Plasmids from freshwater environments capable of IncQ retrotransfer are diverse and include pQKH54, a new IncP-1 subgroup archetype

Anthony S. Haines,¹ Parveen Akhtar,¹ Elton R. Stephens,¹ Karen Jones,¹ Christopher M. Thomas,¹ Caroline D. Perkins,² Jacqueline R. Williams,² Martin J. Day² and John C. Fry²

Correspondence

Christopher M. Thomas
c.m.thomas@bham.ac.uk

¹School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

²Cardiff School of Biosciences, Cardiff University, PO Box 915, Cardiff CF1 3TL, UK

Nine mercury-resistance plasmids isolated from river epilithon were assessed for their ability to retrotransfer the non-conjugative IncQ plasmid, R300B, derivatives of which have commercial uses that may result in accidental or deliberate release into the environment. Retrotransfer frequencies ranging from 2.1×10^{-4} to 1.75×10^{-5} were obtained for five of the nine plasmids – the remaining plasmids showed low or undetectable retrotransfer ability. The majority of the retrotransfer-proficient plasmids could not be classified by the tests used. Classical incompatibility testing with RP4 identified pQKH6, pQKH54 and pQM719 as IncP-1. Hybridization to replicon probes confirmed this for pQKH6 and pQM719 and added pQKH33. PCR with primers designed to amplify *trfA* and *korA* regions of IncP-1 plasmids did not identify any other plasmids. Plasmids pQKH6 and pQM719 but not pQKH54 produced similar *SphI* restriction profiles to the IncP-1 β subgroup. The complete nucleotide sequence of pQKH54 was determined, revealing it to have a complete IncP-1 backbone but belonging to a new distinct subgroup which was designated IncP-1 γ . The results emphasize the ubiquity and diversity of IncP-1 plasmids in the environment but demonstrate that plasmids of as yet unknown groups are also able to retrotransfer IncQ plasmids efficiently.

Received 20 February 2006

Revised 24 May 2006

Accepted 4 June 2006

INTRODUCTION

A range of conjugative plasmids can facilitate the movement of genes in the opposite direction to classical transfer events by a process known as retrotransfer or retromobilization. This process, which was first observed in homologous and intergeneric matings using the IncP-1 plasmid pULB113 (Mergeay *et al.*, 1987), enables capture of chromosomal genes or non-conjugative plasmids by the host bearing the conjugative plasmid. The mechanism of this process, whether it occurs as an indirect consequence of conjugation or is a distinct genetically determined process, has proved controversial (Blanco *et al.*, 1991; Top *et al.*, 1992; Heinemann & Ankenbauer, 1993a, b; Perkins *et al.*, 1994; Ramos-González *et al.*, 1994; Szpirer *et al.*, 1999), but for IncP-1 plasmid RK2 it has been demonstrated that the plasmid must transfer into the recipient before it can mobilize the captured plasmid back to the donor (Sia *et al.*,

1996). Consistent with this model of retromobilization involving two unidirectional steps, it has been proposed that low surface exclusion is important in the mobilizing plasmid (Szpirer *et al.*, 1999), presumably because strong surface exclusion would disrupt mating pairs before transfer back from the recipient had occurred. In addition it seems likely that retromobilization specificity is determined by the VirD/TraG-like coupling proteins (Gomis-Ruth *et al.*, 2002). However, it is not clear how widespread this ability is among uncharacterized plasmids and whether this phenomenon is environmentally important.

Attempts have also been made to link retrotransfer to certain plasmid incompatibility groups. So far, retrotransfer has been demonstrated for the plasmid groups IncP-1 (Mergeay *et al.*, 1987; Powell *et al.*, 1989; Blanco *et al.*, 1991; Heinemann & Ankenbauer, 1993a, b; Smit *et al.*, 1993; Perkins *et al.*, 1994; Hill *et al.*, 1995; Top *et al.*, 1995), IncM and IncN (Thiry *et al.*, 1984; Mergeay *et al.*, 1985), IncF and IncW (Heinemann & Ankenbauer, 1993a, b; Włodarczyk & Piechucka, 1995) and IncP-9 (Ramos-González *et al.*, 1994; Ronchel *et al.*, 2000) in a variety of different bacteria as well as some plasmids that do not belong to well-established groups (Szpirer *et al.*, 1999). The majority of these groups

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of pQKH54 is AM157767.

A supplementary table of biologically relevant reading frames identified in the pQKH54 sequence is available with the online version of this paper.

are recognized as broad host range, possessing mechanisms which allow them to transfer extensively (Mergeay *et al.*, 1990; Szpirer *et al.*, 1999). IncP-1 plasmids have been specifically implicated in retrotransfer and can transfer to an exceptionally wide range of cell types, showing DNA mobilization to Gram-positive bacteria (Trieu-Cuot *et al.*, 1987), cyanobacteria (Kreps *et al.*, 1990) and yeasts (Heinemann & Sprague, 1989).

Allocating plasmids to specific incompatibility groups often proves difficult. Traditional methods of testing are laborious and rely on transfer of a known incompatibility group plasmid into the strain bearing the plasmid under study and checking for displacement of either plasmid (Stanisich, 1988). Displacement occurs as a consequence of two plasmids sharing similar replication control and/or partitioning systems (Kittell & Helinski, 1993; Novick, 1987). So far about 30 incompatibility groups are known for plasmids studied in *Enterobacteriaceae* (Jacob, 1977) and 14 in *Pseudomonas* species (Boronin, 1992). Specific DNA sequences corresponding to genes involved in replication control have been identified for the *Enterobacteriaceae* plasmid groups (Couturier *et al.*, 1988; Davey *et al.*, 1984) and have proved useful in characterizing plasmids of medical importance by replicon typing.

Few studies have assessed the potential of plasmids from natural environments to carry out retrotransfer. However, some retrotransfer-proficient plasmids showing

broad-host-range characteristics have been isolated from natural sources (Hill *et al.*, 1992). In this study we aimed to determine whether plasmids capable of retrotransfer within an aquatic environment are limited to incompatibility groups known already to be capable of retrotransfer. The only known plasmid group identified was the IncP-1 family, although members of this group represented a minority of the plasmids tested. One plasmid, pQKH54, which appeared to be IncP-1 by some tests but not others was characterized by determining its complete genome sequence and proved to belong to a new subgroup of this ubiquitous plasmid family, which we have designated IncP-1 γ .

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2 respectively. Strains were maintained on nutrient agar (NA) or L agar (LA) and grown in nutrient broth (NB) or Luria Broth (LB) containing the appropriate selective markers (Sambrook *et al.*, 1989). Other media used are mentioned in context. Supplements were as follows: nalidixic acid (Nal), 200 $\mu\text{g ml}^{-1}$; rifampicin (Rif), 100 $\mu\text{g ml}^{-1}$; streptomycin (Sm), 1000 $\mu\text{g ml}^{-1}$; kanamycin (Km), 50 $\mu\text{g ml}^{-1}$; tetracycline (Tc), 40 $\mu\text{g ml}^{-1}$; mercuric chloride (Hg), 27.5 $\mu\text{g ml}^{-1}$ in nutrient agar or 13.5 $\mu\text{g ml}^{-1}$ in casein hydrolysate medium. An exception was matings involving *Cytophaga heparina*, where Hg concentrations of 10 $\mu\text{g ml}^{-1}$ were used.

Purification of plasmid DNA and restriction analysis. Plasmids of >100 kb were extracted by the method of Wheatcroft & Williams (1981). For smaller plasmids the alkaline SDS procedure

Table 1. Bacterial strains

Abbreviations: Sm^r, streptomycin resistant; Nal^r, nalidixic acid resistant; Rif^r, rifampicin resistant; Sp^r, spectinomycin resistant; Trp, tryptophan; Ilv, isoleucine, leucine, valine; Met, methionine; 3CB, 3-chlorobenzoate.

