Host range diversification within the IncP-1 plasmid group

Hirokazu Yano,^{1,2†} Linda M. Rogers,^{1,2} Molly G. Knox,² Holger Heuer,³ Kornelia Smalla,³ Celeste J. Brown^{1,2} and Eva M. Top^{1,2}

Correspondence

Eva M. Top
evatop@uidaho.edu

¹Institute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, ID 83844, USA

²Department of Biological Sciences, University of Idaho, Moscow, ID 83844, USA

³Julius Kühn-Institut – Federal Research Centre for Cultivated Plants (JKI), Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

Broad-host-range plasmids play a critical role in the spread of antibiotic resistance and other traits. In spite of increasing information about the genomic diversity of closely related plasmids, the relationship between sequence divergence and host range remains unclear. IncP-1 plasmids are currently classified into six subgroups based on the genetic distance of backbone genes. We investigated whether plasmids from two subgroups exhibit a different host range, using two IncP-1 γ plasmids, an IncP-1 β plasmid and their minireplicons. Efficiencies of plasmid establishment and maintenance were compared using five species that belong to the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. The IncP-1 β plasmid replicated and persisted in all five hosts in the absence of selection. Of the two IncP-1 γ plasmids, both were unable to replicate in alphaproteobacterial host *Sphingobium japonicum*, and one established itself in *Agrobacterium tumefaciens* but was very unstable. In contrast, both IncP-1 γ minireplicons, which produced higher levels of replication initiation protein than the wild-type plasmids, replicated in all strains, suggesting that poor establishment of the native plasmids is in part due to suboptimal replication initiation gene regulation. The findings suggest that host ranges of distinct IncP-1 plasmids only partially overlap, which may limit plasmid recombination and thus result in further genome divergence.

Received 5 April 2013

Accepted 2 September 2013

INTRODUCTION

Bacterial plasmids are genetic elements that replicate separately from the host chromosome (Funnell & Phillips, 2004). In addition to encoding the machinery necessary for their replication control, they also often encode the functions required for their stable maintenance and horizontal transfer, and various host-beneficial traits such as antibiotic resistance, metal resistance, virulence and catabolic activity (Liebert *et al.*, 1999; Nojiri *et al.*, 2004; Schlüter *et al.*, 2007; Heuer & Smalla, 2012). Approximately 50 % of plasmids sequenced so far are thought to be self-transmissible or mobilizable by a co-residing plasmid in the cell (Garcillán-Barcia *et al.*, 2011). Plasmids thus facilitate rapid adaptation of bacteria to changing environments by mediating horizontal gene transfer, as exemplified by the alarming rise in multi-drug-resistant pathogens (Wellington *et al.*, 2013).

To fully understand the reservoirs and the trajectories of antibiotic resistance gene spread (Allen *et al.*, 2010), we need to better understand the factors that affect the range of bacteria in which a plasmid can transfer, replicate and persist even in the absence of antibiotics. A plasmid's host range has been defined at three levels: (i) the range of hosts to which the plasmid transfers, referred to as transfer host range; (ii) the range of hosts in which the plasmid can replicate, replication host range; and (iii) the range of hosts in which the plasmid can persist in the population in the absence of selection for the plasmid, previously called 'long-term' host range (De Gelder *et al.*, 2007). This long-term or persistence host range is determined by a combination of factors such as efficiency of replication, segregational loss rate, post-segregational killing, conjugative transfer rate and plasmid cost (Stewart & Levin, 1977; Slater *et al.*, 2008). It has been shown that the transfer host range is sometimes wider than the replication host range (Guiney, 1982; Guiney *et al.*, 1984; Heinemann & Sprague, 1989), and the replication host range is in turn clearly wider than the persistence host range (De Gelder *et al.*, 2007; Sota *et al.*, 2010). We also showed that the range of hosts in which a plasmid has resided over evolutionary

†Present address: Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo 180-8639, Japan.

Abbreviations: CDS, coding sequence; IS, insertion sequence; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline.

time can be inferred by comparing the genomic signatures of plasmids with those of all sequenced bacterial chromosomes. Strikingly, the genomic signatures (di-, tri- or tetranucleotide relative abundance) of plasmids with a narrow host range were very similar to those of the species known to carry them, while broad-host-range plasmids had signatures that showed similarity to either many different hosts or almost none (Suzuki *et al.*, 2008, 2010).

Self-transmissible plasmids belonging to incompatibility group P-1 (IncP-1 plasmids) were originally discovered in clinical bacterial isolates (Jobanputra & Datta, 1974; Jacoby *et al.*, 1976). Currently, it is recognized that the IncP-1 plasmids are ubiquitous in environmental bacteria residing in soil, sewage, marine sediments and manure (Dahlberg *et al.*, 1997; Schlüter *et al.*, 2007; Binh *et al.*, 2008; Bahl *et al.*, 2009; Gomes *et al.*, 2010; Heuer & Smalla, 2012). IncP-1 plasmids are generally considered to have a broad host range because representative plasmids of this group are able to transfer and replicate in hosts belonging to three proteobacterial subclasses, namely *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (Schmidhauser & Helinski, 1985; De Gelder *et al.*, 2005; Shintani *et al.*, 2010; Yano *et al.*, 2012). Since Jakobson & Guiney (1983) proposed to separate plasmids RP4 and R751 as different subgroups (α , β) based on the sequence similarity in the transfer origin (Jakobson & Guiney, 1983), six IncP-1 subgroups (α , β , γ , δ , ϵ and ζ , see Fig. 1a) have been proposed based on the results of phylogenetic analysis for plasmid backbone genes (Jobanputra & Datta, 1974; Haines *et al.*, 2006; Sen *et al.*, 2010, 2013; Bahl *et al.*, 2007; Norberg *et al.*, 2011). To date, more than 55 IncP-1 plasmid sequences have been deposited in the NCBI database. Interestingly, the natural hosts of IncP-1 γ , IncP-1 ϵ and IncP-1 ζ plasmids are unknown because these plasmids have been discovered solely by exogenous plasmid isolation, a method that captures plasmids by mating the bacterial community of an environmental sample with a laboratory strain (Haines *et al.*, 2006; Norberg *et al.*, 2011; Bahl *et al.*, 2007; Heuer *et al.*, 2012). A recent comparison of the genomic signatures of IncP-1 plasmid backbone genes with those of all available bacterial chromosome sequences suggested that plasmids from different IncP-1 subgroups had acquired signatures of hosts belonging to diverse taxonomic groups (Norberg *et al.*, 2011). These divergent plasmids were thus thought to have evolved in different hosts. However, it remains to be elucidated whether genetically divergent IncP-1 plasmids really have partially or completely overlapping host ranges.

IncP-1 γ plasmids pKS208 and pMBUI1 were recently isolated by exogenous plasmid isolation methods. Plasmid pKS208 was captured in a biparental mating between a *Pseudomonas putida* recipient strain and bacteria from a hospital sewage treatment plant in Belgium, selecting for gentamicin resistance (Heuer *et al.*, 2002). Plasmid pMBUI1 was obtained in a triparental mating between *Escherichia coli* and pond water in Moscow, Idaho, after selection for the ability to mobilize plasmid pBBR1-MCS (Brown *et al.*, 2013). Recent phylogenetic analysis of their plasmid backbone genes indicated that both pKS208 and

pMBUI1 belong to the IncP-1 γ subgroup, which until recently comprised only one completely sequenced plasmid, the archetype pQKH54 (Haines *et al.*, 2006; Sen *et al.*, 2013).

In this study, we address the hypothesis that sequence divergence among plasmids of the IncP-1 group is associated with host specialization. The specific objective was to determine experimentally whether the *in vitro* plasmid host range differs between plasmids from distinct IncP-1 subgroups. We briefly describe the genomic features of the new members of the IncP-1 γ subgroup, and then compare their transfer, replication and persistence host range with that of a known broad-host-range plasmid of the IncP-1 β group using multiple experimental approaches.

