Factors Affecting Conjugal Transfer of Plasmids Encoding Mercury Resistance from Pure Cultures and Mixed Natural Suspensions of Epilithic Bacteria

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Sixty-five pure cultures of epilithic bacteria were examined for their ability to transfer mercury resistance to *Pseudomonas aeruginosa*; five isolates transferred plasmids encoding mercury resistance with frequencies ranging from 8.4×10^{-8} to 2.8×10^{-3} per recipient. Two of the plasmids, pQM3 and pQM4, encoded narrow-spectrum mercury resistance, pQM3 also encoded streptomycin resistance, and both plasmids were broad host range. Maximum transfer frequencies of epilithic plasmids from pure cultures occurred over the range 10–25 °C at 3.5 g C l⁻¹ and with donor to recipient ratios of 0.4–30. Transfer occurred over a range of pH values (pH 5.0–8.0) but the effect of pH was most significant at non-optimal temperature. Anaerobiosis inhibited transfer of one epilithic plasmid, pQM1, but not that of pQM3. Plasmids encoding mercury resistance were also transferred from mixed natural suspensions of epilithic bacteria (MNS) to *Pseudomonas* spp. on agar in the laboratory. Transfer from MNS occurred over a wide range of environmentally relevant conditions with maximum frequencies (2×10^{-5} per recipient) after 24 h, at 25 °C, pH 5.5–8.0 and on a medium containing 10 g C l⁻¹. The optimal initial cell density of MNS and recipient was 1.7×10^5 c.f.u. cm⁻² and highest frequencies were obtained with donor to recipient ratios ranging from 1.2×10^{-1} to 1.7×10^{-3} . Most of the plasmids (54%) from MNS transferred from their original *P. aeruginosa* transconjugants to a *Pseudomonas putida* strain, with frequencies ranging from 1.1×10^{-6} to greater than 1.0×10^{-1} per recipient. The majority (80%) of the plasmids were larger than 300 kb and all of these large plasmids encoded UV resistance in addition to mercury resistance. Twenty-one plasmids > 300 kb were analysed by restriction digests and were shown to be similar, with only minor structural alterations. One of these alterations was associated with the acquisition of streptomycin resistance. Overall, these results suggest that the epilithic bacteria examined possess the potential to transfer mercury resistance within the epilithon under a wide range of environmentally relevant conditions.

INTRODUCTION

Plasmids have been detected in bacteria isolated from a variety of aquatic environments. Frequencies of plasmid incidence in marine isolates range from 23% to 46% (Glassman & McNicol, 1981; Hada & Sizemore, 1981; Simon *et al.*, 1982; Kobori *et al.*, 1984). A survey of plasmids in freshwater epilithic bacteria (Burton *et al.*, 1982) showed that 10–15% of isolates contained at least one plasmid, depending on whether the samples were from unpolluted or polluted sites.

Bacteria isolated from water have been shown to transfer plasmids in pure-culture matings (Shaw & Cabelli, 1980; Toranzo *et al.*, 1984; Gauthier *et al.*, 1985) and a number of reports have

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Abbreviation: MNS, mixed natural suspensions of epilithic bacteria.

investigated the influence of various factors on conjugal plasmid transfer. The effects of temperature and pH have been studied (Shenderov, 1971; Singleton & Anson, 1981, 1983; Altherr & Kasweck, 1982; Kelly & Reaney, 1984; Gauthier *et al.*, 1985) but few reports have dealt with the effects of mating time, nutrient status, or donor to recipient ratio (Beringer, 1974; Gauthier *et al.*, 1985), and reports on the effect of anaerobiosis on conjugation have been conflicting (Anderson, 1975; Burman, 1975; Graves & Riggs, 1980). Most of these studies used pure cultures of bacteria: genetic work with mixed natural communities is rare (Bale *et al.*, 1988).

The epilithon of freshwater rivers contains a dense population of closely spaced, fixed bacteria (Lock *et al.*, 1984), which makes it an ideal community for studying plasmid transfer in aquatic environments. The aim of this work was to isolate epilithic bacteria containing conjugative plasmids and to determine the optimal conditions for transfer of these plasmids to *Pseudomonas* strains, which are commonly-occurring aquatic bacteria (Nuttall, 1982; Jones *et al.*, 1986). We also undertook to determine the optimal conditions for the transfer of plasmids from mixed natural suspensions of epilithic bacteria (MNS), with the aim of understanding the environmental factors most likely to determine the rates of plasmid transfer in natural aquatic habitats. The transferred plasmids were then characterized further to discover whether one particular type was transferred most often. Work in this laboratory has already demonstrated the presence of a conjugative plasmid encoding mercury resistance in the epilithon (Bale *et al.*, 1987, 1988), so the mercury-resistance phenotype was also used in this study.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are shown in Table 1. All strains were routinely maintained on standard plate count agar (PCA; Oxoid CM463) containing appropriate selective agents and incubated at 30 °C. Rifampicin-resistant mutants of these strains were used as recipients in mating experiments. Liquid cultures were grown in 10 ml nutrient broth (NB; Oxoid CM3) for 18 h in an orbital incubator at 30 °C, except for epilithic isolates, which were incubated at 20 °C.

Isolation of epilithic strains and preparation of epilithic suspensions. Stones were collected from a shallow area in the lower reaches of the organically polluted River Taff, South Wales (Mawle *et al.*, 1985). Epilithic suspensions were prepared by scrubbing the stones with a sterile toothbrush in 100 ml sterile distilled water followed by stomaching (Burton *et al.*, 1982). This procedure gave the MNS used subsequently. Viable counts were performed on PCA incubated at 20 °C for 4 d. Mercury-resistant strains were isolated by plating serial dilutions of epilithic suspension onto PCA + HgCl₂ (27 µg ml⁻¹), and pseudomonad-like mercury-resistant strains by plating onto a pseudomonad selective medium (PSM), made from pseudomonas agar base (Oxoid CM559) with a cetrimide/nalidixic acid supplement (Oxoid SR102) + HgCl₂ (27 µg ml⁻¹). Plates were incubated at 20 °C for 4 d. Epilithic isolates were identified using API20B kits (API Laboratory Products) designed for aerobic, heterotrophic bacteria, and a probabilistic identification matrix for Gram-negative, aerobic, non-fermentative bacteria (Holmes *et al.*, 1986).