Strain	Bacterial subgroup	Characteristic	Source or reference
<i>Pseudomonas putida</i>	<i>Gammaproteobacteria</i>		
PaW340		Sm ^r Nal ^r Trp ⁻	Jeenes & Williams (1982)
UWC1		Rif ^r	McClure <i>et al.</i> (1989)
UWC3		Rif ^r Ilv ⁻	Hill <i>et al.</i> (1992)
UWC7		Nal ^r	Perkins <i>et al.</i> (1994)
UWCB20		Rif ^r Tn5::tdnC*	McClure <i>et al.</i> (1990)
<i>Escherichia coli</i>	<i>Gammaproteobacteria</i>		
LE392		supE44 supF58 lacY1 galK2 metB1 trpR55 hsdR514 galT22 λ^- F ⁻	Sambrook <i>et al.</i> (1989)
DH10B		F ⁻ mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR endA1 thi-1 recA1 gyrA96 relA1 hsdR17 supE44 Δ (ara-leu)7697 galU galK rpsL nupG	Sambrook <i>et al.</i> (1989)
DH5 α		F ⁻ ϕ 80dlacZ Δ M15 endA thi-1 recA1 gyrA96 relA1 hsdR17 supE44 deoR Δ (lacZYA-argF)U169	Hanahan (1983)
<i>Alcaligenes eutrophus</i> JMP222	<i>Betaproteobacteria</i>	3CB ⁺ Rif ^r , prototrophic	Don & Pemberton (1981)
<i>Agrobacterium tumefaciens</i> GMI 9023	<i>Alphaproteobacteria</i>	Sm ^r Rif ^r Sp ^r , prototrophic	Rosenberg & Huguet (1984)
<i>Cytophaga heparina</i>	<i>Bacteroidetes</i>	Km ^r , prototrophic	NCIMB 9290

*Produces a yellow product, 2-hydroxymuconic semialdehyde, on spraying with catechol.

Table 2. Plasmids

Abbreviations: ND, not determined; Pn, penicillin; Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Hg, mercury; Tm, trimethoprim; Sm, streptomycin; Sul, sulphonamide; Tra⁺, conjugative; Mob⁺, mobilizable; R, rifampicin resistance mutant of specified strain. Exogenous isolation recipients: UWC1, UWC3, *Pseudomonas putida*; PAO2002R, PAO348R, *Pseudomonas aeruginosa*; LE392R, *Escherichia coli*. Only those strains actually used in this study, as opposed to during the previously described isolation of these plasmids, are listed in Table 1.

Plasmid	Size (kb)	Known characteristics	Known host range*	Exogenous isolation conditions			
				Temp. (°C)	Recipient	Selection	Source or reference
RP4	60	Pn ^r Tc ^r Km ^r Tra ⁺	BHR	—	—	—	Datta <i>et al.</i> (1971)
pQM899†	66	Pn ^r Tc ^r Hg ^r Tra ⁺	BHR	—	—	—	Bradley (1992)
pUZ8	58	Km ^r Tc ^r Hg ^r	BHR	—	—	—	Hedges & Matthew (1979)
R751	53	Tm ^r Tra ⁺	BHR	—	—	—	Jobanputra & Datta (1974)
pJP4	80	Hg ^r Tra ⁺	BHR	—	—	—	Don & Pemberton (1985)
pSS50	53	Hg ^r Tra ⁺	BHR	—	—	—	Hooper <i>et al.</i> (1989)
R300B	8.7	Sm ^r Sul ^r Mob ⁺	BHR	—	—	—	Barth & Grinter (1974)
pUC18	2.686	Ap ^r	NHR	—	—	—	Norlander <i>et al.</i> (1983)
pGEMT-Easy	3.015	Ap ^r	NHR	—	—	—	Promega
pCR4Blunt-TOPO	3.957	Ap ^r Km ^r	NHR	—	—	—	Invitrogen
Epilithic plasmids							
pQKH6	60	Hg ^r Tra ⁺	ABG	30	UWC3	Mob ⁺	Hill <i>et al.</i> (1992)
pQM705	60	Hg ^r Tra ⁺	G	20	LE392R	Hg ^r	Bradley (1992)
pQM719	59	Hg ^r Tra ⁺	G	20	LE392R	Hg ^r	Bradley (1992)
pQKH54	80	Hg ^r Tra ⁺	ABG	15	UWC3	Mob ⁺	Hill <i>et al.</i> (1992)
pQM85	100	Hg ^r Tra ⁺	BG	10	UWC1	Hg ^r	Fry & Day (1990)
pQKH33	>300	Hg ^r Ap ^r Tc ^r Km ^r Tra ⁺	ND	20	UWC3	Mob ⁺	Hill <i>et al.</i> (1992)
pQM1	254	Hg ^r UV ^r Tra ⁺ IncP13	BG	20	PAO2002R	Hg ^r	Bale <i>et al.</i> (1987)
pQM62	256	Hg ^r Tra ⁺	G	15	UWC1	Hg ^r	Bale <i>et al.</i> (1988)
pQM125	>300	Hg ^r Tra ⁺	ND	20	PAO348R	Hg ^r	Rochelle <i>et al.</i> (1989)
pQM714	70	Hg ^r Tra ⁺	G	20	LE392R	Hg ^r	Bradley (1992)

*16S rRNA classification subgroups of Proteobacteria to which plasmids can transfer. BHR, broad host range, can be transferred to a wide range of Gram-negative bacteria (A=α, B=β, and G=γ 16S rRNA subgroups of the Proteobacteria listed in Table 1). ND, Not determined.

†Hg^r transposon Tn501 inserted into the Km^r gene of RP4 to produce plasmid pQM899.

was used (Birnboim & Doly, 1979; Kado & Liu, 1981). Further purification was by CsCl gradient for larger-scale preparations (Sambrook *et al.*, 1989). Both standard and pulsed-field gel electrophoresis (PFGE) were carried out to obtain accurate size measurements for restriction bands. PFGE was performed by the CHEF DR II system with 0.5 × TBE (Sambrook *et al.*, 1989) at 8 °C using 1.2 % pulsed-field-grade agarose gels (Bio-Rad pulsed-field certified). Gels were run for 18 h, 150 V at 4 s pulse times, followed by 6 h, 110 V at 2.5 s pulse times. Ethidium-bromide-stained gels were scanned using a scanning densitometer and similarity between plasmid profiles was calculated by matching of peak positions using GelManager for Windows (BioSystematica). Dendrogram plots were produced as a result of clustering by UPGMA (unweighted pair group method using averages) on *SphI* digests of each plasmid, performed in buffers according to the manufacturer's instructions. The multiple regression method (Rochelle *et al.*, 1985) was used to calculate fragment sizes based on migration of known molecular mass markers.

Conjugative transfer. Overnight NB cultures of donor (100 µl) and recipient (1000 µl) strains were mixed and mating allowed to proceed on a 0.45 µm cellulose nitrate filter which was placed on a NA plate for 24 h at 20 °C or 30 °C. The bacteria were then resuspended in 2–5 ml 1/10 × NB and c.f.u. enumerated by standard techniques on NA at 30 °C containing appropriate antibiotics (Bale

et al., 1987). Frequencies of conjugal transfer and retrotransfer were determined from isogenic matings between *Pseudomonas putida* UWC20, bearing the conjugative plasmids, and UWC7(R300B). Transconjugants were selected on NA + Nal + Sm + Hg and retro-transconjugants on NA + Rif + Sm + Hg. In mobilization UWC7 was the donor, containing both the conjugative plasmid and R300B. Transfer of R300B to UWC20 was selected on NA + Rif + Sm. For plasmid host-range studies, PaW340 bearing the Hg^r plasmid was mixed with a range of recipients. Casein hydrolysate medium (Oxoid L41), which is deficient in tryptophan, was used to select against PaW340, with additions of Hg to select for transconjugants. Donors were enumerated on *Pseudomonas* selective medium (Oxoid CM559) plus ceftrimide and nalidixic acid (Oxoid CN selective supplement SR102) and Hg. Representative colonies of putative transconjugants and retrotransconjugants were checked for the relevant plasmids. Transfer frequencies were compared by analysis of variance using log₁₀-transformed data to ensure homogeneity of variance and normality of error distribution. All means are expressed as geometric means and transfer frequencies as transconjugants per recipient. Mean values were compared by least significant difference (LSD) or minimum significant difference (MSD), using the Tukey–Kramer test for the latter, as appropriate.