METHODS

Bacterial strains, plasmids and media. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli*, *P. putida* and *Agrobacterium tumefaciens* strains were grown in Luria–Bertani broth (LB) (Sambrook & Russell, 2001). *Sphingobium japonicum* and *Cupriavidus pinatubonensis* strains were grown in 1/3-LB (per litre H₂O: 3.3 g Bacto tryptone, 1.7 g yeast extract and 5 g NaCl), and 1/10-TSB [one-tenth dilution of Bacto trypticase soy broth (BD Bioscience)], respectively. Solid media were made by addition of 1.5% agar to LB (referred to as LBA), 1/3-LB (1/3-LBA), or 1/10-TSB (1/10-TSBA). *E. coli* strains were incubated at 37 °C for DNA cloning experiments. After introduction of IncP-1 replicons, all strains were grown at 30 °C. If necessary, the media were supplemented with antibiotics at the following concentrations: for *E. coli*, 50 µg kanamycin (Km) ml⁻¹ and 10 µg tetracycline (Tc) ml⁻¹; for *P. putida*, 50 µg Km ml⁻¹ and 10 µg Tc ml⁻¹; for *C. pinatubonensis*, 25 µg Km ml⁻¹ and 2.5 µg Tc ml⁻¹; for *S. japonicum*, 25 µg Km ml⁻¹ and 2.5 µg Tc ml⁻¹; and for *A. tumefaciens*, 50 µg Km ml⁻¹. Streptomycin- (Sm) and rifampicin- (Rif) resistant strains were obtained by streaking the ancestral strains on appropriate solid media containing 1000 µg Sm ml⁻¹ or 100 µg Rif ml⁻¹, respectively. Agar plates containing 200 µg Sm ml⁻¹ or 100 µg Rif ml⁻¹ were used to select donor, recipient or transconjugant cells in conjugation experiments.

C. pinatubonensis MK2 was constructed by transformation of strain JMP134 (Lykidis *et al.*, 2010) with unstable IncP-1 β plasmid pMS0506 (Sota *et al.*, 2010), and the subsequent screening of spontaneously arising pMS0506-free segregants. Absence of plasmids in MK2 was confirmed by PCR and Southern hybridization for total DNA, using the *trfA2* gene of pBP136 as target sequence. Plasmid pMBUI1T17 was constructed by *in vitro* transposon tagging of pMBUI1 using the EZ-Tn5 <TET-1> transposon insertion kit (Epicentre), and the subsequent screening of a transfer-proficient clone. Minireplicons of IncP-1 plasmids were constructed as described below.

General molecular techniques. DNA cloning was performed using enzymes from New England Biolabs, GeneJET Gel extraction kit (Fermentas) and heat-shock transformation of *E. coli* DH5 α cells (Sambrook & Russell, 2001). A Qiagen Plasmid Midi kit was used to purify intact IncP-1 plasmids from *E. coli* and *P. putida* strains. Phusion DNA polymerase (New England Biolabs) was used to amplify DNA segments of IncP-1 plasmids. Oligonucleotides used in this study were synthesized by Sigma-Aldrich.

Conjugation experiments were carried out using nitrocellulose filters on solid media with four replicates. Mating mixtures were prepared by mixing 1 ml each of overnight cultures of donor and recipient strains, and washing and resuspending the cells in 40 µl saline. The

Fig. 1. Diversity of IncP-1 plasmids. (a) Phylogenetic tree of 55 IncP-1 plasmids based on the 819 bp conserved region in *trfA2*. The tree was generated using the maximum-likelihood method for the alignment made by the program MUSCLE and Kimura-Nei method in MEGA5 (Tamura *et al.*, 2011). Triangles indicate branches composed of multiple plasmids. Bar, 0.05 changes per position. (b) Similarities of replication initiation proteins and relaxases (Tral) between representative plasmids of each IncP-1 subgroup (RK2, IncP-1 α ; R751, pBP136, pB1 and pB12, IncP-1 β ; pQKH54, pMBUI1 and pKS208, IncP-1 γ ; pAKD4, IncP-1 δ ; pKJK5 and pAKD34, IncP-1 ϵ ; pMCFB1, IncP-1 ζ). Amino-acid sequence identities were determined using BLASTP, and are represented in the heat map.

mixtures were placed on the filters (0.45 μ m pores; Whatman/GE Healthcare) on LBA, 1/3-LBA or 1/10-TSBA without antibiotics and incubated at 30 $^{\circ}$ C for 24 h. Cells were recovered from the filters in 1.5 ml saline and serial dilutions were plated on appropriate selective media to determine the number of donors, recipients and transconjugants.

For preparation of electrocompetent cells of *E. coli*, *C. pinatubonensis* and *A. tumefaciens*, 1 ml aliquots of overnight bacterial cultures were diluted in 100 ml of fresh media, grown to an OD₆₀₀ of 0.8, washed with a 10% glycerol solution and resuspended in 2 ml of the solution. Electrocompetent cells of *P. putida* were prepared from overnight culture by washing cells with 300 mM sucrose. Electrocompetent cells of *S. japonicum* were prepared from bacterial colonies grown for 2 days as described previously (Yano *et al.*, 2012). Cell suspensions (100 μ l) containing 10⁸–10⁹ cells were mixed with plasmid DNA for electroporation. As an exception, *E. coli* TransforMax EC100 electrocompetent cells (Epicentre) were directly used for the transformation assay shown in Fig. 3(a). Electroporation was conducted using a 1 mm gap cuvette and Gene Pulser Xcell (Bio-Rad) at 1.8 kV for *E. coli* and *S. japonicum*, 2.0 kV for *P. putida* and *C. pinatubonensis*, and 2.4 kV for

A. tumefaciens. After electroporation cells were incubated in their appropriate liquid medium for 2 h, and subsequently plated on agar media without antibiotics to count the number of total viable cells, and on media with the appropriate antibiotic to count the number of transformants.

All intact IncP-1 plasmids used for transformation assay were purified from *P. putida* KT2440, and the amount of DNA was normalized to 0.036 pmol (1 μ g of pBP136Km equivalent), according to the results of densitometry analysis for the serially diluted linearized DNA in ethidium bromide-stained agarose gels. The same method was used to normalize the quantity of minireplicons. Thus, 0.043 pmol (150 ng of pLR303R equivalent) of minireplicon purified from *E. coli* EC100 was used in each electroporation assay. Statistical significance was determined by ANOVA and the Dunnett's test in R (R Development Core Team, 2010).

Construction of IncP-1 minireplicons. Fragments of 2.0–2.3 kb from the *trfA* start codon to the end of the conserved G+C-rich region in the *oriV* of the three IncP-1 plasmids were PCR-amplified using the following primer sets: for mini-pKS208, SphSDpKS208trfAF

Table 1. Strains and plasmids used in this study

Strain (class) or plasmid	Genotype and relevant characteristics*	Reference/source
<i>E. coli</i> strains (<i>Gammaproteobacteria</i>)		
BW25113	F ⁻ <i>rrnB3</i> Δ <i>lacZ4787</i> <i>hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i>	Baba <i>et al.</i> (2006)
BW25113S	Spontaneous Sm ^r mutant of BW25113; Sm ^r	This study
BW25113R	Spontaneous Rif ^r mutant of BW25113; Rif ^r	This study
DH5 α	F ⁻ <i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA</i> <i>glnV44</i> ϕ 80 <i>lacZ</i> Δ M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	Sambrook & Russell (2001)
EC100	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i>	Epicentre, Madison, WI, USA
<i>P. putida</i> strains (<i>Gammaproteobacteria</i>)		
KT2440	Type strain, ATCC 47054	Bagdasarian <i>et al.</i> (1981)
KT2440S	Spontaneous Sm ^r mutant of KT2440; Sm ^r	This study
KT2440R	Spontaneous Rif ^r mutant of KT2440; Rif ^r	This study
<i>C. pinatubonensis</i> strains (<i>Betaproteobacteria</i>)		
JMP134	Soil isolate	Lykidis <i>et al.</i> (2010)
MK2	JMP134 derivative cured of plasmid pJP4	This study
MK2S	Spontaneous Sm ^r mutant of MK2; Sm ^r	This study
<i>A. tumefaciens</i> strain (<i>Alphaproteobacteria</i>)		
C58rif	Spontaneous Rif ^r mutant of type strain C58; Rif ^r	Wood <i>et al.</i> (2001)
<i>S. japonicum</i> strains (<i>Alphaproteobacteria</i>)		
UT26S	Type strain, JCM 17232	Nagata <i>et al.</i> (2011)
UT26SS	Spontaneous Sm ^r mutant of UT26S; Sm ^r	This study
Plasmids		
pBP136Km	IncP-1 β replicon; Tra ⁺ , Km ^r	Kamachi <i>et al.</i> (2006)
pHY925	pTXB1:: <i>trfA2</i> , Ap ^r	Yano <i>et al.</i> (2012)
pHY997	p15A replicon carrying Tn1000 resolution system (<i>res-tnpR</i>); Cm ^r	This study
pKS208	IncP-1 γ replicon; Mob ⁺ , Ap ^r Km ^r Gm ^r	Heuer <i>et al.</i> (2002)
pLR301R (mini-pKS208)	pSW29T:: (<i>trfA</i> _{pKS208} - <i>oriV</i> _{pKS208} - <i>res-tnpR</i>); Mob ⁺ , Km ^r	This study
pLR302R (mini-pMBUI1)	pSW29T:: (<i>trfA</i> _{pMBUI1} - <i>oriV</i> _{pMBUI1} - <i>res-tnpR</i>); Mob ⁺ , Km ^r	This study
pLR303R (mini-pBP136)	pSW29T:: (<i>trfA</i> _{pBP136} - <i>oriV</i> _{pBP136} - <i>res-tnpR</i>); Mob ⁺ , Km ^r	This study
pMBUI1	IncP-1 γ replicon; Tra ⁺	Brown <i>et al.</i> (2013)
pMBUI1T17	pMBUI1 <i>tao53.1</i> :: <i>tetA</i> ; Tc ^r	This study
pMS0506	IncP-1 β replicon, pBP136 Δ (<i>trbB</i> - <i>traM</i>):: (<i>oriT</i> _{RP4} - <i>kan</i>); Mob ⁺ , Km ^r	Sota <i>et al.</i> (2010)
pRK2013	ColE1 replicon carrying RK2 conjugation genes	Figurski & Helinski (1979)
pSW29T	R6K replicon, promoterless suicide vector; Mob ⁺ , Km ^r	Demarre <i>et al.</i> (2005)