Plasmid detection. Plasmids were detected using the method of Kado & Liu (1981) with the modifications of Rochelle *et al.* (1986). Plasmid sizes were determined using the size standards and multiple regression method described by Rochelle *et al.* (1986). In our laboratory this method gave average 95% confidence intervals of ±4.4% of the mean plasmid size.

Detection of transferable mercury resistance in epilithic isolates and other pure cultures. Mercury-resistant and pseudomonad-like mercury-resistant isolates were screened for the presence of plasmids. Sixty-five of these isolates containing plasmids ≥30 kb were used as donors in mating experiments with a variety of *P. aeruginosa* strains (PAO2002, PU21, PAO348) as recipients. Equal volumes (1 ml) of donor and recipient NB cultures were mixed and filtered through 0.22 µm cellulose acetate membrane filters (25 mm diam.; Oxoid N25/22UP). The filters were incubated face up on the surface of PCA plates for 24 h at 20 °C, then the cells on the filter were resuspended by vortex mixing in 10 ml of 1/10-strength NB and serial dilutions were plated onto selective media. Recipient counts were performed on PCA + rifampicin (100 µg ml⁻¹) and transconjugant counts on PCA + rifampicin (100 µg ml⁻¹) + HgCl₂ (27 µg ml⁻¹). Selective plates were incubated at 20 °C for 4 d. The presence of plasmids in the transconjugants was confirmed by gel electrophoresis of DNA samples.

Detection of plasmid transfer from epilithic suspensions. The MNS (1 ml) was mixed with 1 ml of a broth culture of recipient bacteria and poured onto the surface of a very dry PCA plate. The liquid rapidly soaked into the agar. The incubation conditions generally used for these mating plates were 20–25 °C, 24 h, pH 7.0, on PCA plates (3.2 g C l⁻¹). After incubation, the growth on the plates was harvested, resuspended in 10 ml 1/10-strength NB and then plated onto selective media. PSM + rifampicin (100 µg ml⁻¹) was used for recipient counts and

Table 1. *Bacterial strains used in this study*

Species	Strain	Genotype/phenotype*	Reference/source†
<i>Pseudomonas aeruginosa</i>	PAO2002	<i>argH32 str-39 chl-2 rec-1</i>	Chandler & Krishnapillai (1974)
	PU21	<i>ilvB112 leu-1 str-1</i> Rif ^r	Jacoby (1974)
	PAO348	<i>met-28 trp-6 lys-12 pro-82 his-114 ilv-226 leu-13 hsdS20</i> ; FP ⁻	V. Krishnapillai (pers. comm.)
<i>P. putida</i>	PP3	Prototrophic	Senior <i>et al.</i> (1976)
	KT2440	<i>hsdR1 hsdM⁺</i> ; prototrophic	Bagdasarian <i>et al.</i> (1981)
	PAR153	Hg ^r ; prototrophic; P ⁺	Epilithic isolate
<i>P. fluorescens</i>	10038	Prototrophic	NCTC 10038
	AR41	Prototrophic; P ⁻	Epilithic isolate
	AR17	Hg ^r ; prototrophic; P ⁻	Epilithic isolate
<i>P. cepacia</i>	PAR161	Hg ^r Sm ^r ; prototrophic; P ⁺	Epilithic isolate
	PAR167	Hg ^r Sm ^r ; prototrophic; P ⁺	Epilithic isolate
<i>P. maltophilia</i>	10499	Met ⁻	NCTC 10499
	AR27	Met ⁻ P ⁻	Epilithic isolate
<i>Alteromonas</i> sp.	PAR174	Hg ^r ; prototrophic; P ⁺	Epilithic isolate
<i>A. putrefaciens</i>	10695	Prototrophic	ATCC 10695
<i>Escherichia coli</i>	J53	<i>proB22 metF63</i>	Bachmann (1987)
	LE392	<i>supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 hsdR514</i> ; λ^- F ⁻	Maniatis <i>et al.</i> (1982)
<i>Klebsiella pneumoniae</i>	8172	Prototrophic	NCTC 8172
<i>Proteus vulgaris</i>	6213	Prototrophic	NCIB 6213
<i>Acinetobacter calcoaceticus</i>	BD413	Prototrophic	NCIB 11826
Unidentified Gram-negative rod	PAR166	Hg ^r ; prototrophic; P ⁺	Epilithic isolate

* P⁺, contains at least one plasmid; P⁻, plasmid free.

† NCTC, National Type Culture Collection; NCIB, National Collection of Industrial Bacteria; ATCC, American Type Culture Collection.

PSM + rifampicin + HgCl₂ (27 µg ml⁻¹) for transconjugant counts. Selective plates were incubated at 30 °C for 48 h. Transfer of other resistance markers was also tested using the following selective agents in place of HgCl₂: potassium tellurite (127 µg ml⁻¹), streptomycin (300 µg ml⁻¹), kanamycin (400 µg ml⁻¹), and gentamicin (150 µg ml⁻¹). Liquid matings were performed by adding 1 ml MNS to 1 ml of a broth culture of recipient bacteria and incubating at 20 °C for 24 h before plating onto selective media. Transfer frequencies were expressed in all mating experiments as the mean number of transconjugants per recipient.

Confirmation and characterization of transconjugants. The identities of *P. aeruginosa* PAO348 transconjugants from MNS matings were confirmed by replica plating onto minimal salts agar (Bale *et al.*, 1987) + glucose (1 g l⁻¹) supplemented with different combinations of the following amino acids (100 µg ml⁻¹): methionine, tryptophan, lysine, histidine, proline, valine, leucine. Resistance markers other than mercury were determined by replica plating onto PCA containing individual antimicrobial agents (100 µg ml⁻¹): streptomycin, gentamicin, tetracycline, carbenicillin (1 mg ml⁻¹), potassium tellurite (127 µg ml⁻¹). *P. aeruginosa* PAO348 transconjugants were used as donors in pure-culture matings with *P. putida* KT2440 to determine the ability of the plasmids from MNS to re-transfer. To test for UV resistance, dilution series of plasmid-containing and plasmid-free strains were irradiated on the surface of a UV transilluminator (1.3 mW cm⁻² at 302 nm). After irradiation, all plates were incubated in the dark and resistance was determined by comparing the percentage survival of the strains.