Probes used in hybridization studies. Probes for replicon typing as defined by Couturier *et al.* (1988) were prepared by digestion

of purified plasmid DNA with appropriate restriction enzymes, separated by gel electrophoresis and eluted from low-melting agarose using the GeneClean II kit (Bio Inc.). Probes for the following incompatibility groups were used: IncY (1245 bp), IncT (304 bp), IncX (942 bp), IncL/M (800 bp), IncU (950), IncQ (357 bp), IncFIA (917 bp), IncFIB (1202 bp), IncB/O (1600 bp), rep9 (539 bp), IncHI1 (2250 bp), IncHI2 (1800 bp) and IncP (750 bp). Probes were denatured in a boiling water bath for 10 min and labelled by random priming using dioxigenin (DIG)-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. Additional probes were prepared from the plasmids R751 and pQKH6. R751 (Thorsted *et al.*, 1998) DNA was digested with *Pst*I, producing five fragments corresponding to: (i) Tra2/Tra3/*trfA* region; (ii) Tra1/*oriT*/primase; (iii) Tn5090/404, encoding trimethoprim resistance; (iv) *oriV*/Tn4321; and (v) *korA*/*korB*. These were separated by gel electrophoresis and purified as specified using the Glassmax DNA Isolation Spin Cartridge system (Gibco-BRL). As Tn4321 has shown some homology to the mercury-resistance transposon Tn501 it was separated from the *oriV* fragment by digestion with *Eco*RI and purification of the two smallest fragments. Plasmid pQKH6 DNA was digested with *Eco*RI and the largest six fragments were separated by the same technique. These probes were denatured as above and labelled with 32 P using Amersham Multiprime according to the manufacturer's instructions. After labelling, unincorporated 32 P was removed using Sephadex G50 columns (Sambrook *et al.*, 1989).

Southern blotting and colony hybridization. Extracted plasmid DNA was digested with *Eco*RI and separated by gel electrophoresis with appropriate replicon controls. Chromosomal DNA of the plasmid host strain was extracted (Austubel *et al.*, 1989) and included to show any background hybridization. Gels were washed and blotted overnight onto nylon membrane (Hybond-N, Amersham) using standard procedures (Sambrook *et al.*, 1989). DNA was fixed by UV irradiation. Membranes were hybridized with DIG-labelled incompatibility probes following the Boehringer Mannheim DIG Nucleic Acid Detection kit instructions. The chemiluminescent detection technique was carried out using Lumigen PPD. Membranes were exposed to X-ray film at room temperature for 30 min to 3 h. For colony hybridization NB cultures were used to inoculate circular membranes on tryptone-based medium, incubated overnight at 30 °C; then the cells were lysed and the DNA was denatured, neutralized and fixed by standard techniques (Sambrook *et al.*, 1989). After washing and prehybridization, 32 P-labelled probes were used to detect related sequences and signals detected by exposure to X-ray film.

PCR amplification and sequencing of selected IncP-1 regions. Degenerate oligonucleotide primers for IncP-1 control regions were designed previously (Thomas & Thorsted, 1994). A 241 bp *trfA* product (based on plasmid RK2) was expected using the forward primer 5'-CGA AAT TCR TRT GGG AGA AGTA-3' and reverse primer 5'-CGY TTG CAA TGC ACC AGG TC-3'. For the *korA* gene, a 294 bp (based on RK2) sequence was amplified by the forward primer 5'-ATG AAG AAA CGG CTN ACC GA-3' and reverse primer 5'-TTC CTG TTT YYT CTT GGC GTC-3'. All primer sequences were compared with GenBank nucleotide sequence data for possible homologies with other non-target sequences. Total DNA extracts were from 1.5 ml overnight cultures as described by Austubel *et al.* (1989). Amplification and analysis of products used standard procedures (Sambrook *et al.*, 1989). Products were sequenced directly using the PCR primers or cloned into pGEMT-Easy and then at least three clones were sequenced from universal primers to eliminate errors that might have occurred during amplification.

IncP-1 incompatibility testing. Incompatibility tests were performed in *P. putida* strains. Transfer frequencies into plasmid-bearing bacteria at 30 °C for both RP4 and the mercury-resistance

plasmids were determined as described above, selecting for the incoming plasmid only (Stanisich, 1988). After overnight incubation, four colonies were removed from each of the transconjugant plates, either NA + Rif + Km in the case of RP4 transfer or NA + Sm + Hg for mercury-resistance plasmid transfer. Cells were resuspended in 2 ml 1/10 × NB, and 40 µl aliquots of each colony suspension were pipetted into 48 wells of a microtitre plate followed by replica plating to NA plates and separate NA plates containing individual and both selective markers. After overnight incubation at 30 °C replica plating from growth on NA plates was repeated to determine if growth in non-selective conditions would allow the plasmids to segregate. This was repeated to give over 100 generations of unselected growth. Failure to grow on one or other of the selective plates then indicated incompatible plasmids. DNA was regularly extracted and checked for the correct plasmids and absence of interplasmid recombination. To differentiate surface exclusion effects from true incompatibility, control matings were performed with RP4 and mercury-resistance plasmids into plasmidless recipients of PaW340 or UWCB20. Additional controls were performed by mating known IncP-1 plasmids together and examining the degree of segregation.

Sequencing of pQKH54 and sequence accession number.

Subclone libraries of pQKH54 in pUC18 vector were initially generated by cloning *Sph*I fragments, and partial *Taq*I or *Sau*3AI digests of the two large *Sph*I fragments. These were sequenced using an ABI373 automated sequencer. Later, subclones were generated using randomly sheared fragments with the TOPO Shotgun Subcloning kit from Invitrogen, using an ABI3700 sequencer. Finishing reads used custom primers on clones or PCR products. A few PCR products were cloned using the Promega pGEM-T Easy vector system. The sequence was produced and assembled using the Phred/Phrap/Consed package (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998, 2001). Analysis and annotation of the sequence used Artemis and ACT (Rutherford *et al.*, 2000) and WU-BLAST2 (<http://blast.wustl.edu/>). The nucleotide sequence of pQKH54 has been submitted to the EMBL database (GenBank/EMBL/DBJ accession no. AM157767). After consultation with the IS database in Toulouse, France (<http://www-is.biotoul.fr/>), the two new IS elements identified in the DNA sequence have been designated ISUnCu3 (position 13002 to 15597 in the sequence) and ISUnCu4 (position 39749 to 40861 in the sequence) because the identity of the original bacterial host for the plasmid was not known and uncultured.

Construction of phylogenetic tree. To generate the phylogenetic tree for IncP-1 plasmids, protein sequences were aligned using PILEUP (Wisconsin Package, Devereux *et al.*, 1984) and the alignments concatenated. A neighbour-joining tree was generated using CLUSTALX, with gaps excluded and correction for multiple substitutions.

RESULTS

Retrotransfer matings

Conjugation frequencies of all the plasmids used in this study were relatively high, ranging from about 10^0 to 10^{-3} transconjugants per recipient (Table 3). Five of the nine plasmids isolated from epilithon showed significant retro-transfer frequencies, ranging from 2.1×10^{-4} to 1.8×10^{-5} , which were generally 2–3 orders of magnitude lower than the respective conjugation frequencies. The highest retro-transfer frequency detected among the plasmids studied (3.6×10^{-3}) was shown by the IncP-1 β plasmid pSS50, which was 15–30 fold higher than the epilithic plasmids pQKH6, pQM705 and pQM719. For plasmids pQM1,

Table 3. Summary of characteristics of plasmids studied in this work

Plasmid	Hybridization* R751 probes	Hybridization† pQKH6 probes	IncP-1 by replicon probing‡	Excluded by/excludes RP4§	PCR <i>trfA/korA</i>	IncP-1β by restriction profile¶	Transfer/Retrotransfer /Mobilization#	Host range††
RP4	Be	a	+	ND	+ / +	—	10 ⁻¹ /10 ⁻⁴ /10 ^{-1**}	ND
R751/pJP4	ABCDE	AB	+	+ / +	+ / +	+	ND	ABG
pSS50	ABDE	ABCE	+	ND	+ / +	+	10 ⁻¹ /10 ⁻³ /10 ⁻²	ABG
pQKH6	ABCDE	ABCDE	+	+ / +	+ / +	+	10 ⁻¹ /10 ⁻⁴ /10 ^{-2**}	ABG
pQM719	ABCdE	AbcE	+	+ / +	+ / —	+	10 ⁻¹ /10 ⁻⁴ /10 ^{-2**}	ABG
pQKH54	bde	ABCDE	—	+ / +	— / —	—	10 ⁰ /10 ⁻⁵ /10 ^{-2**}	aBG
pQM85	B	E	—	— / —	— / —	—	10 ⁻² /10 ⁻⁴ /10 ⁻⁴	BG
pQM705	B	None	—	— / —	— / —	—	10 ⁻¹ /10 ⁻⁴ /10 ⁻⁴	G
pQM62	Cd	e	—	— / —	— / —	ND	10 ⁰ /10 ⁻⁸ /10 ^{-7**}	G
pQM125	b	cE	—	— / —	— / —	ND	10 ⁻¹ /10 ⁻⁷ /10 ^{-6**}	ND
pQM714	None	cE	—	— / —	— / —	ND	10 ⁻³ /UD/10 ^{-4**}	B
pQM1	None	e	—	— / —	— / —	ND	10 ⁻³ /UD/10 ⁻⁷	BG
pQKH33	D	e	+	ND	— / +	ND	ND	<i>P. putida</i>

ND, Not done; UD, undetectable.