*Antibiotic resistances: Sm, streptomycin; Rif, rifampicin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline; Gm, gentamicin; Ap, ampicillin. Mob⁺, mobilizable; Tra⁺, self-transmissible.

(AATTGCATGCATAAGGAGGTATACACCATGGATCATCAAGAC-TTCGACG) and EcopKS208oriVR (AATGAATTCTCGGGGGGATGGGAGAGCCG); for mini-pMBUI1, SphSDpBMU1trfAF (AATTGCATGCATAAGGAGGTATACACCATGGAACAACAAGATTTGATGAGG) and EcopMBUI1oriVR (AATGAATTCTCGGGGGGATGGGAGGAGGCG); and for mini-pBP136, SphSDpBP136trfAF (AATTGCATGCATAAGGAGGTATACACCATGACGAACAACGAGTTCAACG) and EcopBP136oriVR (AATGAATTCTCGGGGGGATGGGAGGAGGCG). The borders of the conserved region in the *oriV* in each plasmid were defined by the CLUSTAL W alignment (Larkin *et al.*, 2007) of the sequences of the three plasmids. The PCR products were then digested with *SphI* and *EcoRI*, and cloned into the *SphI*-*EcoRI* sites of a promoterless vector pSW29T (Demarre *et al.*, 2005). This gave rise to minireplicons pLR301 (mini-pKS208), pLR302 (mini-pMBUI1) and pLR303 (mini-pBP136). Construct pHY997 is a pSTV28 (Takara Bio) derivative carrying a resolution system of Tn1000 from the F plasmid and two transcriptional terminators (H. Yano, unpublished). After DNA sequences of the *trfA*-*oriV* region on pLR301, pLR302 and pLR303 were confirmed, the resolution gene cassette of pHY997 was

inserted into the *EcoRI* site of the minireplicons. This was done to reduce their segregational loss in the wild-type (*recA*⁺) strains, where the plasmid copies in the cell can dimerize through homologous recombination. This gave rise to pLR301R, pLR302R and pLR303R (see Fig. 3b).

Plasmid persistence assay. Plasmid persistence or stability assays were performed as previously described (Sota *et al.*, 2010) with the following modifications. Briefly, all cultures were passaged in serial batch cultures for 10 generations per day. *E. coli*, *P. putida* and *A. tumefaciens* were grown in 5 ml LB medium, and *C. pinatubonensis* in 5 ml TSB; *S. japonicum* was grown in a 20 ml volume of 1/3-LB, and cultures were passaged only every other day. Plating and colony replication were done on the corresponding agar media with and without the appropriate antibiotics.

Proteins and Western blotting. TrfA₂pBP136 was purified by affinity chromatography from the cell lysate of *E. coli* Rosetta 2 (DE3) harbouring pHY925 as described previously (Yano *et al.*, 2012). Quantitative Western blotting for whole-cell extracts was conducted

using the same anti-TrfA₂_{pBP136} rabbit antiserum as described previously (Yano *et al.*, 2012). This antibody barely reacted with TrfA₁_{pMBUI1}, probably because of its highly divergent amino-acid sequence. IRDye800-conjugated goat-anti-rabbit IgG (Rockland) was used as a secondary antibody. Densitometry analysis for the blots was performed using the Odyssey infrared imaging system (Li-COR) according to the manufacturer's protocols. The quantity of TrfA proteins in cell extracts was determined based on standard curves with $R^2 > 0.99$. The Western blotting experiment was performed at least three times for each host–plasmid combination.

Bioinformatic analysis. A phylogenetic tree was generated using maximum-likelihood based on DNA sequences aligned by MUSCLE in MEGA5 (Edgar, 2004; Tamura *et al.*, 2011). The program BLAST (Altschul *et al.*, 1990) was used for similarity analysis. GenomeMatcher (Ohtsubo *et al.*, 2008) was used for the pairwise BLASTN analysis to identify the insertion and deletion polymorphisms in IncP-1 plasmids. DNA sequences compared are obtained from the following RefSeq or GenBank accession numbers: pA81, NC_006830; pADK17, JN106168; pADP-1, NC_004956; pAKD1, JN106164; pAKD14, JN106165; pAKD15, JN106166; pAKD16, JN106167; pAKD34, JN106169; pAKD25, JN106170; pAKD26, JN106171; pAKD29, JN106172; pAKD31, JN106173; pAKD33, JN106174; pAKD34, JN106175; pAKD4, GQ983559; pALIDE02, NC_014911; pAMMD1, NC_008385; pAOBO02, NC_008766; pB1, JX469829; pB10, NC_004840; pB12, JX469826; pB3, NC_006388; pB4, NC_003430; pB8, NC_007502; pBP136, AB237782; pBS228, NC_008357; pC1-1, HQ891317; pCNB1, NC_010935; pDS3, JX469834; pEMT3, JX469827; pEST4011, NC_005793; pG527, JX469830; pI2, NC_016978; pIJB1, NC_013666; pJP4, NC_007337; pJKK5, NC_008272; pKS208, JQ432564; pKS212, JX469831; pLME1, JF274988; pMBUI1, JQ432563; pMCBF6, EF107516; pNB8c, JF274990; pQKH54, NC_008055; pRSB222, JX46982; pRSB223, JX469828; pTB11, NC_006352; pTB30, NC_016968; pTP6, NC_007680; pUO1, NC_005088; pWDL7, GQ495894; pWEC911, JX469833; pYS1, JX469832; R751, NC_001735; RK2, NC_001621.

RESULTS

General features of the pKS208 and pMBUI1 genomes

Plasmids pKS208 and pMBUI1 consist of 50 604 and 44 303 bp, respectively. Their gene products generally showed highest identity to the gene products of the archetype IncP-1 γ plasmid pQKH54. Although most IncP-1 plasmids contain *trfA* genes that encode two forms of the replication initiation protein TrfA [referred to as TrfA1 for the long form (362–479 aa) and TrfA2 for the short form (ca. 280 aa)], the three IncP-1 γ plasmids do not have the secondary internal translational start site in their *trfA* genes, which only seem to encode the long form (373 aa for pMBUI1 and pQKH54; 362 aa for pKS208). Phylogenetic analysis using the sequence that is homologous to *trfA2* places these two plasmids within the same clade as pQKH54, with pKS208 more distantly related to the other two plasmids (Fig. 1a). In addition, pMBUI1 and pQKH54 are closely related to each other in terms of the number of shared genes, gene product identity and local DNA G + C content (Fig. 2); for example, TrfA_{pMBUI1} and TrfA_{pQKH54} are 98 % identical over the entire length of the protein, while TrfA_{pMBUI1} and TrfA_{pKS208} show only 79 % identity. Amino-acid sequence identities of TrfA proteins between IncP-1 subgroups were 48–79 % (Fig. 1b). This

extent of diversity was also true for the relaxase TraI (Fig. 1b), which has also been used to infer plasmid phylogeny for plasmid classification purposes (Garcillán-Barcia *et al.*, 2009).

