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Isolation and Screening of Plasmids from the Epilithon Which Mobilize Recombinant Plasmid pD10

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This study examined the potential of bacteria from river epilithon to mobilize a recombinant catabolic plasmid, pD10, encoding 3-chlorobenzoate degradation and kanamycin resistance. Fifty-four mobilizing plasmids were exogenously isolated by triparental matings between strains of *Pseudomonas putida* and epilithic bacteria from the River Taff (South Wales, United Kingdom). Frequencies for mobilization ranged from 1.7×10^{-8} to 4.5×10^{-3} per recipient at 20°C. The sizes of the mobilizing plasmids isolated ranged from 40 kb to over 200 kb, and 19 of 54 were found to encode mercury resistance. Plasmid-encoded resistance to tetracycline and streptomycin was also found but not resistance to UV light or various heavy metals. Eight plasmids of epilithic bacteria, analyzed by comparing restriction fragmentation patterns, showed significant differences between those isolated from different independent matings. Optimal temperatures for mobilization of pD10 were between 15 and 25°C. Four mercury resistance plasmids were found to be broad host range, transferring mercury resistance and mobilizing pD10 readily to representative species of β- and γ-purple bacteria. In general, frequencies of pD10 mobilization by plasmids of epilithic bacteria were 2 to 3 orders of magnitude lower than conjugal transfer frequencies. Thus, there is a high potential for exchange of recombinant genes introduced into the epilithon by mobilization between a variety of bacterial species.

Industrial chemicals are continuously being released into the environment. The biodegradation of many of these priority pollutants by enriched, isolated microorganisms has been demonstrated in the laboratory (9, 14). However, although catabolized slowly by microorganisms in situ, these substances persist in and pollute the environment. In many cases, xenobiotic degradation has been found to be encoded by transmissible plasmids (48), and thus there is a potential use for these plasmids in waste treatment. In addition, cloning and manipulation of catabolic genes has significantly increased the potential biotechnological applications for genetically engineered microorganisms, particularly in the bioremediation of polluted sites. The development of catabolic gene probe technology has enhanced detection of specific DNA sequences in environmental samples, thereby potentially providing a reliable way of monitoring recombinant genes in the environment (36, 50, 51).

The potential beneficial uses for recombinant catabolic genes can only be realized when their fate and possible transfer in the environment are better understood. Many studies have focused on plasmid transfer in aquatic environments (44, 52). Previous studies in this laboratory have examined conjugal transfer of natural plasmids of epilithic bacteria under a range of environmental conditions, in laboratory microcosms (40) and in unenclosed, in situ experiments in the River Taff, South Wales, United Kingdom (4). The use of nonconjugative plasmids may limit interbacterial transfer, but mobilization by other self-transmissible plasmids may occur (29, 55) either by conjugative mobilization or by cointegration. Most studies investigating mobilization of recombinant plasmids in natural ecosystems have excluded the indigenous microflora (19, 34), although mobilization has also been shown in the presence of the natural bacteria in a model activated-sludge unit (32). The nonconjugative, 3-chlorobenzoate (3CB)-degrading, recombinant plasmid pD10 was mobilized in situ by activated-sludge-derived mobilizing plasmids to the indigenous bacteria. Plasmid pD10 is derived from RSF1010 (of the *Escherichia coli* incompatibility group IncQ) which can be mobilized to a variety of gram-negative species if transfer functions are provided (6, 54) by a conjugative mobilizing plasmid (16).

In this article, we describe experiments designed to evaluate the potential for transfer of pD10 (22.7 kb) by mobilization between microorganisms inhabiting river epilithon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Two auxotrophs of strain UWC1 (one requiring isoleucine, leucine, and valine; the other requiring threonine) were isolated after plasmid transposon Tn*10* mutagenesis (27) using the suicide donor vector pLG223. Auxotrophs were identified by screening on minimal media and then characterized by auxanography (11). Spontaneous loss of tetracycline resistance (Tc^r) (associated with Tn*10* deletion) resulted in the isolation of stable auxotrophic derivatives of strain UWC1, designated UWC3 (Ilv^-) and UWC4 (Thr^-), into which plasmid pD10 was introduced by electroporation. A spontaneous rifampin-resistant (Rif^r) mutant of PaW340 (22) was isolated for use in this study and designated UWC5.

Plasmid pD10 is a recombinant plasmid encoding the essential steps in chlorocatechol metabolism on genes *tfdC*, *tfdD*, and *tfdE*. This plasmid enables the host strain to utilize 3CB as the sole source of carbon and energy (13). Natural plasmids (isolated from the River Taff) able to mobilize pD10 were designated pQKH1 to pQKH54 (Table 3).