*R751 *Pst*I probes: A, trb-trfA; B, tra-oriT; C, Tn402; D, korAB; and E, oriV. Upper-case letter means strong hybridization; lower-case letter means weak hybridization.

†pQKH6 *Pst*I probes: A, 38·4 kb; B, 10·1 kb; C, 7·0 kb; D, 3·1 kb; E, 2·8/2·7 kb. Upper-case letter means strong hybridization; lower-case letter means weak hybridization.

‡Hybridization was performed with all the replicon probes described in Methods.

§IncP-1 incompatibility was assessed by determining (a) whether the presence of RP4 reduced the ability of test plasmids to transfer into PaW340 or (b) whether the presence of the test plasmid reduced the ability of RP4 to transfer into PaW340. Results are means from replicate matings ($n=2$). Two-way or one-way ANOVA of transfer frequencies showed significant difference between plasmid ($P<0\cdot001$) and transfer mechanism ($P<0\cdot001$).

||PCR was performed as described in Methods.

¶*Sph*I digestion was carried out as described in the text.

#All plasmids showed a significant difference between conjugation and retrotransfer frequencies. UWC7(R300B) is classified as the donor strain in retrotransfer mating. **Indicates a significant difference between the mobilization and retrotransfer frequencies by MSD comparisons.

††Host range is indicated in the same way as in Table 2. Upper-case letters are used when tested species/strains of that group could receive the plasmid; lower-case letters indicate that transfer frequency into the test strain for that group is significantly reduced. The host range of RP4 was not tested in this study.

pQM62, pQM125 and pQM714, retrotransfer was either undetectable or was just within the limits of detection. All plasmids able to retrotransfer well (at frequencies $>10^{-5}$) could also mobilize R300B at frequencies of $>10^{-4}$. Plasmids showing low or undetectable retrotransfer generally had a lower mobilization frequency. An exception was pQM714, which showed similar conjugation and mobilization frequencies but retrotransfer could not be detected.

Plasmid host range

Host range was tested using *P. putida* PaW340 as donor against the various representative species listed in Table 1 as recipients: *Escherichia coli*, *Alcaligenes eutrophus*, *Agrobacterium tumefaciens* and *Cytophaga heparina*. Transfer to the *C. heparina* strain could not be detected for any of the plasmids tested (Table 3). However, the RP4 derivative plasmid pQM899 and the IncP-1β plasmids showed a broad host range, being transferred and maintained in all subgroups of the Proteobacteria. Epilithic plasmids pQKH6

and pQM719 showed a pattern of transfer similar to that of the IncP-1β plasmids pJP4 and pSS50, with higher transfer frequencies to *A. tumefaciens*. The transfer frequency of pQKH54 was somewhat lower to this alphaproteobacterial species but high transfer frequencies, as shown by pQKH6 and pQM719, were detected to the gamma- and betaproteobacteria subgroups. The remaining plasmid tested, pQKH33, only showed a higher transfer frequency to *P. putida*, with transfer to the other strains at or below the limit of detection.

Classification of plasmids by hybridization with labelled probes

The only replicon probe that gave a positive signal was that for IncP-1, which showed strong Southern hybridization with plasmids pQKH6, pQM719 and pQKH33 at both high and low stringency using RP4 as a positive control (Table 3). No other probes reacted with any of the plasmids under study, even at low stringency, with the probes for the

broad-host-range plasmid groups IncN, IncW and IncQ. Plasmid pQM1 has previously been shown to belong to the IncP-13 group of the pseudomonads (Bale *et al.*, 1988) so is unlikely to produce a signal by *E. coli* replicon typing.

Plasmids belonging to the IncP-1 β subgroup have previously been differentiated from the IncP-1 α subgroup based on characteristic *Sph*I fragment pattern (Burlage *et al.*, 1990). After *Sph*I digestion a number of identical bands were observed between pQKH6 and pQM719 and the IncP-1 β plasmids R751 and pSS50 (data not shown), amounting to a similarity of 50–60% as indicated in the dendrogram in Fig. 1, which shows just those plasmids that we suspected of being IncP-1 and an outlier, pQM705. RP4, which belongs to the IncP-1 α subgroup, did not show these characteristic fragments, indicating a lack of similarity of pQKH6 and pQM719 to the IncP-1 α subgroup. Plasmid pQKH33 was not examined because it was much larger than the other plasmids (>300 kb) but additional resistance markers it carries to Ap, Tc and Km are common to the IncP-1 α subgroup and suggest that it may belong to this group. No significant similarities were observed in the *Sph*I digestion patterns of pQKH54, pQM85, pQM705 and the known IncP-1 β plasmids.

All the plasmids were probed with the fragments from R751 and pQKH6 (Table 3). As expected from the results above, pSS50, pQKH6 and pQM719 hybridized with almost all of the R751 fragments. Regions of common homology were shown for the fragments containing the *Tra*I and *oriV*

Fig. 1. Dendrogram showing degree of similarity of various plasmids isolated from the epilithon to the IncP-1 β plasmids R751 and pSS50 based on the similarity of their restriction patterns after digestion with *Sph*I. See Methods for details.

regions of the different IncP-1 subgroup plasmids RP4 and R751. Of the remaining plasmids only RP4, pQKH54 and pQM62 hybridized strongly with more than one R751 fragment. Plasmids pQM719 and pSS50 also showed a high degree of similarity with the majority of pQKH6 fragments, all of which hybridized to pQKH54. The other epilithic plasmids failed to hybridize with most of the pQKH6 fragments with the exception of the smallest two fragments (5/6). Hybridization of these latter fragments with 9 out of 10 (now including pQKH33) epilithic plasmids and pNMM1 indicated that they encode mercury-resistance genes possessing some homology to *Tn501* present on this vector; such genes are widespread and abundant in the environment (Barkay *et al.*, 2003).

IncP-1 incompatibility testing

Since the only group identified by hybridization is IncP-1 we determined whether this genetic relatedness was manifest as classical incompatibility. Transfer of the epilithic plasmids to PaW340(RP4) identified three, pQKH6, pQM719 and pQKH54, which could displace RP4 (Table 3). Reduction in transfer due to surface exclusion was also exerting an effect, as transfer of these plasmids to a plasmidless PaW340 recipient was up to 250-fold higher. As results for the IncP-1 plasmid pJP4 were very similar, pQKH6, pQM719 and pQKH54 were classified as IncP-1 plasmids by this approach. The other plasmids showed no sign of incompatibility to RP4 in this direction although a small degree of surface exclusion was detected for pQM1 and pQM125. Displacement of the epilithic plasmids by RP4 (Table 3) only identified one plasmid, pQKH6, as belonging to the IncP-1 group. Plasmids pQM1, pQM62, pQM125 and pQM714 were again clearly compatible. For the remaining plasmids, pQM705, pQM719, pQKH54 and pQM85, it took some time before a compatible coexistent relationship could be established and both plasmids were clearly visible with no signs of cointegrate formation after extraction and electrophoresis. This suggested a degree of similarity between the replication control genes of these plasmids. Unfortunately pQKH33 could not be tested by this procedure due to its additional markers, Ap, Tc and Km, which prevented selection for RP4.

PCR using *trfA* and *korA* primers

PCR amplification using the *trfA* primers was detected in only 2 out of 10 (including pQKH33) of the plasmids isolated from epilithon (Table 3). A 241 bp product was obtained for plasmid controls from the IncP-1 α group, RP4 and pUZ8, and the IncP-1 β subgroup, R751, pSS50. A product of the same size was detected for plasmids pQKH6 and pQM719 but not for any of the other plasmids under study. Amplification using the *korA* primers again resulted in two plasmids producing a similar sized product to the controls, namely pQKH6 and pQKH33. Plasmid pQM719 showed no product, supporting results of colony hybridization with the *korA/korB* R751 probe, which gave only a weak signal, whereas pQKH33 gave a strong hybridization signal

with this probe and a *korA* PCR product (Table 3). The origin of the PCR products for *trfA* and *korA* products from R751, pSS50 and pQKH6 was confirmed by sequencing. Sequence alignment showed that pSS50 and pQKH6 were indistinguishable on this basis and that their closest sequenced IncP-1 β relative was pJP4 (Smith & Thomas, 1987; Trefault *et al.*, 2004), to which they showed >99 % identity, whereas they were clearly different from R751, as expected from the described diversity for this IncP-1 subgroup (Droge *et al.*, 2000).