Comparative analysis of the three IncP-1 γ plasmids showed that pQKH54 and pMBUI1 share a 44 kb conserved region (Fig. 2). This region, which we here designate the IncP-1 γ plasmid backbone, contains 56 coding sequences (CDSs), 42 of which are also conserved in other IncP-1 subgroups. We could not attribute functions to the 14 IncP-1 γ -specific CDSs, except for the resolvase gene (*rsv* in Fig. 2), which may be responsible for plasmid dimer resolution. Plasmid pKS208 lacks several of the coding sequences conserved between pQKH54 and pMBUI1 (*kliABC*, *trbW*, *tao53*, *kleE*, part of *kfrC*) but carries additional CDSs of unknown function (*orf37*, *orf53*, *orf63*). Compared with pMBUI1, pQKH54 shows three insertions in the backbone that are associated with transposons or insertion sequence (IS) elements, and it carries mercury resistance genes (Fig. 2). Plasmid pMBUI1 does not seem to carry any mobile genetic elements or accessory genes, and can thus be considered cryptic. Two of the antibiotic resistance genes in pKS208 (*bla* and *aacA* in Fig. 2) are located next to the *intI* gene encoding a class I integron integrase, suggesting that they were acquired as part of integron gene cassettes. In addition, two directly repeated IS elements (IS15deltaII) are located next to the kanamycin resistance gene (*aphA*). The putative composite transposon composed of two IS15deltaII and *aphA* is not flanked by the direct repeats that are typically produced upon transposition of this family of IS elements, suggesting *aphA* was not acquired through a simple insertion of this transposon. Note that *traC* of pKS208 is 507 bp shorter than that of the other two IncP-1 γ plasmids (see gap in the DNA G + C content plot in the *traC* region in Fig. 2).

To facilitate the host range analysis of pMBUI1, a tetracycline resistance gene was inserted into gene *tao53.1* of pMBUI1, which is located outside of the IncP-1 backbone and encodes a hypothetical protein (Fig. 2). The resulting plasmid was named pMBUI1T17.

Replication host range of the intact IncP-1 plasmids

First, the replication host ranges of the two new IncP-1 γ plasmids and the IncP-1 β plasmid pBP136 were compared by conjugation assays. *P. putida* was first used as donor because it was considered a permissive host of IncP-1 γ plasmids, as two of the three sequenced IncP-1 γ plasmids were originally captured in this species by exogenous plasmid isolation (archetype pQKH54 and pKS208). In a second set of matings, *E. coli* was used as the donor. Five strains belonging to different phylogenetic clades were used as recipients: strains of *E. coli* and *P. putida*, both *Gamma-proteobacteria*, *C. pinatubonensis* from the *Betaproteobacteria*, and *A. tumefaciens* and *S. japonicum* from the *Alphaproteobacteria*. We could not detect self-transfer of

Fig. 2. Insertion and deletion polymorphism in the IncP-1 γ plasmids. Insertions and deletions specific to pQKH54 or pKS208 are shown above the IncP-1 γ backbone. pMBUI1 was assumed to have no insertions or deletions. Pentagons indicate coding sequences. Local DNA G + C content was calculated every 50 bp with a 1000 bp window size. Functions of accessory genes: *mer*, mercury resistance; *aph*, kanamycin resistance; *aacA*, gentamicin resistance; *bla*, beta-lactam antibiotics resistance.

pKS208 even between isogenic *P. putida* strains (Table 2), possibly because the plasmid does not have a complete *traC* gene. Therefore, its host range was determined in the presence of a helper plasmid pRK2013 that carried transfer genes of IncP-1 α plasmid RK2 (Figurski & Helinski, 1979).

Both IncP-1 γ plasmids and the IncP-1 β plasmid were transferred from *P. putida* to *E. coli* and *P. putida*, but no transfer could be detected to the betaproteobacterium *C. pinatubonensis* (Table 2). As this *C. pinatubonensis* strain is known to be a suitable host for other IncP-1 plasmids, including a pBP136 replicon, we repeated the matings using *E. coli* as the donor. Now all three plasmids were transferred to *C. pinatubonensis*. The lack of transferability from *P. putida* may be due to the inability of the recipient to form productive mating pairs with this donor. When the alphaproteobacteria were used as recipients, no transfer was detected for either of the IncP-1 γ plasmids from *P. putida* as donor, whereas pBP136Km was transferred successfully. When *E. coli* was the donor, transfer of the IncP-1 γ plasmids to *A. tumefaciens* was detected, but at low to extremely low frequencies, and no transfer to *S. japonicum* was observed. In contrast, IncP-1 β plasmid pBP136Km transferred to both hosts at high frequencies (Table 2). The results suggest that IncP-1 γ plasmids and IncP-1 β plasmids have an overlapping but different host range, and that either poor transfer or poor establishment of the IncP-1 γ plasmids is limiting their apparent host range.

To compare the plasmids' replication functions alone, we next determined the host range of the three plasmids by transformation assays. The three plasmid DNAs were prepared from *P. putida* KT2440, and used as donor

DNA for electroporation. Strains transformed were the ancestral non-resistant forms of the recipients used in the conjugation experiments except for *E. coli*, where the restriction system-free strain EC100 was used because transformation of BW25113 with plasmids obtained from *P. putida* barely yielded transformants. All three plasmids established in *P. putida* and *C. pinatubonensis* at indistinguishable efficiencies (Fig. 3a). One of the IncP-1 γ plasmids, pMBUI1T17, established in *E. coli* at a lower efficiency than pBP136Km, while the other, pKS208, established as efficiently as pBP136Km. As for the two alphaproteobacterial hosts, pMBUI1T17 did not establish in either one while pKS208 established in *A. tumefaciens*, but less efficiently than pBP136Km (Fig. 3a), and not at all in *S. japonicum*. This is consistent with the results from conjugation assays (Table 2). Our results suggest that the replication activity of intact IncP-1 γ plasmids in alphaproteobacterial strains is poor, and the replication host range of the IncP-1 γ plasmids only partially overlaps with that of the IncP-1 β plasmids.

Persistence host range of the intact IncP-1 plasmids

We investigated if the established plasmids could be stably maintained over 100 generations in the absence of selection for the plasmid (Fig. 4). IncP-1 β plasmid pBP136Km was highly stable in *E. coli*, *C. pinatubonensis* and *A. tumefaciens*, and showed a moderate instability in *P. putida* and *S. japonicum*. The two IncP-1 γ plasmids were more stable than pBP136Km in *P. putida* during the first 60 generations. Plasmid pMBUI1T17 showed moderate instability in *E. coli* and *C. pinatubonensis*, whereas

Fig. 3. Replication host range of pBP136, pMBUI1 and pKS208. (a) Efficiencies of establishment of the three intact IncP-1 plasmids in five hosts. Strains were *E. coli* EC100, *P. putida* KT2440, *C. pinatubonensis* MK2, *A. tumefaciens* C58rif and *S. japonicum* UT26. BDL, below detection limit. Statistical significance was determined by ANOVA followed by Dunnett's test: * $P < 0.05$, ** $P < 0.001$. (b) Structure of IncP-1 minireplicons. Flags indicate promoters and pins indicate terminators. The three plasmids are identical except for the region from the start codon of *trfA* to the end of the G+C-rich region in *oriV*. (c) Efficiencies of establishment of the three minireplicons in the same five hosts as in (a), except that the *E. coli* strain used was BW25113. Note that pSW29T alone did not establish in any host. (d) Western blots showing the levels of TrfA proteins produced from intact plasmids and minireplicons in three hosts in exponential growth phase. Cell extract quantities were normalized based on their protein contents using the Bradford assay (Bradford, 1976).

pKS208 was highly stable (Fig. 4). This moderate instability could thus be associated with plasmid-specific genetic determinants rather than subgroup-specific ones. Although pKS208 replicated adequately in *A. tumefaciens*, it was highly unstable in this host. This indicates not only that IncP-1 γ plasmids establish themselves poorly in alphaproteobacterial hosts, but also that they are not maintained well in these populations after establishment.