A tetracycline-resistant derivative of pD10 was constructed for the host range study. A Tc^r interposon Ω fragment with *Eco*RI ends was obtained from an *Eco*RI digest of pHP45Ω-Tc (15) and ligated into plasmid pD10 at

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype[a]	Source or reference
P. putida strain		
UWC1	Rif^r	33
UWC3	Rif^r Ilv^-	This study
UWC4	Rif^r Thr^-	This study
UWC5	Rif^r Trp^- Sm^r	This study
Plasmids		
pLG223::Tn*10*	Tc^r	5
pD10	Km^r $3CB^+$	33
pD10::Ω-Tc	Km^r $3CB^+$ Tc^r	This study
pHP45::Ω-Tc	Tc^r Ap^r	15
pQM1	Hg^r UV^r	3
pQKH6	Hg^r	This study
pQKH9	Hg^r	This study
pQKH14	Hg^r	This study
pQKH33	Hg^r Sm^r Tc^r	This study
pQKH42		This study
pQKH47	Sm^r	This study
pQKH48		This study
pQKH54	Hg^r	This study

[a] Phenotype abbreviations used: ILV, isoleucine, leucine, valine; Thr, threonine; Trp, tryptophan.

the unique *Eco*RI site. Plasmid pD10::Ω-Tc was then transformed into *E. coli* C600 (43). The narrow-host-range mobilizing plasmid R64.11 was subsequently used to mobilize pD10::Ω-Tc into *Pseudomonas putida* UWC5.

Media for growth and maintenance of bacteria. Bacterial strains were maintained on minimal media with defined carbon sources as described by Slater et al. (47). Carbon sources were used at the following concentrations: succinate, 1 or 10 g $liter^{-1}$; glucose, 1 or 15% (wt/vol); 3CB, 5 mM. Amino acids were added at the following concentrations: tryptophan, 25 μg ml^{-1}; isoleucine, 20 μg ml^{-1}; leucine, 20 μg ml^{-1}; valine, 20 μg ml^{-1}; histidine, 20 μg ml^{-1}; and methionine, 20 μg ml^{-1}. For culturing of strain UWC3, additions of isoleucine, leucine, and valine were made. Antibiotic concentrations used were as follows: kanamycin, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}; $HgCl_2$, 27 μg ml^{-1} in standard plate count agar (PCA; Oxoid CM643) and 13 or 6.5 μg ml^{-1} in minimal media. *Pseudomonas* selective medium (Oxoid CM559; Oxoid Ltd., London, England) containing supplements of cetrimide and nalidixic acid (Oxoid SR102) with appropriate antibiotics was used in the selection of transconjugants. PCA agar was used for the filter plate matings. Liquid cultures were grown in nutrient broth (Oxoid CM3) overnight by using an orbital shaker at the required temperatures. All antibiotics were purchased from Sigma Chemical Co., Dorset, United Kingdom. Otherwise, chemicals were of the highest quality available.

Isolation of epilithic mobilizing plasmids. Typical flat smooth stones were taken from the shallow reaches of the River Taff (31). Mixed natural suspensions from the epilithon

TABLE 2. Strains and selective media used in the host range study[a]

Recipient strain	Relevant genotype or phenotype	Selective media[a]	Reference or source[b]
E. coli LE392	*supE44 supF58 lacY1 galK2 metB1 trpR55 hsdR514 galT22* λ^- F^-	R: M9 met C: M9 met $Hg_{6.75}$ M: M9 met Km	30
A. eutrophus JMP222	$3CB^+$ Sm^r; prototrophic	R: S C: S $Hg_{13.5}$ M: S Km	12
P. maltophilia	Met- Km^r	R: S_{10} met C: S_{10} met $Hg_{13.5}$ M: S_{10} met Tc	NCTC 10499
A. calcoaceticus	His^- Rif^r	R: S_{10} his C: S_{10} his $Hg_{13.5}$ M: S_{10} his Km	NCIB 11826
P. putida UWC3	Ilv^- Rif^r	R: S ilv C: S ilv $Hg_{13.5}$ M: S ilv Km	This study
P. cepacia	Km^r; prototrophic	R: Glu C: Glu $Hg_{13.5}$ M: Glu Tc	NCTC 10661
P. aeruginosa PAO2002	Km^r; prototrophic	R: S_{10} C: S_{10} $Hg_{13.5}$ M: S_{10} Tc	8
Flavobacterium sp.	Km^r; prototrophic	R: Glu_{15} C: Glu_{15} $Hg_{6.75}$ M: Glu_{15} Tc	46

[a] Selective media were used for selection of recipients (R), transconjugants from conjugal transfer (C), and transconjugants from mobilization of pD10 (M). Abbreviations: M9, M9 salts (28); S, minimal medium (47) supplemented with succinate (1 [S] or 10 [S_{10}] g $liter^{-1}$); Glu, minimal medium (47) supplemented with glucose (1 [Glu] or 15% [Glu_{15}] [wt/vol]); met, methionine; his, histidine; ilv, isoleucine-leucine-valine. Hg was added at 13.5 ($Hg_{13.5}$) or 6.75 ($Hg_{6.75}$) mg $liter^{-1}$.
[b] NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria.