DNA sequencing and the IncP-1 backbone of pQKH54

The results described above indicated that pQKH54 is related to IncP-1 plasmids but differs with respect to incompatibility reactions, backbone restriction fragment profiles and sequence similarity since it gave negative results with the Couturier IncP-1 probe and the PCR test with *trfA* and *korA* primers (Table 3). This suggested that pQKH54 may represent a new branch of the IncP-1 family. Therefore its DNA sequence was determined as described in Methods, resulting in a complete genome of 69 966 bp. The results are summarized in supplementary Table S1, available with the online version of this paper. They confirmed that pQKH54 is indeed a typical IncP-1 plasmid on the basis that it carries all the key gene blocks of the IncP-1 group: the minimal replicon *oriV* and *trfA*; the transfer origin *oriT* and associated transfer gene blocks *tra* and *trb*; the central control/partitioning region *korA-korB*, and associated auxiliary operons *kfr*, *kle* and *klc*. In general the promoter regions for these transcriptional units are typical of other IncP-1 plasmids and have obvious candidate operators for the IncP-1 global regulators, so it seems that the essential features of the regulatory network coordinating the genes for the basic plasmid functions of replication, stable inheritance and transfer are intact.

The G + C content of the backbone is generally lower than for the other subgroups. If the *trb* region is excluded beyond the end of *trbL* (as the majority of these genes are unique to pQKH54), then the mean G + C content of the backbone is 56.5 mol%, while it is 63.1 mol% for RK2 (Pansegrau *et al.*, 1994), 66.6 mol% for R751 (Thorsted *et al.*, 1998) and 62.0 mol% for pEST4011 (Vedler *et al.*, 2004). Thus it appears that pQKH54 has spent a considerable proportion of its recent evolutionary history in species with a lower G + C content than the other branches of the IncP-1 family. While the overall structure of the backbone is conserved, there are several differences at the gene level, with genes common to the other IncP-1 groups either being deleted or replaced, or a novel gene inserted. In particular a new operon apparently comprising three small genes was identified upstream of, and reading towards, the *klc* region. The predicted promoter has KorC and KorA binding sites. Therefore this appears to be part of the IncP-1 KorA/C regulon for this plasmid. Of the *kle* region, only *kleE* remains, preceded by a new gene we have called *kleH*. *klcB* is divided into two, and may be inactivated but may also

simply provide the multiple functions associated with this region by way of two polypeptides rather than one, since there is a reasonable putative ribosome-binding site preceding the second ORF, *klcB2*. The downstream end of the *trb* operon is the most drastically changed. Starting from the promoter end of the operon, a new gene, *trbQ*, replaces *trbM*. *trbR* replaces *trbO*, with *O_B7* (typically at the start of *trbO*) being found at the end of *trbR*; a completely new terminus for the operon follows *trbP*, consisting of four or five genes. An IS element appears to have inactivated what may be the last gene. The novel genes in this region are generally of a lower G + C content than the rest of the backbone. The *tra* operon carries only two new genes: a small potential regulator *traP* (on the basis of similarity to a phage regulatory protein) replacing *traA*, and a new gene *traQ* (that has no significant matches to anything in the database) between *traF* and *G*.

In the standard IncP-1 replication origin the iterons to which the Rep protein binds are organized as groups of 1, 3, 5 and 1 (in reverse), whereas in pQKH54 they are 1, 2, 5 and 2 (in reverse). Only the group of 5 is essential in RK2 (Thomas *et al.*, 1981; Stalker *et al.*, 1981). The number of auxiliary (non-essential) iterons is thus constant, which may be important since this has been shown to affect copy number control and incompatibility (Cross *et al.*, 1984; Shah *et al.*, 1995). The consensus sequence for the iterons has also changed – most significantly with the GC of the conserved TGAGGGGC motif being replaced by AT (Fig. 2), which may correlate with changes in the putative HTH motif of TrfA observed in pairwise alignments (data not shown).

The degree of DNA backbone sequence similarity to archetypal IncP-1 plasmids ranges from 69 and 67 % (for RK2 and R751 respectively) for 2.3 kb of the central control region, through 65 and 66 % for up to 15 kb of the *trb* region, to 62 and 64 % for up to 15 kb of the *tra* region. This explains why detection of similarity to IncP-1 plasmids by the hybridization methods described above, which depends on base pairing to a defined DNA sequence, was only partially successful. The equivalent comparison between RK2 and R751 themselves showed 74, 73 and 70 % sequence identity for these three regions. Phylogenetic analysis was carried out with the sequences of key genes (separately and pooled) from the regulon (Fig. 3), which showed clearly that pQKH54 is well separated from the IncP-1 α and β groups. We therefore designated this plasmid as belonging to IncP-1 γ . The existence of this third group has already been referred to as ‘personal communication’ on the basis of conference presentations (Adamczyk & Jagura-Burdzy, 2003). Because of this prior designation, another recently described IncP-1 plasmid, pEST4011, was designated IncP-1 δ (Vedler *et al.*, 2004) after the authors of that paper had compared their sequence with that of TrfA of pQKH54 supplied by us. One key point is that pQKH54 lacks evidence of the blocks of inverted repeats and restriction sites typical of the inter-backbone segment regions of IncP-1 β plasmids

Fig. 2. Sequence logos of iterons for representatives of the four known IncP-1 subgroups. In a DNA sequence logo (Schneider & Stephens, 1990; Crooks *et al.*, 2004), the height of each base represents its conservation, with the information content of each position measured in bits. There are some minor variations between the groups, which may to some extent be due to neutral mutations. However, the consensus for pQKH54 shows a very clear difference of the last two bases. This suggests that this replicon is the most distant member of the IncP-1 group yet found, confirming the phylogenetic tree (Fig. 3), and possibly explaining the only partial incompatibility phenotype against RP4.

(Thorsted *et al.*, 1998). Comparison with IncP-1 α and δ plasmids indicates that these repeats are confined to the β group, which raises further questions about their origin and functional significance. It seems that they are a recent acquisition rather than an ancestral remnant.

Genetic cargo carried by pQKH54

There are two locations of IncP-1 plasmids where insertions into the backbone are typically found – between *oriV* and *trfA* and between the *tra* and *trb* operons. At these two areas, pQKH54 carries a total of around 27 kb of genetic load, or apparently non-backbone material. The mercury resistance of pQKH54 is encoded in a region between *tra* and *trb*, in a cassette which is virtually identical to the mercury-resistance portion of Tn5053 (Kholodii *et al.*, 1993,1995). The rest of this mobile element is mosaic in structure, with only a terminal match to Tn5053, suggesting that it is probably not self-transposable, being dependent on another element to provide the transposase function. Most remarkably, it contains a segment that is a very close match to part of *traC* of the IncP-1 β plasmid R751. This appears to have been acquired by the transposon through an inexact transfer or recombination event from an ancestor of pADP-1 because this plasmid contains the appropriate conjunction of transposable element and IncP-1 backbone. A possible scheme for how this might have occurred is shown in Fig. 4. This proposed scheme is important because it suggests that IncP-1 plasmids are sufficiently common in the environment as to have a reticulated history. Adjacent to this, in the mosaic region, are two more apparently complete genes. One of these shows some similarity to a sterol desaturase, while divergent from this is an AraC-family regulator (Gallegos *et al.*, 1997).

Fig. 3. Phylogenetic tree for IncP-1 plasmids currently available in public databases, using several proteins. Primary sequences of KorA, IncC2, KorB, KorC and KfrC (Upf54.4) were joined together and then aligned for IncP-1 plasmids plus a plasmid of the IncU group, which carries versions of these genes (Rhodes *et al.*, 2004). The alignment was then used to build the phylogeny. The plasmids pB10 and pJP4 are not included since they appear to be chimeric products of recombination between pB4-like and R751 subgroup plasmids.

Fig. 4. Proposed lineage of the mercury-resistance element of pQKH54 explaining how it may have acquired a segment of IncP-1 backbone from an insertion as in pADP1, resulting in duplication of the *traC* region in pQKH54. Insertion or recombination events are shown as lines which converge to a point; lines indicating the resultant sequence do not. Mobile elements are shown as patterned boxes, as is gene *traC*. Terminal inverted repeats are shown as outward-facing triangles. Elements whose existence we have inferred are shown interrupted with diagonal stripes. Not to scale. The events are as follows. A. IS1071 inserts into Tn5053. B. With a second copy of IS1071 it transposes into an IncP-1 β plasmid like pADP1 adjacent to *traC*. C. Some sort of aberrant transposition or recombination brings heterogeneous DNA adjacent to *traC*. This DNA has or acquires another copy of Tn5053. D. The majority of this Tn5053 is deleted by homologous recombination between two copies of $\kappa\gamma$, a short 'parasitic' mobile element. Also, in either this or the preceding stage, two small deletions occur internal to IS1071. E. This leaves an element which can presumably be mobilized by Tn5053 transposase supplied *in trans*.