Factors affecting conjugation. The standard incubation conditions for pure-culture matings were 20 °C, 24 h, pH 7.0, on PCA plates (3.5 g C l⁻¹). The amount of organic nutrients in PCA was calculated as g C l⁻¹ based on the percentage carbon in the individual nutrients of PCA. Different nutrient status regimes were attained by using plates containing varying amounts of PCA. Thus, 0 g C l⁻¹ was Bacto-agar (Difco 0140-01) with no added carbon in the form of PCA. The values do not take into account any available carbon that may have been present in the agar. The effects of temperature, pH, mating time, nutrient status, donor to recipient ratio, and anaerobiosis on plasmid transfer from epilithic isolates to *P. aeruginosa* were investigated. The basic mating protocol was as described above except that selective plates in experiments with pure cultures were incubated at 30 °C for 48 h. An epilithic plasmid encoding mercury resistance (pQM1, 254 kb; Bale *et al.*, 1987) and the antibiotic-resistance plasmid RP1 (Lowbury *et al.*, 1969) were also studied in the pure-culture experiments. *P. aeruginosa* PAO2002 was

used as the donor for pQM1 and RP1 and *P. aeruginosa* PU21 was the recipient. For broth matings, 1 ml volumes of NB cultures of donor and recipient bacteria were mixed and incubated without filtering.

Anaerobic matings. The basic mating protocol was performed in a strict anaerobic environment (glove box isolator, 100% N₂) with filters containing donors and recipients incubated on PCA plates containing 0.5% KNO₃ as the terminal electron acceptor (Van Hartingsveldt *et al.*, 1971); all media that were incubated anaerobically contained 0.5% KNO₃. Selective plates were placed in anaerobic jars (BBL Microbiology Systems), set up inside the anaerobic cabinet, and then transferred to a 20 °C (pQM1, pQM3) or 30 °C (RP1) incubator.

Phenotypic tests for conjugative plasmids. Transferred mercury-resistance plasmids were tested for additional phenotypes using Mastring-S antibiotic discs (M26 and M47; Mast Laboratories) on DST agar (Oxoid CM261) incubated at 20 °C for 48 h. Organomercurials were used at the following concentrations in PCA (µg ml⁻¹): fluorescein mercuric acetate (85.0), merbromin (375), phenylmercuric acetate (168), and thimerosal (40.5). The media were inoculated with broth cultures of the epilithic donor, the mercury-resistant transconjugant and the plasmid-free recipient.

Endonuclease digestion. Bacteria from which plasmids were to be isolated for restriction digests were grown in 50 ml NB for 16 h at 30 °C in an orbital incubator. Plasmids were isolated from cell lysates by sucrose gradient centrifugation (Wheatcroft & Williams, 1981). Restriction enzymes were obtained from Northumbria Biologicals, and digests were carried out as recommended by the supplier.

Statistical analysis. All transfer frequencies reported are means of at least two determinations. One-way analysis of variance followed by Tukey's test was used to compare differences between means using the SPSS-X computer package (Hull & Nie, 1981). All transfer frequencies were transformed by log₁₀ *x* to ensure normality of distribution and homogeneity of variances. Minimum significant ranges (MSR) were calculated by the Tukey-HSD method (Sokal & Rohlf, 1981). All significant differences are quoted for *P* < 0.05.

RESULTS AND DISCUSSION

Isolation and characteristics of conjugative plasmids in pure cultures

The mean ($\pm 95\%$ confidence limits) density of recoverable viable bacteria in the epilithon was $8.8 \pm 6.7 \times 10^6$ c.f.u. cm⁻² (*n* = 22) and the mean proportion of bacteria resistant to mercury was $2.5 \pm 0.7\%$ (*n* = 15). Plasmids were more common in the mercury-resistant epilithic bacteria: the frequencies were 47% (*n* = 129) in unselected bacteria and 70% (*n* = 79) in mercury-resistant isolates.

The smallest conjugative plasmid reported so far is 28 kb (pULG14; Thiry, 1984), so only plasmids of 30 kb or larger were considered to be potentially conjugative in this study. Forty-four mercury-resistant isolates containing at least one plasmid ≥ 30 kb were used as donors in membrane-filter matings with *P. aeruginosa* PAO2002, PU21, and PAO348 as recipients. None of these isolates transferred mercury resistance (lowest detectable frequency = 1.0×10^{-9}). The isolates included *Pseudomonas* spp., *Alcaligenes* spp., *Acinetobacter* spp., *Klebsiella* spp., and *Citrobacter* spp., all of which have been isolated previously from aquatic sources (Nuttall, 1982; Jones *et al.*, 1986).

The proportion of pseudomonad-like mercury-resistant isolates in the epilithon was $7.2 \times 10^{-5}\%$ of the total viable count, and 66% (*n* = 32) of these contained at least one plasmid ≥ 30 kb. Five of these isolates transferred mercury resistance to PAO348 with frequencies ranging from 8.4×10^{-8} to 2.8×10^{-3} (Table 2). Four of these five isolates contained at least one plasmid which was also detected in the mercury-resistant PAO348 transconjugants. The exception, PAR166, contained a single plasmid, pQM7, which was never detected in the PAO348 transconjugants. The plasmid either integrated into the PAO348 chromosome once it transferred, or was unable to replicate in PAO348 but carried a transposable DNA sequence encoding mercury resistance which inserted into the chromosome. Such transpositional systems have been reported before (Bale *et al.*, 1988) and might be important in natural bacterial populations (Kelly & Reanney, 1984).

Mercury resistance has been reported to be transferable from 5% of soil isolates (Kelly & Reanney, 1984), from 26% of marine bacteria (Gauthier *et al.*, 1985), and from 9% of bacteria isolated from freshwater fish (Toranzo *et al.*, 1984). Our results are similar, as 24% of the pseudomonad-like isolates transferred mercury resistance to *P. aeruginosa*, although transfer did not occur from random isolates (<2%).

Table 2. Characteristics of epilithic isolates transferring mercury resistance to *P. aeruginosa* PAO348

Donor and recipient organisms were mixed and deposited onto membrane filters. The filters were incubated on PCA for 24 h at 20 °C; selective plates for donors, recipients and transconjugants were incubated at 20 °C for 4 d. The presence of plasmids in the transconjugants was confirmed by gel electrophoresis. Isolates were identified using API20B kits and a probabilistic identification matrix (Holmes *et al.*, 1986). Further details are given in Methods.