TABLE 3. Effect of isolation temperature of mobilizing plasmids on frequency of mobilization from UWC5(pD10) containing mobilizing plasmid (donor) to UWC3 (recipient) at 20°C

Isolation temp (°C)	Mobilizing plasmid pQKH:	Mobilization frequency/recipient[a]
30	1	5.1×10^{-6}
	2	4.2×10^{-5}
	3	1.2×10^{-4}
	4	9.4×10^{-6}
	5	2.4×10^{-4}
	6	1.4×10^{-5}
	7	1.6×10^{-3}
	8	3.3×10^{-5}
	9	2.9×10^{-5}
	14	8.0×10^{-5}
	15	6.1×10^{-6}
	16	2.5×10^{-5}
	17	2.5×10^{-6}
	18	1.6×10^{-4}
	19	3.6×10^{-5}
	20	4.4×10^{-5}
	21	2.5×10^{-5}
20	25	1.0×10^{-4}
	26	1.4×10^{-6}
	27	1.4×10^{-6}
	28	1.5×10^{-6}
	29	1.5×10^{-4}
	30	4.7×10^{-5}
	31	6.3×10^{-5}
	32	1.3×10^{-4}
	33	5.0×10^{-4}
	34	3.1×10^{-6}
15	41	1.4×10^{-5}
	42	2.9×10^{-3}
	43	9.7×10^{-4}
	44	2.9×10^{-3}
	45	1.2×10^{-4}
	47	2.1×10^{-3}
	48	5.0×10^{-3}
	54	7.6×10^{-4}

[a] Matings were carried out at 20°C for 24 h. Transconjugant counts were made on plates containing succinate, isoleucine-leucine-valine, and kanamycin; recipient counts were made on plates containing succinate and isoleucine-leucine-valine, and plates were incubated at 20°C for 48 h. Geometric mean frequency values for each isolation temperature are as follows: 30°C, 3.6×10^{-5}; 20°C, 2.2×10^{-5}; 15°C, 7.6×10^{-4}.

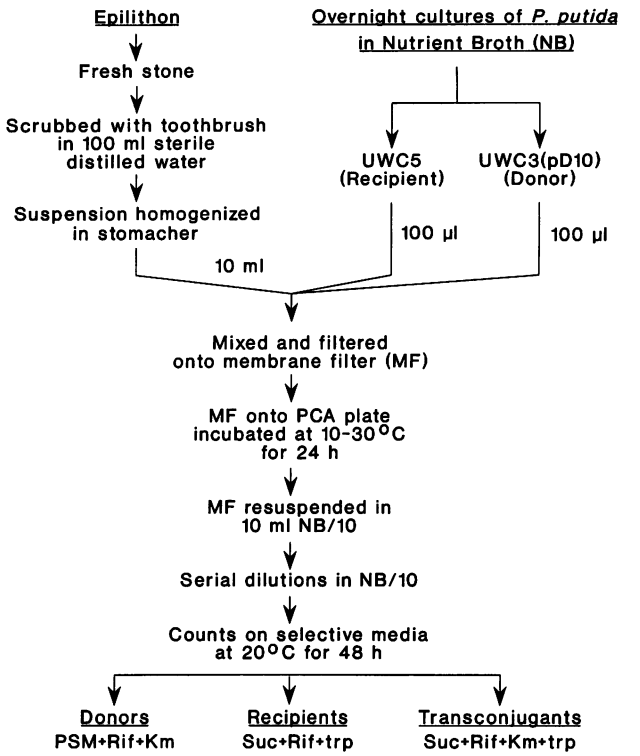

FIG. 1. Schematic diagram for exogenous isolation of mobilizing plasmids from epilithon by triparental mating. Abbreviations: PSM, *Pseudomonas* selective medium; Rif, rifampin; Km, kanamycin; Suc, succinate; trp, tryptophan; NB, nutrient broth.

were prepared by scrubbing the stones with a sterile toothbrush in 100 ml of sterile distilled water and then homogenizing the suspension in a stomacher (7). Mobilizing plasmids from the epilithon were isolated by exogenous isolation (17). Figure 1 shows a schematic diagram for exogenous isolation. In this study, natural plasmids were isolated solely by their ability to mobilize pD10 and not for any phenotypic markers which they might possess and were not isolated in the hosts in which they occurred in nature. For the exogenous isolation of natural plasmids, 10-ml samples of freshly obtained mixed natural suspension were mixed separately with 100-µl overnight cultures of *P. putida* UWC5 and UWC3(pD10). Cell suspensions were then filtered onto a 0.22-µm-pore-size cellulose nitrate membrane filter (45-mm diameter; Whatman Ltd., Maidstone, United Kingdom), which was subsequently incubated on PCA (at 30°C for 24 h, 20°C for 24 h, or 15°C for 6 days). The filter was then transferred to 10 ml of 1/10 strength nutrient broth, and cells were resuspended by vortex mixing. Putative plasmid-containing recipients were selected on plates containing succinate, rifampin, kanamycin, and tryptophan. Recipient counts were made on plates containing succinate, rifampin, and tryptophan and donor counts were made on plates containing *Pseudomonas* selective media, rifampin, and kanamycin.

Control matings were done to show that strains UWC5, UWC3, and UWC4 did not contain any mobilizing elements, and that they did not contain plasmids prior to the triparental mating was confirmed by gel electrophoresis. Putative mobilizing plasmids were confirmed by gel electrophoresis to show their physical presence and by the mobilization of pD10 from UWC3(pD10) containing the isolated plasmids to UWC5.