Nearby on one side of this transposon is an IS (ISUnCu3), while adjacent to this element is a Tn5053-related resolvase (Kholodii *et al.*, 1993). This probably represents the remnants of a previous insertion. Between *oriV* and *trfA* there is a large segment of non-backbone material. Despite a region of homology including a two-component transposase on pB4 and an adjacent orphan gene, we were unable to identify matching termini for a transposon in this region. Neighbouring the pB4 homologous region is what appears to be an operon of genes and a divergently expressed AraC-family regulator.

DISCUSSION

This work has clearly shown that while all the plasmids studied are capable of conjugal transfer, not all are able to mobilize R300B efficiently and that there is not a consistent relationship between the efficiency of mobilization and that of retrotransfer of R300B. This confirms that retrotransfer is plasmid-specific and not a property of all conjugation systems (Guiney, 1993). However, at least for IncP-1 plasmids, the self-transmissible plasmid must move from the donor to the recipient before promoting

retromobilization (Sia *et al.*, 1996), so one would predict that retromobilization should obey the specificity rules of the TraG/VirD4 coupling protein family (Llosa *et al.*, 2003). This does hold to the extent that plasmids that retromobilize efficiently are also generally good at mobilization, although it might be expected that mobilization frequency would be higher than retromobilization, which is not the case for pQM85 and pQM705 (Table 3). Therefore additional factors may be involved and, as with certain examples of mobilization, retrotransfer may reveal plasmid-pair specific properties (Willetts, 1981; Willetts & Crowther, 1981). Where mobilization is efficient but retromobilization is not, it may be that surface exclusion, as suggested by Szpirer *et al.* (1999), can explain the results, so that pQM719 expresses proteins that cause the mating bridge to break down before the reverse transfer can occur. Detailed comparison of pQM719 with other IncP-1 plasmids may reveal differences in surface exclusion proteins that explain why it does not behave as expected from its apparent classification.

Reinforcing their importance as agents of retromobilization, the screening identified new IncP-1 plasmids, although not all of them initially gave clear-cut results. For pQM719, the

mating technique used to examine plasmid incompatibility did not produce bidirectional incompatibility, which may be due to a second replicon in this plasmid. This would be consistent with pQM719 not being a completely typical IncP-1 plasmid, as already suggested in the previous paragraph. However, *SphI* restriction profiles, colony hybridization and the PCR suggest that both pQM719 and pQKH6 are related to the IncP-1 β subgroup (Table 3) and have host ranges similar to the IncP-1 β plasmids examined.

By contrast pQKH54 showed inconsistent plasmid grouping by the phenotypic and genotypic tests used, suggesting a degree of similarity with IncP-1 plasmids but insufficient sequence identity to react with the *repP* probe from the Couturier collection (Couturier *et al.*, 1988). Similar problematic results were obtained for incompatibility testing of plasmids pQM705 and pQM85 (Table 3), indicating that although similarities may exist to the replication and maintenance genes of RP4, enough differences are present to allow maintenance of both systems after continuous growth in non-selective conditions. Explanations for such asymmetric incompatibility include the occurrence of a replicon fusion followed by deletion events to leave P-1 group incompatibility determinants on plasmids with alternative replication systems (Smith & Thomas, 1989). Because the divergence of pQKH54 from RK2 and R751 made it difficult to use hybridization or PCR to assess its relatedness to the IncP-1 group, sequencing the whole genome proved an efficient analytical approach. The sequence revealed a surprisingly conserved IncP-1 backbone. It is therefore surprising that it showed efficient transfer but relatively inefficient retrotransfer (Table 3). The sequence may provide the basis for a detailed analysis of the surface exclusion functions as discussed above.

While pQKH54 clearly belongs to the IncP-1 family it is distinct from IncP-1 β plasmids and appears to be somewhat less broad host range than pQKH6 and pQM719. This would also be consistent with the lower G + C content of the genome since it suggests adaptation to a different group of hosts. The restriction map of pQKH54 predicted from the DNA sequence (data not shown) exhibited clustered regions of sites, coincident with the insertions of transposable elements or phenotypic determinants, a characteristic of all typical IncP-1 plasmids (Thomas & Smith, 1987; Smith & Thomas, 1989). Nevertheless pQKH54 does not possess the clusters of repeats associated with restriction sites (Thorsted *et al.*, 1998; Heuer *et al.*, 2004) that are now clearly associated only with IncP-1 β plasmids. Despite the absence of these putative hot-spots for acquisition of transposable elements the only insertion into the backbone of pQKH54 occurs in one of these regions. The contiguous *oriV*-*rep* region confirms the nature of the ancestral backbone backbone as previously predicted by Smith *et al.* (1993) and first observed by Heuer *et al.* (2004).

Preliminary analysis for pQKH6 (data not shown) indicated one larger clustered region, containing both the

mercury-resistance genes and genetic information homologous to the *oriV* region of RK2. This is consistent with the pQKH6 backbone being only interrupted in one place as found for the recently sequenced plasmids pJP4 (Trefault *et al.*, 2004) and pB3 (Heuer *et al.*, 2004) and as suggested for IncP-1 β plasmids pSS50 and pSS60 (Burlage *et al.*, 1990). While using PCR primers for *trfA* and *korA* did not identify any additional IncP-1 plasmids it did provide a simple basis to assess relatedness of backbone genes, showing that indeed pQKH6 and pSS50 are indistinguishable on the basis of the sequence determined.

The clearest phenotype conferred by pQKH54 is mercury resistance, which has been found previously in IncP-1 β plasmids (Smith & Thomas, 1987). The particular version of *mer* genes related to those in Tn501 is environmentally widespread, having been reported in 35–100 % of Hg^f bacteria isolated from terrestrial environments (Osborn *et al.*, 1993). In addition, the pQKH54 sequence identified four genes in two transposon-associated regions which may encode hitherto unsuspected phenotypic properties. The closest sequence database matches are a choline dehydrogenase, a hydratase, a taurine dioxygenase and a sterol desaturase (Supplementary Table S1). Both operons are divergently transcribed from AraC-family regulators. While we have not studied them experimentally, it is tempting to speculate that these may all be involved in catabolism of bile salts.

Overall the capacity of these aquatic plasmids to retro-transfer the small non-conjugative IncQ plasmid R300B and other IncQ plasmids (Hill *et al.*, 1995) has important implications not only in the release of genetically engineered sequences, which are frequently encoded on non-conjugative cloning vectors (Bolivar *et al.*, 1977), but also in terms of the role of plasmids in promoting horizontal gene transfer in natural microbial communities. This study shows that bacteria harbouring plasmids indigenous to freshwater biofilms are capable of capturing such genes and this can potentially occur from a wide range of bacterial hosts. Almost certainly completely new incompatibility groups have yet to be identified. Evidence for this comes from plasmid pQM85 studied here, which could capture R300B efficiently by retrotransfer, but could not be placed into any known retrotransfer proficient incompatibility group. Plasmids of the IncP-1 family appear to be the commonest for which good identification methods exist (Pukall *et al.*, 1996; Smalla *et al.*, 2000). Our data indicate that within natural aquatic systems a whole spectrum of IncP-1-like plasmids may be present with differing degrees of similarity to clinical IncP-1 plasmids, and probably adapted to particular hosts. This suggests a broad IncP-1 group rather than a closely defined one (Smith & Thomas, 1989) and this point is reinforced by the recent report of a fourth IncP-1 subgroup designated IncP-1 δ (Vedler *et al.*, 2004). Sequencing and classification of plasmids from a whole range of environmental sources is required to determine the true plasmid diversity found in nature and to determine

whether new plasmids are related to known groups or represent completely new sequence families.

ACKNOWLEDGEMENTS

We thank Dr Mark Bailey for helpful advice and supplying the incompatibility probes used. The environmental sampling and initial analysis was supported by a grant from the Natural Environment Research Council. A.S.H. and K.J. and the majority of the DNA sequencing were supported by a project grant from The Wellcome Trust (06083). P.A. was supported by a BBSRC Quota studentship for training in research methods. A proportion of this work was carried out at Vlaamse Instelling voor Technologisch Onderzoek, Belgium, and we thank Dr Max Mergeay and Dr Roger Dijkmans for a productive visit.