Epilithic isolate		Plasmid content of epilithic isolate*	Transfer frequency to PAO348†
<i>P. putida</i>	PAR153	pQM6, 190 kb; Tra ⁺ , Hg	1.0×10^{-7}
<i>P. cepacia</i>	PAR161	pQM2, 110 kb	ND
		pQM3, 78 kb; Tra ⁺ , Hg, FMA, merbromin, Sm	2.8×10^{-3}
<i>P. cepacia</i>	PAR167	pQM9, 110 kb	ND
		pQM8, 78 kb; Tra ⁺ , Hg, FMA, merbromin, Sm	2.6×10^{-4}
Gram -ve rod	PAR166‡	pQM7, 90 kb (Tra ⁺ , Hg)	8.4×10^{-8}
<i>Alteromonas</i> sp.	PAR174	pQM4, 172 kb; Tra ⁺ , Hg, FMA, merbromin	2.0×10^{-5}
		pQM5, 2.5 kb	ND

ND, Transfer of these plasmids was not detected.

* FMA, fluorescein mercuric acetate; Sm, streptomycin.

† Mean of at least three determinations.

‡ Although this strain consistently transferred mercury resistance to PAO348, pQM7 was never detected in the transconjugants.

Table 3. Host ranges of pQM3 and pQM4

Donor and recipient organisms were mixed and deposited onto membrane filters. The filters were incubated on PCA for 24 h at 20 °C; selective plates for donors, recipients and transconjugants were incubated at 30 °C for 48 h. The natural hosts of pQM3 and pQM4, PAR161 and PAR174 respectively, were used as the donors in these matings and rifampicin-resistant mutants of each of the test hosts were used as recipients. Transfer was confirmed by detection of the plasmids in the transconjugants by gel electrophoresis.

Recipient bacterium		Transfer frequency	
		pQM3	pQM4
<i>P. aeruginosa</i>	PAO348	2.8×10^{-3}	2.0×10^{-5}
	PU21	2.2×10^{-8}	$< 4.1 \times 10^{-10}$ *
<i>P. putida</i>	KT2440	1.9×10^{-2}	2.8×10^{-4}
	PP3	3.9×10^{-7}	ND
<i>P. fluorescens</i>	AR17	5.9×10^{-1}	ND
	AR41	5.3×10^{-6}	$< 1.4 \times 10^{-7}$ *
	10038	1.4×10^{-8}	$< 1.5 \times 10^{-9}$ *
<i>P. maltophilia</i>	AR27	$< 1.0 \times 10^{-9}$ *	5.0×10^{-9}
	10499	$< 1.1 \times 10^{-9}$ *	1.6×10^{-8}
<i>A. putrefaciens</i>	10695	ND	3.2×10^{-7}
<i>E. coli</i>	LE392	9.6×10^{-7}	5.2×10^{-3}
	J53	1.7×10^{-6}	4.6×10^{-6}
<i>A. calcoaceticus</i>	BD413	$< 1.7 \times 10^{-9}$ *	$< 2.0 \times 10^{-9}$ *
<i>Proteus vulgaris</i>	6213	$< 5.7 \times 10^{-9}$ *	$< 1.5 \times 10^{-8}$ *
<i>K. pneumoniae</i>	8172	$< 8.5 \times 10^{-9}$ *	$< 7.2 \times 10^{-8}$ *

ND, Not determined.

* Lowest detectable transfer frequency.

Plasmids pQM3 and pQM4 were chosen for further study. Both plasmids encoded resistance to fluorescein mercuric acetate and merbromin but not phenylmercuric acetate or thimerosal (Table 2); thus they encoded narrow-spectrum mercury resistance (Robinson & Tuovinen, 1984). pQM3 also encoded streptomycin resistance. Both plasmids were broad host range, transferring to a range of *Pseudomonas* spp. and *Escherichia coli* but not to *Acinetobacter calcoaceticus*, *Proteus vulgaris* or *Klebsiella pneumoniae* (Table 3). In general, all the mercury-

resistant transconjugants contained either a single 78 kb or a single 172 kb plasmid. However, in *P. aeruginosa* PAO348 and PU21, and *P. fluorescens* AR41, pQM3 underwent structural rearrangements.

The mean ratio of membrane to broth mating transfer frequency was 662 ($n = 4$) for pQM3 and > 1016 ($n = 2$) for pQM4, when the plasmids were transferred from their natural hosts to PAO348. Thus, both plasmids transferred more efficiently on a solid surface than in liquid. This is probably a reflection of the habitat of the natural hosts, on stone surfaces. Not all epilithic plasmids are transferred better on surfaces, however, as pQM1 (Bale *et al.*, 1987) had a plate to broth ratio of 1.9 in these experiments.

Transferable resistances from suspensions of epilithic bacteria

Mercury, streptomycin and kanamycin resistances were transferred from MNS to the restriction-less *P. aeruginosa* PAO348 with frequencies of 2.2×10^{-5} , 2.2×10^{-6} and 7.3×10^{-6} , respectively, but transfer of tellurite or gentamicin resistance was not detected (lowest detectable frequency = 1.0×10^{-8}). Mercury resistance was also transferred to the restriction-less *P. putida* KT2440 (5.1×10^{-4}) but not to the restriction-positive *P. aeruginosa* PU21 ($< 6.8 \times 10^{-10}$).