Electroporation. Recombinant pD10 DNA was introduced into *P. putida* UWC3 and UWC4 by electroporation by using a Bio-Rad Gene Pulsar with a pulse controller and capacitance extender (Bio-Rad Ltd., Hemel Hempstead, United Kingdom). A 50-ml nutrient broth culture was grown to an optical density at 600 nm of 0.6 to 0.9, chilled on ice, and harvested by centrifugation. The pellet was washed twice in 40 ml of 10% glycerol (ice cold) and finally resuspended in 1/200 of the initial culture volume with 10% (wt/vol) glucose and 300 mM sucrose (ice cold). It was then distributed into 30-µl aliquots on ice. The DNA (50 µl) was added and mixed gently. The cell suspension containing DNA was transferred to a prechilled electroporation cuvette and pulsed for 5 ms at 12.5 kV cm^{-1} (25-µF capacitor, 200-Ω parallel resistance). The cell suspension was removed and gently resuspended in 800 µl of prewarmed (30°C) SOC medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl,

10 mM $MgCl_2$, 10 mM $MgSO_4$) (21). The cell suspension was incubated at 30°C for 30 to 45 min, to allow phenotypic expression, before plating out on selective media.

Plasmid detection. Plasmids were isolated by using a modified method of the protocol of Kado and Liu (24, 37), and plasmid sizes were calculated by using the DNA SIZE program (42). Large-scale preparations of mobilizing plasmid DNA for restriction pattern analysis and of smaller plasmids such as pD10 were carried out by using the methods of Wheatcroft and Williams (53) and Clewell and Helinski (10), respectively. Restriction endonucleases were obtained from Northumbria Biologicals Limited, Northumbria, United Kingdom, and were used as specified by the manufacturer.

Mating experiments. Mobilizing frequencies for the naturally isolated plasmids were obtained by membrane filter plate matings on PCA (20 or 30°C, 24 h). Laboratory matings on agar were carried out as described by Bale et al. (2). The donor strain (3 ml), containing exogenously isolated mobilizing plasmid, was mixed with 2 ml of the recipient strain. Donor and recipient viable counts were made by the Miles and Misra drop plating technique (35), and transconjugant counts were done on spread plates. Transfer frequencies were expressed in all mating experiments as the mean number of transconjugants per recipient. Plates used for transconjugant selection were routinely checked for their ability to inhibit the growth of the donor and recipient strains. Transconjugants were routinely confirmed by phenotype, and plasmid preparations were made for visualization by gel electrophoresis (24). Control plates for the mating experiments included donor and recipient strains mixed and filtered as described before but plated out immediately without incubation. This was done to show that mating between donor and recipient strains did not occur at high frequencies on the agar after plating out. The highest count obtained for transconjugants in this way was 1.3×10^{-8} per recipient, compared to the lowest transconjugant count obtained in the mating experiments, 1.4×10^{-6} (Table 3), showing that mating on the spread plates was very low and therefore negligible when compared with the transfer frequencies actually obtained. Four of the recipients used in the host range study (namely, *Pseudomonas maltophilia*, *Pseudomonas aeruginosa*, *Flavobacterium* sp., and *Pseudomonas cepacia*) encoded resistance to kanamycin. Therefore, plasmid pD10::Ω-Tc containing either pQKH6, pQKH9, pQKH14, or pQKH54 was used for mobilization into these strains. Mobilizing plasmid-containing *P. putida* UWC5 (pD10::Ω-Tc) was stable after 10 generations in L broth, and mobilizing frequencies were of the same order of magnitude as those obtained with strain UWC5(pD10).

Resistance markers. Plasmid-encoded resistance to a range of antibiotics was tested in *P. putida* UWC5, by using antibiotic discs (carbenicillin, 60 μg; chloramphenicol, 10 μg; ampicillin, 10 μg; gentamicin, 10 μg; erythromycin, 5 μg) or antibiotic medium no. 3 (AM3, Oxoid CM287), containing tetracycline (100 μg ml^{-1}) or streptomycin (125 μg ml^{-1}). Heavy-metal resistance was detected by growth of selected strains on PCA with one of the following additions: Cr^{2+} (15 mM), Pb^{2+} (5 mM), Co^{+} (5 mM), Zn^{2+} (10 mM), Ni^{2+} (5 mM), or Hg^{2+} (0.1 mM). Plasmid-encoded UV light resistance (UV^r) was tested by irradiating duplicate 10^{-5} dilution plates (50 μl spread plated onto PCA) for up to 40 s and incubating in the dark (30°C, 48 h). The UV light source (output, 220 μJ s^{-1} cm^{-2}) was positioned 17 cm above each plate for irradiation, and estimations of percentage survival were based on the numbers of CFU before irradiation. Appropriate positive and negative controls were done; for example, PaW340 was the positive control in testing for resistance to streptomycin (Sm^r) and tetracycline.

Statistical methods. One-way and two-way analyses of variance were used to compare differences in the means with the Minitab (Version 7) computer package (Minitab Inc., University Park, Pa.). All counts and frequencies were transformed by $\log_{10}x$, which ensured normality of distribution and homogeneity of variances. The minimum significant differences were calculated by using the Tukey-Kramer method (49).

RESULTS

Isolation and characteristics of mobilizing plasmids from epilithon. Triparental matings between *P. putida* UWC3 (pD10), *P. putida* UWC5, and mixed natural suspensions from the epilithon were used to isolate mobilizing plasmids. The use of auxotrophic strains in this mating produced good selection against the donor strain on minimal media. Plating on succinate-kanamycin-rifampin-tryptophan provided effective selection against the epilithon, facilitating efficient isolation of plasmid-containing recipients (UWC5).