REFERENCES

- Adamczyk, M. & Jagura-Burdzy, G. (2003). Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochimica Polonica* **50**, 425–453.
- Austubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A. & Struhl, K. (1989). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bale, M. J., Fry, J. C. & Day, M. J. (1987). Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. *J Gen Microbiol* **133**, 3099–3107.
- Bale, M. J., Fry, J. C. & Day, M. J. (1988). Transfer and occurrence of large mercury resistance plasmids in river epilithon. *Appl Environ Microbiol* **54**, 972–978.
- Barkay, T., Miller, S. M. & Summers, A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol Revs* **27**, 355–384.
- Barth, P. T. & Grinter, N. J. (1974). Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulphonamides. *J Bacteriol* **120**, 618–630.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Blanco, G., Ramos, F., Medina, J. R., Gutierrez, J. C. & Tortolero, M. (1991). Conjugal retrotransfer of chromosomal markers in *Azotobacter vinelandii*. *Curr Microbiol* **22**, 241–246.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.
- Boronin, A. M. (1992). Diversity of *Pseudomonas* plasmids: to what extent? *FEMS Microbiol Lett* **100**, 461–468.
- Bradley, D. J. (1992). *Gene transfer within epilithon of river stones*. PhD thesis, UWCC, Cardiff, UK.
- Burlage, R. S., Bemis, L. A., Layton, A. C., Saylor, G. S. & Larimer, F. (1990). Comparative genetic organization of incompatibility group P degradative plasmids. *J Bacteriol* **172**, 6818–6825.
- Couturier, M., Bex, F., Bergquist, P. L. & Maas, W. K. (1988). Identification and classification of bacterial plasmids. *Microbiol Rev* **52**, 375–395.
- Cross, M. A., Warne, S. R. & Thomas, C. M. (1986). Analysis of the vegetative replication origin of broad-host-range plasmid RK2 by transposon mutagenesis. *Plasmid* **15**, 132–146.
- Crooks, G. E., Hon, G., Chandonia, J. M. & Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res* **14**, 1188–1190.
- which has full text at: <http://weblogo.berkeley.edu/Crooks-2004-GR-WebLogo.pdf>.
- Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B. & Richmond, M. H. (1971). Properties of an R-factor from *Pseudomonas aeruginosa*. *J Bacteriol* **108**, 1244–1249.
- Davey, R. B., Bird, P. I., Nikolett, S. M., Praszkie, J. & Pittard, J. (1984). The use of mini-gal plasmids for rapid incompatibility grouping of conjugative R plasmids. *Plasmid* **11**, 234–242.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence-analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Don, R. H. & Pemberton, J. M. (1981). Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J Bacteriol* **145**, 681–686.
- Don, R. H. & Pemberton, J. M. (1985). Genetic and physical map of the 2,4-dichlorophenoxyacetic acid degradative plasmid pJP4. *J Bacteriol* **161**, 466–468.
- Droge, M., Puhler, A. & Selbitschka, W. (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol Gen Genet* **263**, 471–482.
- Ewing, B. & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* **8**, 186–194.
- Ewing, B., Hillier, L., Wendt, M. C. & Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* **8**, 175–185.
- Fry, J. C. & Day, M. J. (1990). Plasmid transfer in the epilithon. In *Bacterial Genetics in Natural Environments*, pp. 55–80. Edited by J. C. Fry & M. J. Day. London: Chapman & Hall.
- Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K. & Ramos, J. L. (1997). AraC/XylS family of transcriptional regulators. *Microbiol Mol Biol Rev* **61**, 393–410.
- Gomis-Ruth, F. X., de la Cruz, F. & Coll, M. (2002). Structure and role of coupling proteins in conjugal DNA transfer. *Res Microbiol* **153**, 199–204.
- Gordon, D., Abajian, C. & Green, P. (1998). Consed: a graphical tool for sequence finishing. *Genome Res* **8**, 195–202.
- Gordon, D., Desmarais, C. & Green, P. (2001). Automated finishing with Autofinish. *Genome Res* **11**, 614–625.
- Guiney, D. G. (1993). Broad host range conjugative and mobilizable plasmids in Gram-negative bacteria. In *Bacterial Conjugation*, pp. 75–103. Edited by D. B. Clewell. New York: Plenum.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Hedges, R. W. & Matthew, M. (1979). Acquisition by *Escherichia coli* of plasmid-borne β -lactamases normally confined to *Pseudomonas* spp. *Plasmid* **2**, 269–278.
- Heinemann, J. A. & Ankenbauer, R. G. (1993a). Retrotransfer in *Escherichia coli* conjugation: bidirectional exchange or *de novo* mating. *J Bacteriol* **175**, 583–588.
- Heinemann, J. A. & Ankenbauer, R. G. (1993b). Retrotransfer of IncP plasmid R751 from *Escherichia coli* maxicells – evidence for the genetic sufficiency of self-transferable plasmids for bacterial conjugation. *Mol Microbiol* **10**, 57–62.
- Heinemann, J. A. & Sprague, G. F. (1989). Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature* **340**, 205–209.
- Heuer, H., Szczepanowski, R., Schneiker, S., Puhler, A., Top, E. M. & Schluter, A. (2004). The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1 β group without any accessory genes. *Microbiology* **150**, 3591–3599.