Replication host range of minireplicons

Poor establishment and poor maintenance of IncP-1 γ plasmids in alphaproteobacterial hosts can be due to non-optimal levels of the replication proteins (Swack *et al.*, 1987; Sevastyanovich *et al.*, 2005), or non-productive interactions between host proteins and either the initiation proteins (Maestro *et al.*, 2002, 2003) or the *oriV* gene (Caspi *et al.*, 2000). The observed difference in the host range between the two IncP-1 subgroups may also be due to a bias of plasmids size, as smaller plasmids can transform and transfer more efficiently than larger

plasmids with the same replicon. To address these possibilities, minireplicons of similar size were constructed from the three plasmids under study (Fig. 3b) and their establishment efficiencies were compared by electroporation assays (Fig. 3c). These replicons carried the *oriV* and *trfA* of the respective plasmids, and the latter was constitutively expressed under the influence of the *aph* promoter on the vector backbone in the absence of any of the native plasmids' regulatory genes. Replicon mini-pBP136 produced ca. 3.8-, 3.1- and 3.4-fold the normal levels of TrfA produced by pBP136Km in *E. coli*, *P. putida* and *A. tumefaciens*, respectively (Fig. 3). Plasmid mini-pKS208 seemed to produce more than 3-fold the normal level of TrfA in *P. putida* and *A. tumefaciens*, yet none in *E. coli*. However, we were unable to clearly determine the TrfA levels for IncP-1 γ plasmids pKS208 (Fig. 3d) and pMBUI1 (data not shown) due to the poor reactivity of the anti-TrfA_{pBP136} antiserum to the IncP-1 γ TrfA proteins.

In *P. putida* no difference in establishment efficiency was observed among the three minireplicons (Fig. 3c), as expected from the conjugation and transformation

Table 2. Plasmid transfer frequencies

Donor	Recipient (class*)	Frequency† (mean ± SEM)
<i>P. putida</i>		
KT2440R (pBP136Km)	<i>E. coli</i> BW25113S (γ)	$(1.1 \pm 0.3) \times 10^{-2}$
KT2440R (pMBUI1T17)	<i>E. coli</i> BW25113S (γ)	$(2.5 \pm 0.2) \times 10^{-1}$
KT2440R (pKS208), DH5α (pRK2013)‡	<i>E. coli</i> BW25113S (γ)	$(5.4 \pm 1.6) \times 10^{-5}$
KT2440R (pBP136Km)	<i>P. putida</i> KT2440S (γ)	0.6 ± 0.1
KT2440R (pMBUI1T17)	<i>P. putida</i> KT2440S (γ)	0.4 ± 0.1
KT2440R (pKS208), DH5α (pRK2013)	<i>P. putida</i> KT2440S (γ)	$(4.2 \pm 0.4) \times 10^{-5}$
KT2440R (pBP136Km)	<i>C. pinatubonensis</i> MK2S (β)	$<1.0 \times 10^{-7}$
KT2440R (pMBUI1T17)	<i>C. pinatubonensis</i> MK2S (β)	$<1.0 \times 10^{-7}$
KT2440R (pKS208), DH5α (pRK2013)	<i>C. pinatubonensis</i> MK2S (β)	$<1.0 \times 10^{-7}$
KT2440S (pBP136Km)	<i>A. tumefaciens</i> C58rif (α)	$(1.7 \pm 0.3) \times 10^{-3}$
KT2440S (pMBUI1T17)	<i>A. tumefaciens</i> C58rif (α)	$<1.0 \times 10^{-7}$
KT2440S (pKS208), DH5α (pRK2013)	<i>A. tumefaciens</i> C58rif (α)	$<1.0 \times 10^{-7}$
KT2440R (pBP136Km)	<i>S. japonicum</i> UT26SS (α)	$(5.2 \pm 1.6) \times 10^{-6}$
KT2440R (pMBUI1T17)	<i>S. japonicum</i> UT26SS (α)	$<1.0 \times 10^{-8}$
KT2440R (pKS208), DH5α (pRK2013)	<i>S. japonicum</i> UT26SS (α)	$<1.0 \times 10^{-8}$
<i>E. coli</i>		
BW25113R (pBP136Km)	<i>C. pinatubonensis</i> MK2S (β)	$(2.0 \pm 0.3) \times 10^{-1}$
BW25113R (pMBUI1T17)	<i>C. pinatubonensis</i> MK2S (β)	$(8.0 \pm 1.5) \times 10^{-4}$
BW25113R (pKS208), DH5α (pRK2013)	<i>C. pinatubonensis</i> MK2S (β)	$(8.0 \pm 0.9) \times 10^{-5}$
BW25113S (pBP136Km)	<i>A. tumefaciens</i> C58rif (α)	$(7.0 \pm 1.4) \times 10^{-2}$
BW25113S (pMBUI1T17)	<i>A. tumefaciens</i> C58rif (α)	$<1.0 \times 10^{-5}$ §
BW25113S (pKS208), DH5α (pRK2013)	<i>A. tumefaciens</i> C58rif (α)	$(8.7 \pm 5.9) \times 10^{-5}$
BW25113R (pBP136Km)	<i>S. japonicum</i> UT26SS (α)	$(1.0 \pm 0.5) \times 10^{-1}$
BW25113R (pMBUI1T17)	<i>S. japonicum</i> UT26SS (α)	$<1.0 \times 10^{-7}$
BW25113R (pKS208), DH5α (pRK2013)	<i>S. japonicum</i> UT26SS (α)	$<1.0 \times 10^{-7}$

*α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria.
†Plasmid transfer frequencies are represented as the number of transconjugants over donors. Matings were performed on cellulose filters. <, Frequency was lower than that of resistant mutants for either donor or recipient. Km at 50 µg ml⁻¹ was used for selection of both pBP136Km and pKS208 in all matings, except for the triparental mating with *E. coli* as recipient, where Ap at 100 µg ml⁻¹ was used to select for pKS208 in the presence of pRK2013 (Km^R); Tc was used to select for pMBUI1T17, at 10 µg ml⁻¹ for *E. coli* and *P. putida*, and 2.5 µg ml⁻¹ for the other strains.
‡Without helper plasmid the transfer frequency of pKS208 was below the detection limit (<10⁻⁷).
§Tc-resistant clones were obtained at higher frequency than background levels on plates with 100 µg Rif ml⁻¹ and 2.5 µg Tc ml⁻¹, but the majority of them did not possess pMBUI1T17 according to the results of PCR targeting the *trfA* gene. However, replating of mating mixtures with a higher Tc concentration (10 µg ml⁻¹ instead of 2.5 µg ml⁻¹) eliminated growth of the recipient and resulted in 20 putative transconjugants ml⁻¹ of mating mixture; four out of four clones tested contained pMBUI1T17 based on PCR, resulting in a very low transfer frequency per donor of 6.1 × 10⁻⁹.

Fig. 4. Plasmid persistence in five hosts. Strains used were the same as those listed in Fig. 3(c). The data points and error bars indicate means and SEM, respectively, obtained from three replicate experiments. Note that some plasmid–host combinations could not be tested as pMBUI1T17 did not replicate in either alphaproteobacterium, and pKS208 not in *S. japonicum*.

efficiencies. The numbers of transformants were greater for the minireplicons, suggesting that an increase in gene expression can have a positive effect on replication (Fig. 3c, compare with Fig. 3b). In *E. coli*, however, the establishment efficiency of mini-pKS208 was significantly lower than that of the other two minireplicons (Fig. 3c). Based on Western blotting results for *E. coli*, the TrfA levels of mini-pKS208 were not significantly higher than that of pKS208, while in *P. putida* and *A. tumefaciens* they were (Fig. 3d). Currently, we have no mechanistic explanation for the unexpectedly low levels of TrfA of mini-pKS208 and its low establishment efficiency. In *A. tumefaciens* the similarity in establishment efficiencies among the three minireplicons was unexpected as the intact IncP-1 γ plasmids did not establish as efficiently in this host as pBP136Km (compare Fig. 3c and Fig. 3a). Together with the higher levels of TrfA produced by mini-pKS208 (Fig. 3d), these findings suggest that the regulation of replication genes of the native IncP-1 γ plasmids was limiting the plasmid's establishment in *A. tumefaciens*, and not the TrfA and *oriV* functions themselves. In the other alphaproteobacterium, *S. japonicum*, the two IncP-1 γ minireplicons also established well – in contrast to their native counterparts – but the efficiency was still lower than that of mini-pBP136. We postulate that in *S. japonicum* the TrfA and/or *oriV* functions of IncP-1 γ plasmids do not allow proper replication. We are unable to compare TrfA levels between the minireplicons and their wild-type counterparts because the latter did not replicate in this host. However, if TrfA levels were greater for the minireplicons than for the native plasmids, that increase apparently could only partially override the inadequate replication efficiency. In conclusion, while there are differences between strains and plasmids, based on overall establishment efficiencies, the alphaproteobacteria are not as good a host for the IncP-1 γ plasmids as for the IncP-1 β plasmids. This can be explained in part by suboptimal regulation of the IncP-1 γ plasmid replication initiation genes.

