P. (1985). Salmon and sea trout in the Taff – past, present and future. *Nature in Wales New Series* **4** (102), 36–45.
- NUTTALL, D. (1982). The populations, characterisation and activity of suspended bacteria in the Welsh River Dee. *Journal of Applied Bacteriology* **53**, 49–59.
- REANNEY, D. C., ROBERTS, W. P. & KELLY, W. J. (1982). Genetic interactions among microbial communities. In *Microbial Interaction and Communities*, vol. 1, pp. 287–322. Edited by A. T. Bull & J. H. Slater. London: Academic Press.
- ROCHELLE, P. A., FRY, J. C., DAY, M. J. & BALE, M. J. (1986). An accurate method for estimating sizes of small and large plasmids and DNA fragments by gel electrophoresis. *Journal of General Microbiology* **132**, 53–59.
- RUÍZ-SAINZ, J. E., CHANDLER, M. R., JUNENEZ-DÍAZ, R. & BERINGER, J. E. (1984). Transfer of a host range plasmid from *Rhizobium leguminosarum* to fast-growing bacteria that nodulate soybeans. *Journal of Applied Bacteriology* **57**, 309–315.
- SENIOR, E., BULL, A. T. & SLATER, J. H. (1976). Enzyme evolution in a microbial community growing on the herbicide Dalapon. *Nature, London* **263**, 476–479.
- SIMON, A. & RIDGE, E. H. (1974). The use of ampicillin in a simplified selective medium for the isolation of fluorescent pseudomonads. *Journal of Applied Bacteriology* **37**, 459–460.
- SINGLETON, P. & ANSON, A. E. (1981). Conjugal transfer of R-plasmid R1drd-19 in *Escherichia coli* below 22 °C. *Applied and Environmental Microbiology* **42**, 789–791.
- SOKAL, R. R. & ROHLF, F. J. (1981). *Biometry*, 2nd edn. San Francisco: Freeman.
- TALBOT, H. W., YAMAMOTO, D. K., SMITH, M. W. & SEIDLER, R. J. (1980). Antibiotic resistance and its transfer among clinical and non-clinical *Klebsiella* strains in botanical environments. *Applied and Environmental Microbiology* **39**, 97–104.
- TIMONEY, J. F. (1981). R-plasmids in pathogenic Enterobacteriaceae from calves. In *Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids*, pp. 547–555. Edited by S. B. Levy, R. C. Clowes & E. L. Koenig. London: Plenum Press.
- WILLETTTS, N. (1984). Conjugation. *Methods in Microbiology* **17**, 33–60.