For the purposes of this study, plasmids were required which could mobilize pD10 efficiently at 20°C (a temperature representative of the mean maximum annual river temperature). The mobilization efficiency of pD10 by plasmids isolated at 30°C showed a marked reduction from 30 to 20°C (namely, 1.6×10^{-5} to 9.8×10^{-6} for pQKH1 and 3.3×10^{-2} to 1.8×10^{-4} for pQKH2). Therefore, exogenous plasmid isolation by prolonged triparental matings at 10 to 20°C was used to enrich for plasmids capable of transferring under cold conditions. These plasmids would be more likely to mobilize pD10 at a higher frequency at 20°C than mobilizing plasmids isolated at higher temperatures. Mobilizing plasmids were isolated from River Taff epilithon at 30, 20, and 15°C, in independent mating experiments. Attempts to isolate plasmids at 10°C were unsuccessful.

Six of 54 mobilizing plasmids isolated encoded tetracycline resistance, 17 (of 41 tested by transfer from the streptomycin-resistant UWC5 host to UWC3) encoded streptomycin resistance, and 19 encoded mercury resistance (Hg^r). No resistance to other heavy metals tested (Cr^{2+}, Pb^{2+}, Co^{+}, Zn^{2+}, and Ni^{2+}) was found. None of the 34 plasmids tested was found to encode for UV light resistance when compared with percentage survival values of pQM1.

The effect of isolation temperature on the efficiency of mobilization of pD10 at 20°C by the mobilizing plasmids isolated is shown in Table 3. Analysis of these data showed that mobilization frequencies at 20°C were significantly higher ($P = 0.001$) for plasmids isolated at 15°C than for plasmids isolated at 20 and 30°C. Transfer to *P. putida* UWC4 occurred at frequencies similar to those at which transfer into UWC3 occurred ($P = 0.963$; results not shown).

Temperature profiles for mobilization by selected plasmids. In preliminary screening (Table 3), four plasmids, namely, pQKH33 (Tc^r Sm^r Hg^r), pQKH42, pQKH48, and pQKH54 (Hg^r), showed high mobilization efficiency at 20°C, ranging from 5.0×10^{-4} to 5.0×10^{-3} per recipient. Plasmids pQKH42, pQKH48, and pQKH54 were isolated from the same triparental mating at 15°C, whereas plasmid pQKH33 was isolated at 20°C. Figure 2 shows that these plasmids had different temperature optima for mobilization. Plasmids pQKH33 and pQKH48 had fairly narrow temperature optima for mobilization, with the greatest mobilization frequencies seen between 20 and 25°C, followed by a gradual

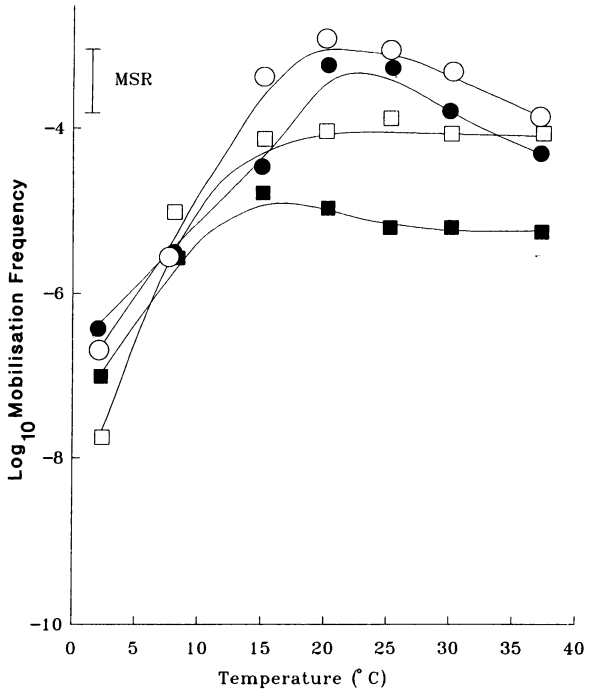

FIG. 2. Effect of temperature on frequency of mobilization (transconjugants per recipient) of pD10 by naturally isolated epilithic mobilizing plasmids pQKH33 (●), pQKH42 (□), pQKH48 (○), and pQKH54 (■). Matings of mobilizing plasmid containing UWC5(pD10) (donor) with UWC3 (recipient) were incubated for 24 h. Selective plates were incubated at 20°C for 48 h. Points indicate mean values of four replicate experiments. MSR, Minimum significant range.

decrease in frequency with the subsequent increase in temperature. In comparison, pQKH42 and pQKH54 had wide temperature optima between 15 and 37°C and showed no significant decrease in mobilization frequency at the higher temperatures.

Comparison of mobilization and conjugal transfer frequencies. An additional phenotypic marker (Hg^r) encoded by three mobilizing plasmids (pQKH6, pQKH9, and pQKH54) was used to compare the efficiency of conjugal transfer with efficiency of mobilization of pD10 (Table 4). Plasmids pQKH6 and pQKH9 were isolated from the same triparental mating at 30°C, and pQKH54 was isolated at 15°C. Conjugal transfer of plasmids pQKH6, pQKH9, and pQKH54 between *P. putida* strains occurred at frequencies between 6.1×10^{-2} and 1.5×10^{-3} (Table 4). Four-way analysis of variance showed that frequencies of transfer and of plasmid pD10 mobilization by these plasmids were not significantly affected by mating temperature ($P = 0.22$) or growth temperature ($P = 0.85$). However, the different plasmids mobilized pD10 at significantly different efficiencies ($P = 0.028$). As expected, pD10 mobilization frequencies by all of these plasmids were 2 to 3 orders of magnitude lower than their respective conjugation frequencies (Table 4).