- Hill, K. E., Weightman, A. J. & Fry, J. C. (1992). Isolation and screening of plasmids from the epilithon which mobilize recombinant plasmid pD10. *Appl Environ Microbiol* **58**, 1292–1300.
- Hill, K. E., Fry, J. C., Weightman, A. J., Day, M. J., Bradley, D. J. & Cousland, B. (1995). Retrotransfer of IncP1-like plasmids from aquatic bacteria. *Lett Appl Microbiol* **20**, 317–322.
- Hooper, S. W., Dockendorff, T. C. & Sayler, G. S. (1989). Characteristics and restriction analysis of the 4-chlorobiphenyl catabolic plasmid, pSS50. *Appl Environ Microbiol* **55**, 1286–1288.
- Jacob, A. E. (1977). Plasmids studied in *Escherichia coli* and other enteric bacteria. In *DNA Insertion Elements, Plasmids and Episomes*, pp. 607–638. Edited by A. I. Bukhari, J. A. Shapiro & S. L. Adhya. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Jeenes, D. J. & Williams, P. A. (1982). Excision and integration of degradative genes from TOL plasmid pWW0. *J Bacteriol* **150**, 188–194.
- Jobanputra, R. S. & Datta, N. (1974). Trimethoprim R factors in enterobacteria from clinical specimens. *J Med Microbiol* **7**, 169–177.
- Kado, C. I. & Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145**, 1365–1373.
- Kholodii, G. Y., Yurieva, O. V., Lomovskaya, O. L., Gorlenko, Z., Mindlin, S. Z. & Nikiforov, V. G. (1993). Tn5053, a mercury resistance transposon with integron's ends. *J Mol Biol* **230**, 1103–1107.
- Kholodii, G. Y., Mindlin, S. Z., Bass, I. A., Yurieva, O. V., Minakhina, S. V. & Nikiforov, V. G. (1995). Four genes, two ends, and a *res* region are involved in transposition of Tn5053: a paradigm for a novel family of transposons carrying either a *mer* operon or an integron. *Mol Microbiol* **17**, 1189–1200.
- Kittell, B. & Helinski, D. R. (1993). Plasmid incompatibility and replication control. In *Bacterial Conjugation*, pp. 223–242. Edited by D. B. Clewell. New York: Plenum.
- Kreps, S., Ferino, F., Mosrin, C., Gerits, J., Mergeay, M. & Thuriaux, P. (1990). Conjugative transfer and autonomous replication of the promiscuous IncQ plasmid in the cyanobacterium *Synechocystis* PCC 6803. *Mol Gen Genet* **221**, 129–133.
- Llosa, M., Zunzunegui, S. & de la Cruz, F. (2003). Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad Sci U S A* **100**, 10465–10470.
- McClure, N. C., Weightman, A. J. & Fry, J. C. (1989). Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated sludge unit. *Appl Environ Microbiol* **55**, 2627–2634.
- McClure, N. C., Saint, C. P., Fry, J. C. & Weightman, A. J. (1990). *The Construction of Broad Host Range Genetic Markers and their Use in Monitoring the Release of Catabolic GEMS to Aquatic Environments*. Copenhagen, Denmark: Proceedings of the 5th European Congress on Biotechnology.
- Mergeay, M., Lejeune, P., Thiry, G. & Faelen, M. (1985). Back transfer: a property of some broad host range plasmids. In *Plasmids in Bacteria*, p. 942. Edited by D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson & A. Hollaender. New York & London: Plenum.
- Mergeay, M., Lejeune, P., Sadouk, A., Gerits, J. & Fabry, L. (1987). Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. *Mol Gen Genet* **209**, 61–70.
- Mergeay, M., Springael, D. & Top, E. (1990). Gene transfer in polluted soils. In *Bacterial Genetics in Natural Environments*, pp. 152–171. Edited by J. C. Fry & M. J. Day. London: Chapman & Hall.
- Norrander, J., Kempe, T. & Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- Novick, R. P. (1987). Plasmid incompatibility. *Microbiol Rev* **51**, 381–395.
- Osborn, A. M., Bruce, K. D., Strike, P. & Ritchie, D. A. (1993). Polymerase chain reaction-restriction fragment length polymorphism analysis shows divergence among *mer* determinants from Gram-negative soil bacteria indistinguishable by DNA-DNA hybridization. *Appl Environ Microbiol* **59**, 4024–4030.
- Pansegau, W., Lanka, E., Barth, P. T. & 7 other authors (1994). Complete nucleotide sequence of Birmingham IncP-alpha plasmids – compilation and comparative analysis. *J Mol Biol* **239**, 623–663.
- Perkins, C. D., Davidson, M. A., Day, M. J. & Fry, J. C. (1994). Retrotransfer kinetics of R300B by pQKH6, a conjugative plasmid from river epilithon. *FEMS Microbiol Ecol* **15**, 33–44.
- Powell, B., Mergeay, M. & Christofi, N. (1989). Transfer of broad host-range plasmids to sulphate-reducing bacteria. *FEMS Microbiol Lett* **59**, 269–274.
- Pukall, R., Tschape, H. & Smalla, K. (1996). Monitoring the spread of broad host and narrow host range plasmids in soil microcosms. *FEMS Microbiol Ecol* **20**, 53–66.
- Ramos-González, M., Ramos-Díaz, M. & Ramos, J. L. (1994). Chromosomal gene capture mediated by the *Pseudomonas putida* TOL catabolic plasmid. *J Bacteriol* **176**, 4635–4641.
- Rhodes, G., Parkhill, J., Bird, C., Ambrose, K., Jones, M. C., Huys, G., Swings, J. & Pickup, R. W. (2004). Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. *Appl Environ Microbiol* **70**, 7497–7510.
- Rochelle, P. A., Fry, J. C., Day, M. J. & Bale, M. J. (1985). An accurate method for estimating sizes of small and large plasmids and DNA fragments by gel electrophoresis. *J Gen Microbiol* **132**, 53–59.
- Rochelle, P. A., Fry, J. C. & Day, M. J. (1989). Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. *J Gen Microbiol* **135**, 409–424.
- Ronchel, M. C., Ramos-Díaz, M. A. & Ramos, J. L. (2000). Retrotransfer of DNA in the rhizosphere. *Environ Microbiol* **2**, 319–323.
- Rosenberg, C. & Huguet, T. (1984). The pATC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumor induction. *Mol Gen Genet* **196**, 533–536.
- Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.-A. & Barrell, B. (2000). Artemis: sequence visualisation and annotation. *Bioinformatics* **16**, 944–945.
- Sambrook, J., Fritsch, E. F. & Manniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, T. D. & Stephens, R. M. (1990). Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res* **18**, 6097–6100.
- Shah, D. S., Cross, M. A., Porter, D. & Thomas, C. M. (1995). Dissection of the core and auxiliary sequences in the vegetative replication origin of promiscuous plasmid RK2. *J Mol Biol* **254**, 608–622.
- Sia, E. A., Kuehner, D. M. & Figurski, D. H. (1996). Mechanism of retrotransfer in conjugation: prior transfer of the conjugative plasmid is required. *J Bacteriol* **178**, 1457–1464.
- Smalla, K., Krogerrecklenfort, E., Heuer, H. & 21 other authors (2000). PCR-based detection of mobile genetic elements in total community DNA. *Microbiology* **146**, 1256–1257.
- Smit, E., Venne, D. & van Elsas, J. D. (1993). Mobilization of a recombinant IncQ plasmid between bacteria on agar and in soil via cotransfer or retrotransfer. *Appl Environ Microbiol* **59**, 2257–2263.

- Smith, C. A. & Thomas, C. M. (1987).** Comparison of the organization of the genomes of phenotypically diverse plasmids of incompatibility group-P – members of the IncP-beta-sub-group are closely related. *Mol Gen Genet* **206**, 419–427.
- Smith, C. A. & Thomas, C. M. (1989).** Relationships and evolution of IncP plasmids. In *Promiscuous Plasmids of Gram-negative Bacteria*, pp. 57–77. Edited by C. M. Thomas. London: Academic Press.
- Smith, C. A., Pinkney, M., Guiney, D. G. & Thomas, C. M. (1993).** The ancestral IncP replication system consisted of contiguous *oriV* and *trfA* segments as deduced from a comparison of the nucleotide sequences of diverse IncP plasmids. *J Gen Microbiol* **139**, 1761–1766.
- Stalker, D. M., Thomas, C. M. & Helinski, D. R. (1981).** Nucleotide-sequence of the region of the origin of replication of the broad host range plasmid RK2. *Mol Gen Genet* **181**, 8–12.
- Stanisich, V. A. (1988).** Identification and analysis of plasmids at the genetic level. In *Plasmid Technology*, pp. 11–47. Edited by J. Grinstead & P. M. Bennett. London: Academic Press.
- Szipirer, C., Top, E., Couturier, M. & Mergeay, M. (1999).** Retro-transfer or gene capture: a feature of conjugative plasmids, with ecological and evolutionary significance. *Microbiology* **145**, 3321–3329.
- Thiry, G., Mergeay, M. & Faelen, M. (1984).** Back-mobilisation of Tra⁻ Mob⁺ plasmids mediated by various IncM, IncN and IncP1 plasmids. *Arch Intern Physiol Biochem* **92**, 64–65.
- Thomas, C. M. & Smith, C. A. (1987).** Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. *Annu Rev Microbiol* **41**, 77–101.
- Thomas, C. M. & Thorsted, P. B. (1994).** PCR probes for promiscuous plasmids. *Microbiology* **140**, 1.
- Thomas, C. M., Stalker, D. M. & Helinski, D. R. (1981).** Replication and Incompatibility properties of segments of the origin region of replication of the broad host range plasmid RK2. *Mol Gen Genet* **181**, 1–7.
- Thorsted, P. A., Macartney, D. P., Akhtar, P. & 9 other authors (1998).** Complete sequence of the IncP beta plasmid R751: implications for evolution and organisation of the IncP backbone. *J Mol Biol* **282**, 969–990.
- Top, E., Vanrolleghem, P., Mergeay, M. & Verstraete, W. (1992).** Determination of the mechanism of retrotransfer by mechanistic mathematical modelling. *J Bacteriol* **174**, 5953–5960.
- Top, E., De Rore, H., Collard, J., Gellens, V., Slobodkina, G., Verstraete, W. & Mergeay, M. (1995).** Retromobilization of heavy metal resistance genes in unpolluted and heavy metal polluted soil. *FEMS Microbiol Ecol* **18**, 191–203.
- Trefault, N., De la Iglesia, R., Molina, A. M., Manzano, M., Ledger, T., Perez-Pantoja, D., Sanchez, M. A., Stuardo, M. & Gonzalez, B. (2004).** Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134(pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. *Environ Microbiol* **6**, 655–668.
- Trieu-Cuot, P., Carlier, C., Martin, P. & Courvalin, P. (1987).** Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. *FEMS Microbiol Lett* **48**, 289–294.
- Vedler, E. M., Vahter, A. & Heinaru, A. (2004).** The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harbouring *tfd* genes for 2,4-dichlorophenoxy-acetic acid degradation. *J Bacteriol* **186**, 7161–7174.
- Wheatcroft, R. & Williams, P. A. (1981).** Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. *J Gen Microbiol* **124**, 433–437.
- Willets, N. (1981).** Sites and systems for conjugal DNA transfer in bacteria. In *Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids*, pp. 207–215. Edited by S. T. Levy, R. C. Clowes & E. L. Koenig. New York: Plenum.
- Willets, N. & Crowther, C. (1981).** Mobilization of the non-conjugative IncQ plasmid RSF1010. *Genet Res* **37**, 311–316.
- Włodarczyk, M. & Piechucka, E. (1995).** Conjugal transfer of plasmid and chromosomal markers between strains of *Thiobacillus versutus*. *Curr Microbiol* **30**, 185–191.