DISCUSSION

Plasmids are ubiquitous mobile genetic elements found in many bacteria. Among the self-transmissible plasmids in the *Proteobacteria*, the IncP-1 group is unique with respect to both the number of available plasmid sequences deposited in GenBank so far and the high sequence diversity within the group. Such a divergence in the plasmid backbone genes within an Inc group (<80 % amino-acid sequence identity between homologues) has also been reported for the groups IncQ (Loftie-Eaton & Rawlings, 2012), IncP-9 (Sevastyanovich *et al.*, 2008) and IncX (Johnson *et al.*, 2012), whereas relatively low divergence was observed among the currently available plasmid sequences of groups IncA/C (Fricke *et al.*, 2009), IncI (Takahashi *et al.*, 2011) and IncP-7 (Yano *et al.*, 2010). Reduced similarity was observed, for an as yet unknown reason, specifically in a partitioning gene in IncI and IncP-7 groups despite high similarity in the replication and

transfer genes within the Inc groups (Takahashi *et al.*, 2011; Yano *et al.*, 2010). This divergence in plasmid backbone sequence within a group of closely related plasmids may be the consequence of adaptive mutations that allow specialization towards specific bacterial hosts. Divergence may be further facilitated by reduced gene flow (recombination) between plasmids due to lack of co-occurrence in the same bacterial cell. This would be equivalent to the concept of reproductive isolation in the evolution of sexually reproducing populations (Wu & Ting, 2004). However, it is not known whether this sequence divergence has any effect on the current potential of these plasmids to transfer, establish themselves and persist in various bacterial hosts. Since it was reported 20 years ago that plasmids in subgroups of the IncH plasmid group showed a different replication host range and thermo-sensitivity in transfer (Maher & Taylor, 1993), few attempts have been made to address the importance of naturally occurring genetic variation within an Inc group to plasmid host range. This is despite greatly increased availability of complete plasmid genome sequences, which now allows these questions to be addressed. In this study, we investigated whether sequence divergence in plasmids of the IncP-1 incompatibility group is associated with host specialization.

Until recently, the IncP-1 γ subgroup consisted of only one plasmid that was completely sequenced and characterized, plasmid pQKH54. This 70 kb plasmid encodes mercury resistance as the only reported accessory trait and was captured from river epilithon in Wales by an exogenous plasmid isolation method (Haines *et al.*, 2006). The authors defined the IncP-1 γ subgroup based on clear divergence of the plasmid's backbone genes from the well-characterized plasmids of the IncP-1 α and IncP-1 β subgroups (Haines *et al.*, 2006; Norberg *et al.*, 2011). Here we describe the first multi-drug resistance plasmid in this IncP-1 γ subgroup, pKS208, as well as the first cryptic plasmid, pMBUI1. While pMBUI1 is closely related to the archetype pQKH54, both in backbone gene synteny and in sequence similarity, pKS208 is clearly more divergent. It is worthwhile mentioning that all three plasmids were isolated by the exogenous isolation method and therefore their natural hosts are unknown. Lee *et al.* (2010) and Stenger & Lee (2011) concluded that two plasmids from *Xylella fastidiosa* as well as others belong to what they called the ' γ -(expanded) subgroup' of IncP-1 plasmids. However, based on our analysis of the plasmids' replication initiation proteins and backbone gene synteny, they are too dissimilar from those of the γ - and other IncP-1 subgroups to be classified with certainty as IncP-1 plasmids (data not shown). Thus, in contrast to their counterparts of the IncP-1 α , IncP-1 β and IncP-1 δ subgroups, which have been found in bacterial isolates, so far no natural or clinical bacterial isolates with IncP-1 γ plasmids have been reported. Similarly, IncP-1 ϵ and IncP-1 ξ plasmids have so far only been obtained through exogenous plasmid isolation. It suggests that this plasmid capture method allows isolation of promiscuous plasmids that are not easily obtained by

cultivation-dependent methods. The question remains in which hosts these plasmids typically reside in various ecosystems.

When the host range of the plasmids was determined by conjugation, self-transfer of pKS208 was not detectable. We speculate that potential in-frame deletions in *traC* or absence of an IncP-1 γ -specific, conserved hypothetical protein gene may be the cause of lack of self-transmissibility of pKS208. The core gene and gene product sequences of pKS208 are considerably different from those of the other IncP-1 γ plasmids (Fig. 1b) (Sen *et al.*, 2013), and so is the DNA G+C content of its *trb* and *tra* operons (Fig. 2). Isolation and characterization of new IncP-1 γ plasmids from various habitats should result in more plasmids like pKS208 and help determine the genetic basis of this conjugation deficiency.

Initial studies on the host range of pQKH54 suggested that this plasmid transferred less efficiently to an *Agrobacterium* host than the IncP-1 β plasmids R751 (β subgroup archetype) and pJP4. This was observed in conjugation assays with a *P. putida* strain as plasmid donor (Haines *et al.*, 2006). By comparing genomic signatures between whole plasmid genome sequences and bacterial chromosomes we previously showed that pQKH54 had the highest genomic signature similarity to three *Pseudomonas* chromosomes. In contrast, the only bacterium with a chromosomal signature rather similar to that of pBP136 was a betaproteobacterium (*Acidovorax* sp.) (Suzuki *et al.*, 2010). Similarly, Norberg *et al.* (2011) subsequently showed that the genomic signature of one of four backbone segments of pQKH54 was most similar to that of the chromosomes of *Gammaproteobacteria*, while pBP136 and most other IncP-1 β plasmids showed backbone signatures that were almost always most similar to those of *Betaproteobacteria*. These findings raised the hypotheses that (i) IncP-1 γ plasmids may have acquired a *Pseudomonas*-like signature because they evolved in these bacteria and are thus well adapted to them, and (ii) they persist more poorly in *Alphaproteobacteria* and *Betaproteobacteria*, as they probably resided in these bacteria for shorter periods. To test this hypothesis we extended the host range analysis for the IncP-1 γ group to two new members of IncP-1 γ group, pKS208 and pMBUI1. We determined the efficiency of plasmid establishment in a quantitative manner and evaluated their persistence in the absence of antibiotics selecting for the plasmids. One phenotype that pKS208 and pMBUI1 have in common is poor establishment and maintenance in alphaproteobacterial hosts when compared with pBP136 (Table 2, Figs 3a and 4). This is in agreement with the previous report of relatively inefficient transfer of pQKH54 to an *Agrobacterium* host, and strongly suggests that IncP-1 γ plasmids have a different host range from the well-studied IncP-1 α and IncP-1 β plasmids (Thomas & Smith, 1987; De Gelder *et al.*, 2007). These partially overlapping host ranges may thus result in reproductive isolation of IncP-1 plasmids, which reduces gene flow between plasmid

subgroups, and facilitates further sequence divergence (Wu & Ting, 2004).

In spite of the hypothesis raised by genomic signature analysis that IncP-1 γ plasmids may have acquired a *Gammaproteobacteria*-like signature because they predominantly evolved in these bacteria, our experimental assays did not show a clear advantage of our two IncP-1 γ plasmids over the IncP-1 β plasmid in establishment (Table 2, Fig. 3a) or persistence (Fig. 4) in two gamma-proteobacterial species. Our empirical knowledge about plasmid host range is limited by the availability of model hosts, and can also be confounded by strain- and species-specific plasmid permissiveness (De Gelder *et al.*, 2007; Heuer *et al.*, 2007, 2010). Future systematic host range analyses using additional model hosts will be important to strengthen the host-specialization hypothesis.