With the possibility of isolating undetected plasmids or transposon-containing plasmids (capable of integrating into the chromosome from the epilithon), it was important to confirm that the mercury resistance plasmids (pQKH6, pQKH9, pQKH14, and pQKH54), visualized by gel electrophoresis, were those mobilizing plasmid pD10. The plasmids were transferred from their original host, UWC5(pD10), into UWC3 by selecting for plasmid-encoded mercury resistance and were then transferred back into strain UWC5(pD10) containing no other plasmids. From this new donor, the mercury-resistant plasmids were subsequently able to mobilize pD10, and frequencies were of the same order of magnitude as those obtained previously with these plasmids.

Structural comparison of mobilizing plasmids from the epilithon. Restriction endonuclease digests of plasmids pQKH6 (60 kb), pQKH9 (57 kb), pQKH14 (52 kb), pQKH33 (>200 kb), pQKH42 (57 kb), pQKH47 (56 kb), pQKH48 (69 kb), and pQKH54 (80 kb) with *Hin*dIII, *Eco*RI, and *Bam*HI showed that the plasmids were different, although distinct similarities were apparent (Fig. 3 shows *Hin*dIII digests of these plasmids). On the basis of restriction fragment sizes, the plasmids were tentatively classified into three groups. Group 1 comprised plasmids pQKH6, pQKH9, and pQKH14, all of which encoded mercury resistance and were isolated from the same exogenous isolation experiment. Each showed distinct differences in their restriction patterns, with plasmids pQKH6 and pQKH14 sharing two common restriction bands. Group 2 comprised plasmids pQKH42, pQKH47, pQKH48, and pQKH54 which were also isolated from the same exogenous mating experiment, although a different one from that of group 1 plasmids. Plasmids pQKH47 and pQKH54 encoded streptomycin and mercury resistance, respectively. No markers were discovered to be associated with the other two plasmids. Figure 3 shows that pQKH42, pQKH47, and pQKH48 had several

TABLE 4. Comparison of mean mobilization frequencies for pD10 and conjugal mercury resistance transfer frequencies (per recipient)[a]

Plasmid	Growth temp[b] (°C)	Frequency/recipient at mating temp of:			
		20°C		30°C	
		Mobilization	Conjugation	Mobilization	Conjugation
pQKH6	20	3.8×10^{-5}	4.2×10^{-2}	3.0×10^{-5}	7.2×10^{-3}
	30	1.5×10^{-5}	1.6×10^{-2}	6.4×10^{-6}	1.2×10^{-2}
pQKH9	20	1.3×10^{-5}	6.1×10^{-2}	1.4×10^{-5}	1.6×10^{-2}
	30	4.2×10^{-5}	6.9×10^{-3}	2.5×10^{-5}	3.1×10^{-2}
pQKH54	20	2.0×10^{-6}	7.4×10^{-3}	4.5×10^{-6}	1.5×10^{-3}
	30	4.6×10^{-5}	3.2×10^{-2}	2.0×10^{-6}	9.3×10^{-3}

[a] Mobilizing plasmid-containing *P. putida* UWC5(pD10) and UWC3 were used as donor and recipient, respectively, and mating pairs were incubated for 24 h. Mobilization of pD10 and transfer of mercury resistance are expressed as frequency per recipient, and mean values of at least two replicate experiments are shown. Minimum significant difference ($\log_{10}x$), 2.66; average coefficient of variation between replicate experiments, 15.7%.

[b] Temperature of growth for donor and recipient strains prior to mating.

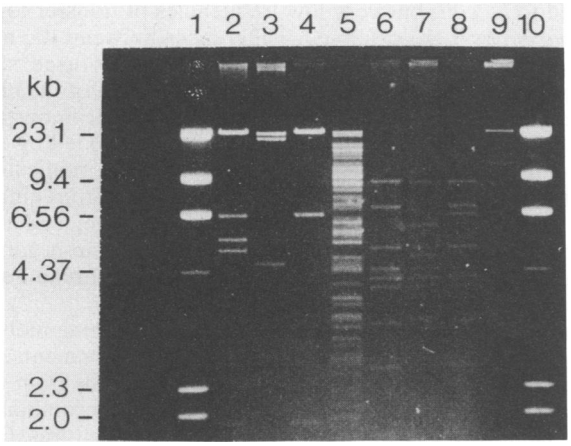

FIG. 3. *Hin*dIII restriction digests of naturally occurring mobilizing plasmids isolated from *P. putida* UWC3. Lanes: 1 and 10, *Hin*dIII digest of λ DNA (fragment sizes are shown on the left); 2, pQKH6; 3, pQKH9; 4, pQKH14; 5, pQKH33; 6, pQKH42; 7, pQKH47; 8, pQKH48; 9, pQKH54.

restriction fragments in common. Plasmid pQKH33 was by far the largest plasmid (>200 kb) examined in this study; it was isolated from an exogenous mating experiment completely separate from that of the other seven plasmids. From Fig. 3, pQKH33 can be seen to have a large number of restriction fragments, making comparison difficult, although it seemed to have a number of fragments in common with the other plasmids isolated.