Effects of temperature on plasmid transfer

The epilithic plasmids pQM1 and pQM3 had maximum transfer frequencies at 20–25 °C (Fig. 1), which was similar to the optimum temperatures reported for two mercury-resistance plasmids isolated from soil (25 °C; Kelly & Reanney, 1984) and for transfer of antibiotic resistance from a sewage isolate (20–25 °C; Altherr & Kasweck, 1982). Maximum frequencies for transfer of mercury resistance from a marine pseudomonad to *E. coli* occurred at 30 °C (Gauthier *et al.*, 1985). These reports suggest that conjugation will be more likely at temperatures higher than those normally expected in temperate aquatic environments. However, our results show that natural plasmids will transfer at environmentally relevant temperatures, because all those studied transferred at 4–6 °C. Transfer of pQM4 occurred maximally at 10–20 °C (2.5×10^{-5}) and transfer was not detected above 30 °C (lowest detectable frequency = 1.0×10^{-9}). pQM1 transfers *in situ* at 6 °C (Bale *et al.*, 1987) but with frequencies 500-fold lower than was demonstrated at the same temperature in these laboratory matings. At 25 °C, pQM3 transferred to *P. aeruginosa* PAO348 with a frequency of 5.8×10^{-3} . When the natural epilithic isolate *P. fluorescens* AR17 was the recipient, the frequency was much higher (5.9×10^{-1}) but the temperature optimum remained the same (Fig. 1). Transfer frequencies of RP1 increased as temperature increased, with an optimum at 37–41 °C; hence our results were similar to those obtained previously for R1drd19 and RP1 (Singleton & Anson, 1981; Kelly & Reanney, 1984).

The transfer of mercury resistance from MNS to PAO348 was studied over a range of physico-chemical conditions. Transfer was dependent on temperature (Fig. 2a), with maximum frequencies (2.2×10^{-5}) occurring at 25 °C. Transfer occurred over the range 8–37 °C but not at 4 °C or 41 °C (lowest detectable frequency = 1.0×10^{-8}). The pattern of temperature sensitivity of transfer of mercury resistance from MNS was similar to that shown from pure cultures. In contrast to our pure culture matings and those of others (George *et al.*, 1981; Walter & Vennes, 1985), no transfer from natural mixtures of bacteria was detected at below 5 °C (Fig. 2a). This failure to detect transfer was probably a reflection of the relatively low densities of potential donors. Since temperature affects bacterial growth rates, 24 h mating at 4 °C was probably insufficient time to allow the donor bacteria to reach a high enough density for transfer.

The incubation temperature of the pure cultures of donors and recipients prior to mating was also important in determining the rates of plasmid transfer (Table 4). The transfer frequency of pQM3 was lowest (1.8×10^{-6}) when the donor (*P. cepacia* PAR161) was grown at 30 °C prior to mating, and the recipient (*P. aeruginosa* PAO348) and mating filter were both incubated at 20 °C. Growing PAO348 at 30 °C gave significantly higher transfer frequencies regardless of the temperature used for growth of PAR161 or for incubation of the mating. Thus, highest

Fig. 1. Effect of temperature on plasmid transfer by conjugation. Plasmids pQM1 (○) and RP1 (■) were transferred from *P. aeruginosa* PAO2002 to *P. aeruginosa* PU21, pQM3 was transferred from *P. cepacia* PAR161 to *P. aeruginosa* PAO348 (○) and *P. fluorescens* AR17 (▲), and pQM4 (□) was transferred from *Alteromonas* sp. PAR174 to *P. aeruginosa* PAO348. MSR, minimum significant range.

Table 4. Effect of the incubation temperature of donor and recipient cultures on the transfer of pQM3

Cultures of donor (*P. cepacia* PAR161) and recipient (*P. aeruginosa* PAO348) were grown in NB at 20 °C or 30 °C, and after filtering the mating mixtures were incubated at 20 °C or 30 °C. Selective plates for donors, recipients and transconjugants were incubated at the same temperature as that used for the mating.

Temp. for growth of recipients (°C)	Temp. for mating (°C)	Transfer frequency	
		Temp. for growth of donors:	
		20 °C	30 °C
20	20	$3.2 \times 10^{-5}\dagger$	$1.8 \times 10^{-6}\ast$
	30	$3.5 \times 10^{-5}\dagger$	$4.8 \times 10^{-5}\dagger\S$
30	20	$3.5 \times 10^{-4}\dagger\S$	$4.8 \times 10^{-4}\dagger$
	30	$9.1 \times 10^{-4}\dagger$	$7.2 \times 10^{-4}\dagger$

* Significantly lower than all other frequencies.

†, ‡, §, Figures with the same symbol were not significantly different.

frequencies were obtained when conditions were optimized for growth of the recipient, indicating that the physiology of bacteria prior to conjugation is important in determining the rates of plasmid transfer.

Effect of pH on plasmid transfer

The influence of pH was investigated at 25 °C (or 22 °C) and 37 °C because it has been reported that the effects of non-optimal pH and temperature on plasmid transfer are synergistic (Singleton & Anson, 1983). A similar synergism was also shown by our results with pure cultures (Fig. 3a) but not with MNS (Fig. 2b). There was no effect on the transfer of pQM1 over the range pH 5.0–8.0 at 25 °C but there was a clear optimum at pH 6.5 at 37 °C. At optimum temperature, pH affected pQM3 and RP1 transfer in similar ways, both plasmids having an optimum at pH 7.0. The effect of pH on pQM3 at 37 °C was more marked but the optimum was still the same. Transfer from MNS was not significantly affected by pH over the range 5.5–8.0 (Fig. 2b) at either 22 °C or 37 °C. Frequencies at pH 5.0 were significantly lower than those above pH 6.5 at 22 °C and significantly lower than those at pH 7.5 at 37 °C. Previous studies have shown plasmids to have pH optima for transfer ranging from 6.0 to 7.5 (Shenderov, 1971;

Fig. 2. Factors affecting the conjugal transfer of mercury-resistance plasmids from MNS to *P. aeruginosa* PAO348 on the surface of agar plates. The parameters investigated were: (a) temperature; (b) pH at 22 °C (●) and 37 °C (○); (c) mating time; (d) concentration of organic nutrients; (e) cell density (donors + recipients) at the start (●) and end (○) of the mating period; (f) donor to recipient ratio at the start (●) and end (○) of the mating period. (▼) In some individual matings no transfer was detected; the lowest detectable frequencies were (1) 1.4×10^{-8} , (2) 2.0×10^{-9} , (3) 5.0×10^{-9} , (4) 9.2×10^{-9} . MSR, minimum significant range.

Harada & Mitsuhashi, 1977; Singleton & Anson, 1983). The pH of the R. Taff epilithon ranged from 6.2 to 7.0 (measured with a surface pH meter) over a 12 month period. Since pH values ranging from 5.5 to 8.0 had no effect on plasmid transfer at 22 °C (Fig. 2b) it is unlikely that pH would be an important influence on plasmid transfer within the epilithon.