Plasmid evolution through recombination requires that plasmids at least temporarily co-occur in the same cell. Although plasmids of the same incompatibility group cannot stably coexist within the same cell line, it has been shown that they can recombine. For example, pB3, pB10, pBP136 and pAOVO02 are found to be chimeric plasmids composed of different types of IncP-1 β plasmid backbone (Schlüter *et al.*, 2003; Norberg *et al.*, 2011). Additionally, recombination was detected between the backbones of IncP-1 δ plasmid pIJB1 and an IncP-1 β plasmid (Sen *et al.*, 2010), between IncP-1 α and IncP-1 δ plasmids, and among IncP-1 β , IncP-1 δ and IncP-1 ϵ plasmids (Sen *et al.*, 2013). This evidence of recombination suggests the presence of gene flow within and between these subgroups. The recombination analysis by Norberg *et al.* (2011) suggested recombination between IncP-1 γ and other subgroup plasmids (possibly with their newly defined IncP-1 ξ subgroup). However, our recent phylogenetic analysis did not detect any incongruence of IncP-1 γ backbone genes and no recombination was detected either, suggesting no or limited recombination between IncP-1 γ plasmids and those from the IncP-1 α , IncP-1 β , IncP-1 δ and IncP-1 ϵ subgroups (Sen *et al.*, 2013). Continued divergent evolution of the IncP-1 γ plasmids due to host specialization and limited recombination may eventually result in a new plasmid 'species' whose backbone genes no longer recombine with those of other IncP-1 subgroup plasmids.

The host range analysis for both minireplicons and the intact plasmids showed that the levels of replication initiation protein may limit plasmid host range. A similar conclusion was obtained for an IncP-9 plasmid pM3 (Sevastyanovich *et al.*, 2005). Unlike other iteron-containing plasmids where the replication initiation protein regulates its own gene expression (Chattoraj *et al.*, 1985; Filutowicz *et al.*, 1985; García de Viedma *et al.*, 1995), the transcription of *trfA* of IncP-1 plasmid is regulated by plasmid-encoded global regulators (KorA, KorB, TrbA) (Jagura-Burdzy *et al.*, 1992; Jagura-Burdzy & Thomas, 1994, 1997). Mutations in non-coding regions and regulatory proteins may thus cause host specialization

and therefore spatial separation of plasmids that results in sequence divergence. Given the rapidly growing concern about the spread of antibiotic resistance genes among Gram-negative pathogens by self-transmissible plasmids (Wellington *et al.*, 2013), current and future studies urgently need to improve our understanding of how plasmid regulatory systems can evolve to influence a plasmid's host range and therefore its ecological and evolutionary trajectory.

ACKNOWLEDGEMENTS

This work was funded by the National Institute of Allergy and Infectious Diseases (NIAID) (NIH grant R01 AI084918). We thank Bernardo González at Universidad Adolfo Ibáñez for kindly providing strain JMP134, Yuji Nagata at Tohoku University for strain UT26S and Didier Mazel at The Pasteur Institute for vector pSW29T. We also thank W. Loftie-Eaton, C. Renfrow, A. Tucker and A. Crabtree at the University of Idaho for their assistance with some of the experiments, and G. Van der Auwera and G. Metzger for useful discussions.

REFERENCES

- Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J. & Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* **8**, 251–259.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. & Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006.0008.
- Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C., Bagdasarian, M. M., Frey, J. & Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.
- Bahl, M. I., Hansen, L. H., Goesmann, A. & Sørensen, S. J. (2007). The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established α , β and δ sub-groups. *Plasmid* **58**, 31–43.
- Bahl, M. I., Burmølle, M., Meisner, A., Hansen, L. H. & Sørensen, S. J. (2009). All IncP-1 plasmid subgroups, including the novel epsilon subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* **62**, 134–139.
- Binh, C. T., Heuer, H., Kaupenjohann, M. & Smalla, K. (2008). Piggy manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol Ecol* **66**, 25–37.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Brown, C. J., Sen, D., Yano, H., Bauer, M. L., Rogers, L. M., Van der Auwera, G. A. & Top, E. M. (2013). Diverse broad-host-range plasmids from freshwater carry few accessory genes. *Appl Environ Microbiol*. In press.
- Caspi, R., Helinski, D. R., Pacek, M. & Konieczny, I. (2000). Interactions of DnaA proteins from distantly related bacteria with the replication origin of the broad host range plasmid RK2. *J Biol Chem* **275**, 18454–18461.
- Chattoraj, D. K., Snyder, K. M. & Abeles, A. L. (1985). P1 plasmid replication: multiple functions of RepA protein at the origin. *Proc Natl Acad Sci U S A* **82**, 2588–2592.
- Dahlberg, C., Linberg, C., Torsvik, V. L. & Hermansson, M. (1997). Conjugative plasmids isolated from bacteria in marine environments show various degrees of homology to each other and are not closely related to well-characterized plasmids. *Appl Environ Microbiol* **63**, 4692–4697.
- De Gelder, L., Vandecasteele, F. P., Brown, C. J., Forney, L. J. & Top, E. M. (2005). Plasmid donor affects host range of promiscuous IncP-1 β plasmid pB10 in an activated-sludge microbial community. *Appl Environ Microbiol* **71**, 5309–5317.
- De Gelder, L., Ponciano, J. M., Joyce, P. & Top, E. M. (2007). Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology* **153**, 452–463.
- Demarre, G., Guérout, A. M., Matsumoto-Mashimo, C., Rowe-Magnus, D. A., Marlière, P. & Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncP α) conjugative machineries and their cognate *Escherichia coli* host strains. *Res Microbiol* **156**, 245–255.
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Filutowicz, M., Davis, G., Greener, A. & Helinski, D. R. (1985). Autorepressor properties of the π -initiation protein encoded by plasmid R6K. *Nucleic Acids Res* **13**, 103–114.
- Fricke, W. F., Welch, T. J., McDermott, P. F., Mammel, M. K., LeClerc, J. E., White, D. G., Cebula, T. A. & Ravel, J. (2009). Comparative genomics of the IncA/C multidrug resistance plasmid family. *J Bacteriol* **191**, 4750–4757.
- Funnell, B. E. & Phillips, G. J. (2004). *Plasmid Biology*. Washington, DC: American Society for Microbiology.
- García de Viedma, D., Giraldo, R., Ruiz-Echevarría, M. J., Lurz, R. & Díaz-Orejas, R. (1995). Transcription of *repA*, the gene of the initiation protein of the *Pseudomonas* plasmid pPS10, is autoregulated by interactions of the RepA protein at a symmetrical operator. *J Mol Biol* **247**, 211–223.
- Garcillán-Barcia, M. P., Francia, M. V. & de la Cruz, F. (2009). The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol Rev* **33**, 657–687.
- Garcillán-Barcia, M. P., Alvarado, A. & de la Cruz, F. (2011). Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol Rev* **35**, 936–956.
- Gomes, N. C., Flocco, C. G., Costa, R., Junca, H., Vilchez, R., Pieper, D. H., Krögerrecklenfort, E., Paranhos, R., Mendonça-Hagler, L. C. & Smalla, K. (2010). Mangrove microniches determine the structural and functional diversity of enriched petroleum hydrocarbon-degrading consortia. *FEMS Microbiol Ecol* **74**, 276–290.
- Guiney, D. G. (1982). Host range of conjugation and replication functions of the *Escherichia coli* sex plasmid Flac: comparison with the broad host-range plasmid RK2. *J Mol Biol* **162**, 699–703.
- Guiney, D. G., Hasegawa, P. & Davis, C. E. (1984). Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*: differential expression of antibiotic resistance phenotypes. *Proc Natl Acad Sci U S A* **81**, 7203–7206.
- Haines, A. S., Akhtar, P., Stephens, E. R., Jones, K., Thomas, C. M., Perkins, C. D., Williams, J. R., Day, M. J. & Fry, J. C. (2006). Plasmids from freshwater environments capable of IncQ retrotransfer are diverse and include pQKH54, a new IncP-1 subgroup archetype. *Microbiology* **152**, 2689–2701.