Host ranges of pQKH6, pQKH9, pQKH14, and pQKH54. Conjugal transfer frequencies of these plasmids were estimated by using the mercury resistance phenotype (Table 5). High transfer frequencies were detected between the *P. putida* donor and all strains except *P. aeruginosa* and *Flavobacterium* sp. (where no transfer was detected). The transfer frequencies to *Acinetobacter calcoaceticus* were at least 2 orders of magnitude lower than to other strains, most noticeably with plasmids pQKH14 and pQKH54.

The mobilization frequencies of pD10 by each plasmid into each recipient strain were estimated by transfer of kanamycin resistance (Km^r) or tetracycline resistance encoded by plasmids pD10 and pD10::Ω-Tc, respectively. Mobilization frequencies to *E. coli* were found to be very high in comparison with those to other strains, especially for plasmids pQKH9 and pQKH14 (Table 5). Mobilization frequencies to *Alcaligenes eutrophus* were also high for all four plasmids. All four plasmids were found to be broad host range for transfer of mercury resistance and for mobilization of pD10, although transfer into *Flavobacterium* sp. and *P. aeruginosa* was not detected. Conjugal transfer to *P. maltophilia* and *P. cepacia* was also detected, but mobilization of pD10::Ω-Tc to these strains was not (Table 5).

DISCUSSION

Exogenous isolation of plasmids may give access to plasmids different from those isolated by antibiotic or other direct selections. In this study, plasmids were exogenously isolated solely by their ability to mobilize the recombinant plasmid pD10. Restriction endonuclease digests (Fig. 3) of mobilizing plasmids isolated in this way indicated that exogenous isolation may be biased, since plasmids from the same matings seemed to be most closely related. These could be deletion derivatives of the same original unstable plasmid and may be, therefore, artifacts of the isolation procedure, but it is also possible that they are naturally occurring variants. Plasmids which are structurally related have been isolated in other studies, namely, endogenously isolated mercury resistance plasmids (23) and exogenously isolated plasmids of epilithic bacteria (3). Evidence for deletions and insertions in one original plasmid is also seen in the transfer of pQM3 from its natural *P. cepacia* host into *P. aeruginosa* (39). The number of independent isolations undertaken and the choice of recipient are obviously important considerations for exogenous isolation, and, therefore, caution is necessary in the interpretation of results from such isolations.

Mercury resistance is a common heavy-metal resistance in bacterial isolates from soil and water environments (25, 18).

TABLE 5. Host ranges of pQKH6, pQKH9, pQKH14, and pQKH54

Recipient[a]	Type of frequency[b]	Transfer frequencies from donor UWC5(pD10) containing various mobilizing plasmids			
		pQKH6	pQKH9	pQKH14	pQKH54
E. coli LE392	tra	1.5×10^{-2}	3.3×10^{-2}	4.4×10^{-2}	4.5×10^{-2}
	mob	3.9×10^{-3}	2.6×10^{-2}	3.5×10^{-2}	1.4×10^{-4}
A. eutrophus JMP222	tra	2.1×10^{-2}	1.1×10^{-2}	1.9×10^{-2}	4.5×10^{-2}
	mob	2.8×10^{-3}	4.0×10^{-4}	2.4×10^{-3}	1.9×10^{-3}
P. maltophilia NCTC 10499	tra	4.8×10^{-3}	4.5×10^{-2}	4.8×10^{-2}	1.6×10^{-4}
	mob	$<2.4 \times 10^{-10}$[c]	$<1.3 \times 10^{-9}$[c]	$<1.2 \times 10^{-9}$[c]	$<1.1 \times 10^{-10}$[c]
A. calcoaceticus NCIB 11826	tra	1.5×10^{-4}	9.0×10^{-4}	1.8×10^{-6}	4.7×10^{-5}
	mob	8.6×10^{-8}	5.0×10^{-7}	8.5×10^{-6}	6.4×10^{-7}
P. putida UWC3	tra	1.2×10^{-2}	3.1×10^{-2}	3.8×10^{-2}	9.3×10^{-3}
	mob	6.4×10^{-6}	2.5×10^{-5}	2.7×10^{-5}	2.0×10^{-6}
P. cepacia NCTC 10661	tra	1.0×10^{-1}	3.2×10^{-2}	2.7×10^{-2}	3.9×10^{-5}
	mob	$<1.4 \times 10^{-9}$[c]	$<3.7 \times 10^{-10}$[c]	$<5.8 \times 10^{-10}$[c]	$<1.7 \times 10^{-10}$[c]

[a] No transfer to *P. aeruginosa* or *Flavobacterium* spp. was detected.

[b] Frequencies shown are for transfer of mercury resistance (tra) and for mobilization of pD10 (mob) per recipient from mobilizing plasmid containing *P. putida* UWC5(pD10) or UWC5(pD10.6). Donors and recipients were grown at 30°C, mating pairs were incubated at 30°C for 24 h, and selective plates were incubated at 30°C for up to 7 days. Minimum significant difference ($\log_{10}x$), 1.25: average coefficient of variation between replicate experiments, 15.6%.