Fig. 3. Effect on conjugal plasmid transfer of (a) pH at 25 °C and 37 °C; (b) mating time; (c) nutrient status; and (d) donor to recipient ratio at the start of the mating. Transfer of pQM1 (●) and RP1 (■) was from *P. aeruginosa* PAO2002 to *P. aeruginosa* PU21; pQM3 (○) was transferred from *P. cepacia* PAR161 to *P. aeruginosa* PAO348, and pQM4 (□) was transferred from *Alteromonas* sp. PAR174 to *P. aeruginosa* PAO348. ▼, Lowest detectable transfer frequency = 2.0×10^{-8} . MSR, minimum significant range.

Effect of mating time on plasmid transfer

Transfer frequencies of RP1 have been reported to reach a maximum after 1 h (Lacy & Leary, 1975) and 24 h (Graves & Riggs, 1980), whereas our results show a maximum frequency after 5 h (Fig. 3b). The rate of RP1 transfer thus depends on the host strains and mating conditions used. Both pQM1 and pQM3 also had maximum transfer frequencies after 5 h; increasing the mating time to 24 h had no significant effect. Transfer of pQM4, however, was not detected within the first 2 h of mating and the transfer frequency continued to increase beyond 5 h (Fig. 3b). Increasing the mating period to 48 h had no significant effect on the transfer of any of the plasmids (results not shown). Transfer from MNS occurred more slowly than from pure cultures, as within the first 3 h of mating there were no transconjugants (Fig. 2c; $< 1.0 \times 10^{-8}$), but low levels were detected after 5 h (3.2×10^{-8}) and maximum frequencies were obtained after 24 h. There was a significant decrease in the transfer frequency between 24 h and 48 h. The donor to recipient ratio after 3 h mating was 1.9×10^{-4} compared to 2.1×10^{-2} after 24 h, suggesting that 3 h mating at 20 °C was not sufficient time for the epilithic donor population to reach a density at which mating aggregates could form. Thus, the combination of temperature and mating time with donor to recipient ratio appears to be important in determining the efficiency of transfer. A similar interaction between mating time and temperature was reported for transfer of tetracycline resistance from bacteria in raw sewage (Fontaine & Hoadley, 1976),

transconjugants being detected after 1 h at 30 °C but not until 3.5 h at 22 °C. Mating time for bacteria within a stable epilithic community should not be a limiting factor on conjugation.

Effect of nutrient concentration on plasmid transfer

Plasmid transfer frequencies were also dependent on the concentration of nutrients in the mating medium. All of the three plasmids tested transferred on agar with no added carbon (Fig. 3c; pQM1, 2.7×10^{-3} ; pQM3, 3.4×10^{-5} ; RP1, 2.1×10^{-5}). The transfer frequencies increased with increasing nutrient concentration, up to 1.8 g C l^{-1} for pQM1 and RP1 and up to 3.5 g C l^{-1} for pQM3. Increasing the nutrient concentration to 10 g C l^{-1} had no significant effect on transfer frequencies in these pure-culture matings. The results for MNS were similar. Transfer from MNS occurred on agar plates with no added carbon (4.8×10^{-7}) and frequencies increased with increasing carbon concentration up to 10 g C l^{-1} (5.7×10^{-5} ; Fig. 2d). Other reports support these findings. For example, the transfer of RP4 between *Rhizobium* strains increased with increasing carbon concentration (Beringer, 1974). The increase of transfer frequencies with nutrient concentration indicates that the physiology of bacteria involved in conjugation is an important determinant. Growth rates of bacteria also increase with increasing nutrient concentration and it has been shown that in *E. coli* there is a positive correlation between multiplication rates of donor and recipient bacteria and fertility (Levin *et al.*, 1979). Plasmid transfer in sterile stream water occurred only when it was amended with dilute nutrient broth (Trevors & Oddie, 1986). Mobilization of pHSV106 in a waste treatment model, however, was not affected by nutrient composition or concentration (Mancini *et al.*, 1987).

Effect of the ratio of donors and recipients on plasmid transfer

It has been reported previously that plasmid transfer frequency is affected by the ratio of donors and recipients in the mating. Gauthier *et al.* (1985) reported that the majority of plasmids from mercury-resistant marine isolates transferred maximally at donor to recipient ratios of 1, and Graves & Riggs (1980) reported that RP1 transfer frequencies were higher at initial ratios ≥ 15 than at ratios ≤ 1 . Our results show maximum transfer frequencies of pQM1, pQM3, and RP1 at initial donor to recipient ratios of 0.4–30, depending on the plasmid, but all three plasmids transferred over the range of ratios used (Fig. 3d). The transfer of mercury resistance from MNS occurred over a wide range of initial donor to recipient ratios (Fig. 2f) but maximum frequencies were at ratios greater than 1.6×10^{-2} . The donor to recipient ratios changed over the course of the matings because of differential growth rates of donors and recipients, but transfer frequencies plotted against ratios at the end of the mating showed the same trends, in both MNS matings (Fig. 2f) and pure-culture matings (not shown).

Effect of cell density on plasmid transfer

The influence of cell density on plasmid transfer was studied for MNS matings only (Fig. 2e). Transfer frequencies were plotted against the cell densities at the start and the end of the 24 h mating period. Maximum frequencies (2.0×10^{-5}) were obtained when the matings contained an initial density (donors + recipients) of $1.7 \times 10^5 \text{ c.f.u. cm}^{-2}$. After 24 h the cell density in this mating had increased to $1.4 \times 10^8 \text{ c.f.u. cm}^{-2}$. The lowest initial density at which transfer was detected was $2.2 \times 10^4 \text{ c.f.u. cm}^{-2}$ (frequency = 6.6×10^{-7}), which increased to a density of $9.7 \times 10^6 \text{ c.f.u. cm}^{-2}$ after 24 h mating. No transfer was detected when the initial density was $4.4 \times 10^2 \text{ c.f.u. cm}^{-2}$, even though the density in this mating had increased to $1.9 \times 10^6 \text{ c.f.u. cm}^{-2}$ after 24 h. We conclude that 24 h mating did not allow the initial population of $4.4 \times 10^2 \text{ c.f.u. cm}^{-2}$ to increase sufficiently for mating aggregates to form. The absence of transfer below a critical cell density, as observed here, has also been reported for previous plasmid transfer studies (Gowland & Slater, 1984; Manceau *et al.*, 1986).

Effect of anaerobiosis on plasmid transfer between pure cultures

Both aerobic and anaerobic zones occur within the epilithon (Lock *et al.*, 1984), so we tested the ability of epilithic plasmids to transfer in anaerobic matings. When donor and recipient pregrowth, mating and selection were performed anaerobically, RP1 showed no significant

Table 5. *Effects of anaerobiosis on plasmid transfer*

Donor and recipient organisms were deposited onto membrane filters. RP1 and pQM1 were transferred from *P. aeruginosa* PAO2002 to *P. aeruginosa* PU21 and pQM3 was transferred from *P. cepacia* PAR161 to *P. aeruginosa* PAO348. Anaerobic matings were performed on PCA + 0.5% KNO₃ for 24 h at 25 °C in an anaerobic cabinet with: (a) anaerobic conditions used for donor and recipient pregrowth and selection subsequent to mating, (b) pregrowth under anaerobiosis but selective plates incubated aerobically, (c) donor and recipient cultures and selective plates incubated aerobically. Standard aerobic matings and aerobic matings on PCA + 0.5% KNO₃ were also carried out.

Mating conditions	Transfer frequency		
	RP1	pQM1	pQM3
Aerobic	2.0×10^{-4} *	4.0×10^{-1} *	3.4×10^{-3} *
Aerobic + 5% KNO ₃	2.3×10^{-4} *	6.2×10^{-1} *	7.3×10^{-3} *
Anaerobic + 5% KNO ₃ (a)	5.8×10^{-4} *	0†	0‡
(b)	ND	0†	6.7×10^{-3} *
(c)	1.4×10^{-5}	0†	1.3×10^{-4}

ND, Not determined.

* Not significantly different (for each plasmid).

† Lowest detectable frequency was 10^{-9} .

‡ Lowest detectable frequency was 10^{-7} .

change in its transfer frequency (Table 5). This contrasts with a previous report (Graves & Riggs, 1980) that RP1 transfer frequencies from *P. aeruginosa* to *E. coli* were 400-fold lower in anaerobic matings compared to aerobic. Under such wholly anaerobic conditions, transfer of pQM1 and pQM3 was not detected. However, pQM3 could transfer anaerobically because when pregrowth and mating were carried out anaerobically, but selective plates were incubated aerobically (Table 5), transfer was as efficient as in aerobic matings. PAO348 and PAO348(pQM3) were streaked onto PCA + 0.5% KNO₃ plates without mercury and incubated anaerobically at 30 °C for 3 d. All PAO348(pQM3) strains ($n = 15$) were unable to grow under these conditions whereas growth of plasmid-free PAO348 was unaffected. Thus, the presence of pQM3 prevented anaerobic growth of PAO348. *P. cepacia* PAR161 was not able to grow anaerobically although it did remain viable over 24 h, thus demonstrating the ability of pQM3 to transfer under anaerobiosis even though its natural host did not grow under these conditions. Unlike that of pQM3, transfer of pQM1 was completely inhibited by anaerobiosis, suggesting an oxygen requirement other than as terminal electron acceptor, as this role was fulfilled by KNO₃ in these experiments. Anderson (1975) reported that transfer of R1 was completely inhibited by anaerobiosis; and inhibition of R1 fertility was shown to be dependent on anaerobic conditions during pregrowth of the donor strain (Burman, 1975), with oxygen tension during recipient pregrowth, mating and selection having no effect.

Overall discussion on factors affecting plasmid transfer

These results demonstrated the presence of broad-host-range conjugative plasmids in the epilithon and showed that they are capable of transfer under environmentally relevant conditions. The plasmids from pure cultures of epilithic bacteria, RP1 and plasmids from MNS showed similar transfer behaviour for all of the variables studied except temperature. The RP1 temperature profiles were very different, all the epilithic plasmids showing much poorer transfer at higher temperatures. The epilithic plasmids therefore had optima for transfer at temperatures which prevail in the environment from which they were isolated. Epilithic bacteria are exposed to a maximum temperature of 21 °C in the R. Taff whereas RP1 was originally isolated from a clinical strain of *P. aeruginosa* (Lowbury *et al.*, 1969). The same hosts were used in the RP1 and pQM1 transfer experiments (Fig. 1), which demonstrates that both the bacterial hosts (Table 3; Bale *et al.*, 1987) and the plasmid itself have roles in determining transfer.

Several of our experiments also show that the host physiology affects plasmid transfer. So, it is probable that factors affecting plasmid transfer in the environment will include the physico-

Fig. 4. Distribution of transfer frequencies of MNS plasmids when transferred from their original transconjugants, *P. aeruginosa* PAO348, to *P. putida* KT2440 in membrane filter matings at 25 °C.

Table 6. *Characteristics of the predominant type of plasmid transferred from MNS*

Plasmids were transferred from MNS to *P. aeruginosa* PAO348 in matings on the surface of PCA plates at 20 °C for 24 h. The plasmids were then transferred from these transconjugants into *P. putida* KT2440 in membrane filter matings. Recipients and transconjugants were selected on minimal salts agar + glucose (1 g l⁻¹) ± HgCl₂ (13.5 µg ml⁻¹). Plasmids were prepared by sucrose gradient centrifugation (Wheatcroft & Williams, 1981) and analysed by *Eco*RI restriction digests. The sizes were calculated by summing the fragment sizes determined by multiple regression (Rochelle *et al.*, 1986).

Plasmid and phenotype	Size (kb)	Fragments (kb) different from pQM120	N*	Transfer frequency†
pQM120 Tra ⁺ Hg Uv	316	—	16	7.9 × 10 ⁻¹
pQM122 Tra ⁺ Hg Uv	331	+6.6 +5.2 +3.1 +2.8 -2.6	1	5.6 × 10 ⁻¹
pQM124 Tra ⁺ Hg Uv	334	+6.6 +5.2 +3.1 +2.8	2	6.2 × 10 ⁻¹
pQM127 Tra ⁺ Hg Uv	324	+5.2 +3.1	1	4.8 × 10 ⁻¹
pQM141 Tra ⁺ Hg Uv	316	Identical to pQM120	1	1.4 × 10 ⁻⁴
pQM181 Tra ⁺ Hg Uv Sm	327	+7.9 +2.8	1	1.1 × 10 ⁻⁶

* Number of plasmids with identical restriction patterns.