[c] Lowest detectable frequency.

Its high incidence in waterways has in some cases been ascribed to industrial pollution. In this study, 19 of 54 plasmids isolated from the epilithon were found to encode mercury resistance. The high proportion of mercury resistance plasmids obtained without direct selection is consistent with the results of Rochelle et al. (41) and Jobling et al. (23), who showed that mercury resistance plasmids are common among aquatic bacteria. Surprisingly, no resistance to UV light was associated with any of the plasmids isolated in this study. UV resistance has been found to be closely associated with mercury resistance in plasmids of epilithic bacteria (3); however, this may be a reflection of the differences in the exogenous isolation methods. In contrast to this study, exogenous isolation of plasmids encoding mercury resistance by Rochelle et al. (41) found that all isolates (of 100 tested) encoded for UV resistance even though direct selection was not made for this phenotype.

Temperature has been shown to significantly influence the frequency of plasmid transfer (38, 41), and our study confirmed that mobilization frequencies were similarly affected. In addition, the temperature at which mobilizing plasmids were isolated was selective. For example, mobilizing plasmids exogenously isolated at 15°C mobilized plasmid pD10 at 20°C at higher frequencies than did plasmids isolated at higher temperatures. This is consistent with the results of Fry and Day (17) who reported that plasmid pQM85, exogenously isolated from the epilithon at 10°C, transferred optimally (with a sharp peak) at the isolation temperature. It would seem reasonable to conclude that temperature is another factor which needs to be considered in exogenous isolation of plasmids from the environment. Figure 2 shows that plasmids pQKH33, pQKH42, pQKH47, and pQKH48 all mobilized at high frequencies at 20°C. Only plasmids pQKH33 and pQKH48 gave temperature profiles with bell-shaped curves similar to, although less accentuated at higher temperatures than, those described in previous studies (1, 41). In contrast, the profiles for pQKH42 and pQKH54 did not decrease significantly from 20 to 37°C, a result which was also seen in a previous study of the mobilization of plasmid pQM17 by RP1 (17). The profile for the mobilization of pHSV106 by *E. coli* χ1784 in L broth also showed this wide optimal temperature range but with a gradual increase in transfer frequency up to 50°C (26). Mobilizing plasmids pQKH6, pQKH9, pQKH14, and pQKH54 were all found to be broad host range and transferred to representatives of the following purple bacteria (56): β-2 (*A. eutrophus* and *P. cepacia*), γ-3 (*P. putida*), γ-4 (*A. calcoaceticus*), and γ-5 (*E. coli*). Conjugal transfer for plasmids pQKH6 and pQKH9 into *P. aeruginosa* has been detected at frequencies of 2.5×10^{-8} and 4.0×10^{-9} per recipient, respectively, on complex media (5a). However, no transfer to *P. aeruginosa* was observed in this study. Such low transfer frequencies may not be detectable on the minimal media used for selection of transconjugants in this study. Transfer to *Flavobacterium* spp. was also not observed. This may also be due in part to the selective media used. However, although RSF1010 (from which pD10 is derived) exhibits a wide host range including *P. aeruginosa* (16), transfer to *Flavobacterium* spp. has not been documented. Therefore, the host range of RSF1010 may be a factor. Frequencies for conjugal transfer of mobilizing plasmids to *P. cepacia* and *P. maltophilia* were found to be high, but no mobilization of pD10::Ω-Tc to these strains was observed. This may also reflect the host range of RSF1010. Frequencies of transfer and mobilization to the different strains used in this study varied considerably. High frequencies for both transfer and mobilization were observed to *E. coli*, but very low frequencies of transfer to *A. calcoaceticus* were observed. Differences between the mobilizing frequencies of the mobilizing plasmids used suggested differences in the efficiency of interaction of the *mob* proteins with the *oriT* of each plasmid (6). Other important factors for broad-host-range replication are encoded by the origin of replication (*oriV*) and *trfA* (*trans*-acting replication functions). In addition, maintenance functions are required for the stable maintenance of broad host range, and this requirement has been shown to vary among gram-negative species (45). However, the molecular basis for broad-host-range transfer is still not fully understood (20).

There are few reports in the literature concerning mobilization of recombinant plasmids in natural environments. In this study, we have successfully isolated plasmids from the epilithon which are capable of mobilizing the recombinant plasmid pD10 at environmentally relevant temperatures. Our observation that a range of plasmids capable of mobilizing plasmid pD10 were readily isolated from epilithic bacteria suggests that transfer of such mobilizing plasmids from indigenous aquatic bacteria to a recombinant *P. putida* strain is highly probable. It follows that recombinant genes, when released to the environment on a mobilizable vector, may be transferred rapidly to the indigenous population, although their selection and survival would depend largely on the advantage(s) which they confer on a new host. This conclusion is supported by evidence from McClure et al. (32), who isolated from the indigenous bacteria of an activated-sludge microcosm, without selection, plasmids which mobilized recombinant catabolic genes to *P. putida* strains. Even chromosomally located recombinant genes may undergo mobilization under environmental conditions, and recent studies show that phage-mediated gene exchange is also an important consideration in freshwater ecosystems (28).

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