† Transferred from *P. aeruginosa* PAO348 to *P. putida* KT2440.

chemical conditions, the host physiology and the types of plasmid present. The synergism between the effects of pH and temperature on transfer suggests that combinations of other variables will also have complex effects. For example, temperature affects growth rate and nutritional requirements of bacteria (Herbert & Bhakoo, 1979), so an interaction between temperature and nutrient status, which also affects growth rate, would be expected. Therefore, rates of transfer will not be determined by a single factor but by a complex interaction of many variables. Thus, accurate predictions of gene transfer in natural environments will be difficult.

Characteristics of plasmids from MNS

Mercury-resistant *P. aeruginosa* PAO348 transconjugants from an MNS mating at 20 °C, pH 7.0, for 24 h, on plates containing 3.5 g C l⁻¹, were tested for resistance phenotypes other than mercury. The only resistance detected was for streptomycin and this was found in only two of the 100 transconjugants tested. All but two of the transconjugants contained single plasmids, the majority (80%) of which were larger than 300 kb. PAO348 did not give reliable or consistent plasmid yields from density gradient centrifugation (Wheatcroft & Williams, 1981), therefore the plasmids were transferred into *P. putida* KT2440, using membrane matings, and from there analysed by restriction digests. Forty-eight of the PAO348 transconjugants (54%) transferred their plasmids to KT2440 with frequencies ranging from 1.1 × 10⁻⁶ to 8.2 × 10⁻¹ (Fig. 4).

Fig. 5. Restriction digests of MNS plasmids transferred from *P. aeruginosa* PAO348 to *P. putida* KT2440. Lanes: (1) *Hind*III digest of λ DNA (fragment sizes are shown on the left), (2) and (8) pQM120, (3) and (9) pQM122, (4) and (10) pQM124, (5) and (11) pQM127, (6) and (12) pQM134, (7) and (13) pQM181. Lanes (2) to (7) were digested with *Eco*RI and (8) to (13) with *Sal*I.

A random sample of 21 of the plasmids that transferred to KT2440 was examined further. Restriction digests with *Eco*RI and *Sal*I showed that the majority (16) of the large (> 300 kb) plasmids were indistinguishable, producing the same digest patterns. *Sal*I was used because complete digestion with this enzyme gave fewer bands than with *Eco*RI and *Hind*III so differences between plasmids showed up more clearly. One of these plasmids (pQM120) was used as a standard for further comparisons (Table 6). This plasmid encoded UV resistance and was found to be about 320 kb in size by *Eco*RI and *Hind*III digests. The remaining five plasmids had alterations of between two and five fragments, accounting for $< 5\%$ of the total plasmid DNA (Figs 5 and 6). Table 6 summarizes the characteristics of the pQM120-type plasmids. With the exception of pQM181 these plasmid alterations had no effect on the detectable phenotype. In addition to mercury and UV resistance, pQM181 encoded streptomycin resistance, which was probably located on the additional 7.9 kb fragment (Fig. 6). It transferred from PAO348 to KT2440 with a frequency of 1.1×10^{-6} . The second plasmid from MNS which encoded streptomycin resistance, pQM194, was 180 kb, sensitive to UV irradiation and did not transfer to KT2440 ($< 1.0 \times 10^{-9}$). pQM141 was indistinguishable from pQM120 but transferred from PAO348 to KT2440 at a much lower frequency (1.4×10^{-4}). The original PAO348 transconjugant contained a second plasmid (200 kb) which did not transfer to KT2440 but presumably inhibited the fertility of pQM141.

The cryptic fragments present in the pQM120-type plasmids may be a result of rearrangements occurring either before or after transfer of the plasmids into PAO348. However, the 7.9 kb fragment encoding streptomycin resistance on pQM181 was probably a result of a rearrangement which occurred within the epilithon because there were no streptomycin resistance genes expressed in the recipient strains used. Streptomycin resistance was also the

Fig. 6. Diagrammatic representation of *EcoRI* digests of some plasmids from MNS in *P. putida* KT2440. Lanes: (1) *HindIII* digest of λ DNA (fragment sizes are shown on the left), (2) pQM120, (3) pQM122, (4) pQM124, (5) pQM181, (6) pQM1, (7) pQM40. Arrows indicate the fragments that differ from the most common digest pattern (pQM120). Thick lines represent fragments of identical size which could not be separated by electrophoresis. The diagrams were constructed from a number of electrophoresis gels, run for varying times to clearly separate the fragments.

only phenotype other than mercury resistance which was detected on plasmids isolated from the pure cultures of epilithic bacteria.

The first mercury-resistance plasmid isolated from the R. Taff epilithon was pQM1 (Bale *et al.*, 1987). Comparison of the *EcoRI* digest patterns of pQM1 and pQM120 (Fig. 6) shows some similarity (58% of the bands). However, the two largest bands of both plasmids were different, and as these accounted for about 30% of the DNA it is unlikely that pQM1 and pQM120 are structurally similar. Another plasmid from epilithic bacteria in the R. Taff, pQM40 (one of the pQM39 group; M. J. Bale, personal communication), is dissimilar from pQM1 (Bale *et al.*, 1988) and also appeared different from pQM120 on the basis of our *EcoRI* digests (Fig. 6). All the other 18 large (250–269 kb) plasmids isolated by Bale *et al.* (1988) gave large numbers of fragments with *SaII* digestion and so were probably unrelated to the pQM120-like plasmids isolated in this study. UV resistance is probably very common in the epilithon, as 80% of the plasmids isolated by Bale *et al.* (1988), and all of those isolated from MNS in the present study conferred this phenotype. Different genera of bacteria from another aquatic habitat (Jobling *et al.*, 1988) have also been found to contain identical and related plasmids.

Our results and other reports (Jobling *et al.*, 1988; Bale *et al.*, 1988) therefore indicate that populations of plasmids encoding mercury resistance within aquatic bacteria have the potential for transfer and structural rearrangements, thus allowing for acquisition and dissemination of phenotypes. The method we describe here for isolating plasmids from MNS could have more general application. It appears to be good at isolating plasmids which transfer at high frequency and so are perhaps actively transferring in nature.

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