- Heinemann, J. A. & Sprague, G. F. J., Jr (1989). Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature* **340**, 205–209.
- Heuer, H. & Smalla, K. (2012). Plasmids foster diversification and adaptation of bacterial populations in soil. *FEMS Microbiol Rev* **36**, 1083–1104.
- Heuer, H., Krögerrecklenfort, E., Wellington, E. M., Egan, S., van Elsas, J. D., van Overbeek, L., Collard, J. M., Guillaume, G., Karagouni, A. D. & other authors (2002). Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol* **42**, 289–302.
- Heuer, H., Fox, R. E. & Top, E. M. (2007). Frequent conjugative transfer accelerates adaptation of a broad-host-range plasmid to an unfavorable *Pseudomonas putida* host. *FEMS Microbiol Ecol* **59**, 738–748.
- Heuer, H., Ebers, J., Weinert, N. & Smalla, K. (2010). Variation in permissiveness for broad-host-range plasmids among genetically indistinguishable isolates of *Dickeya* sp. from a small field plot. *FEMS Microbiol Ecol* **73**, 190–196.
- Heuer, H., Binh, C. T., Jechalke, S., Kopmann, C., Zimmerling, U., Krögerrecklenfort, E., Ledger, T., González, B., Top, E. & Smalla, K. (2012). IncP-1 ϵ plasmids are important vectors of antibiotic resistance genes in agricultural systems: diversification driven by class 1 integron gene cassettes. *Front Microbiol* **3**, 2.
- Jacoby, G. A., Jacob, A. E. & Hedges, R. W. (1976). Recombination between plasmids of incompatibility groups P-1 and P-2. *J Bacteriol* **127**, 1278–1285.
- Jagura-Burdzy, G. & Thomas, C. M. (1994). KorA protein of promiscuous plasmid RK2 controls a transcriptional switch between divergent operons for plasmid replication and conjugative transfer. *Proc Natl Acad Sci U S A* **91**, 10571–10575.
- Jagura-Burdzy, G. & Thomas, C. M. (1997). Dissection of the switch between genes for replication and transfer of promiscuous plasmid RK2: basis of the dominance of *trfAp* over *trbAp* and specificity for KorA in controlling the switch. *J Mol Biol* **265**, 507–518.
- Jagura-Burdzy, G., Khanim, F., Smith, C. A. & Thomas, C. M. (1992). Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. *Nucleic Acids Res* **20**, 3939–3944.
- Jobanputra, R. S. & Datta, N. (1974). Trimethoprim R factors in enterobacteria from clinical specimens. *J Med Microbiol* **7**, 169–177.
- Johnson, T. J., Bielak, E. M., Fortini, D., Hansen, L. H., Hasman, H., Debroy, C., Nolan, L. K. & Carattoli, A. (2012). Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant *Enterobacteriaceae*. *Plasmid* **68**, 43–50.
- Kamachi, K., Sota, M., Tamai, Y., Nagata, N., Konda, T., Inoue, T., Top, E. M. & Arakawa, Y. (2006). Plasmid pBP136 from *Bordetella pertussis* represents an ancestral form of IncP-1 β plasmids without accessory mobile elements. *Microbiology* **152**, 3477–3484.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL_X version 2.0. *Bioinformatics* **23**, 2947–2948.
- Lee, M. W., Rogers, E. E. & Stenger, D. C. (2010). Functional characterization of replication and stability factors of an incompatibility group P-1 plasmid from *Xylella fastidiosa*. *Appl Environ Microbiol* **76**, 7734–7740.
- Liebert, C. A., Hall, R. M. & Summers, A. O. (1999). Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* **63**, 507–522.
- Loftie-Eaton, W. & Rawlings, D. E. (2012). Diversity, biology and evolution of IncQ-family plasmids. *Plasmid* **67**, 15–34.
- Lykidis, A., Pérez-Pantoja, D., Ledger, T., Mavromatis, K., Anderson, I. J., Ivanova, N. N., Hooper, S. D., Lapidus, A., Lucas, S. & other authors (2010). The complete multipartite genome sequence of *Cupriavidus necator* JMP134, a versatile pollutant degrader. *PLoS ONE* **5**, e9729.
- Maestro, B., Sanz, J. M., Faalen, M., Couturier, M., Díaz-Orejas, R. & Fernández-Tresguerres, E. (2002). Modulation of pPS10 host range by DnaA. *Mol Microbiol* **46**, 223–234.
- Maestro, B., Sanz, J. M., Díaz-Orejas, R. & Fernández-Tresguerres, E. (2003). Modulation of pPS10 host range by plasmid-encoded RepA initiator protein. *J Bacteriol* **185**, 1367–1375.
- Maher, D. & Taylor, D. E. (1993). Host range and transfer efficiency of incompatibility group HI plasmids. *Can J Microbiol* **39**, 581–587.
- Nagata, Y., Natsui, S., Endo, R., Ohtsubo, Y., Ichikawa, N., Ankai, A., Oguchi, A., Fukui, S., Fujita, N. & Tsuda, M. (2011). Genomic organization and genomic structural rearrangements of *Sphingobium japonicum* UT26, an archetypal γ -hexachlorocyclohexane-degrading bacterium. *Enzyme Microb Technol* **49**, 499–508.
- Nojiri, H., Shintani, M. & Omori, T. (2004). Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. *Appl Microbiol Biotechnol* **64**, 154–174.
- Norberg, P., Bergström, M., Jethava, V., Dubhashi, D. & Hermansson, M. (2011). The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nat Commun* **2**, 268.
- Ohtsubo, Y., Ikeda-Ohtsubo, W., Nagata, Y. & Tsuda, M. (2008). GenomeMatcher: a graphical user interface for DNA sequence comparison. *BMC Bioinformatics* **9**, 376.
- R Development Core Team (2010). R: A language and environment for statistical computing. <http://www.R-project.org>.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbour, NY: Cold Spring Harbor Laboratory Press.
- Schlüter, A., Heuer, H., Szczepanowski, R., Forney, L. J., Thomas, C. M., Pühler, A. & Top, E. M. (2003). The 64 508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group. *Microbiology* **149**, 3139–3153.
- Schlüter, A., Szczepanowski, R., Pühler, A. & Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev* **31**, 449–477.
- Schmidhauser, T. J. & Helinski, D. R. (1985). Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. *J Bacteriol* **164**, 446–455.
- Sen, D., Yano, H., Suzuki, H., Król, J. E., Rogers, L., Brown, C. J. & Top, E. M. (2010). Comparative genomics of pAKD4, the prototype IncP-1 δ plasmid with a complete backbone. *Plasmid* **63**, 98–107.
- Sen, D., Brown, C. J., Top, E. M. & Sullivan, J. (2013). Inferring the evolutionary history of IncP-1 plasmids despite incongruence among backbone gene trees. *Mol Biol Evol* **30**, 154–166.
- Sevastyanovich, Y. R., Titok, M. A., Krasowiak, R., Bingle, L. E. & Thomas, C. M. (2005). Ability of IncP-9 plasmid pM3 to replicate in *Escherichia coli* is dependent on both *rep* and *par* functions. *Mol Microbiol* **57**, 819–833.
- Sevastyanovich, Y. R., Krasowiak, R., Bingle, L. E., Haines, A. S., Sokolov, S. L., Kosheleva, I. A., Leuchuk, A. A., Titok, M. A., Smalla, K. & Thomas, C. M. (2008). Diversity of IncP-9 plasmids of *Pseudomonas*. *Microbiology* **154**, 2929–2941.
- Shintani, M., Takahashi, Y., Yamane, H. & Nojiri, H. (2010). The behavior and significance of degradative plasmids belonging to Inc

- groups in *Pseudomonas* within natural environments and microcosms. *Microbes Environ* **25**, 253–265.
- Slater, F. R., Bailey, M. J., Tett, A. J. & Turner, S. L. (2008).** Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiol Ecol* **66**, 3–13.
- Sota, M., Yano, H., Hughes, J. M., Daughdrill, G. W., Abdo, Z., Forney, L. J. & Top, E. M. (2010).** Shifts in the host range of a promiscuous plasmid through parallel evolution of its replication initiation protein. *ISME J* **4**, 1568–1580.
- Stenger, D. C. & Lee, M. W. (2011).** Phylogeny of replication initiator protein TrfA reveals a highly divergent clade of incompatibility group P1 plasmids. *Appl Environ Microbiol* **77**, 2522–2526.
- Stewart, F. M. & Levin, B. R. (1977).** The population biology of bacterial plasmids: *a priori* conditions for the existence of conjugationally transmitted factors. *Genetics* **87**, 209–228.
- Suzuki, H., Sota, M., Brown, C. J. & Top, E. M. (2008).** Using Mahalanobis distance to compare genomic signatures between bacterial plasmids and chromosomes. *Nucleic Acids Res* **36**, e147.
- Suzuki, H., Yano, H., Brown, C. J. & Top, E. M. (2010).** Predicting plasmid promiscuity based on genomic signature. *J Bacteriol* **192**, 6045–6055.
- Swack, J. A., Pal, S. K., Mason, R. J., Abeles, A. L. & Chattoraj, D. K. (1987).** P1 plasmid replication: measurement of initiator protein concentration *in vivo*. *J Bacteriol* **169**, 3737–3742.
- Takahashi, H., Shao, M., Furuya, N. & Komano, T. (2011).** The genome sequence of the incompatibility group I γ plasmid R621a: evolution of IncI plasmids. *Plasmid* **66**, 112–121.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).** MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.
- Thomas, C. M. & Smith, C. A. (1987).** Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. *Annu Rev Microbiol* **41**, 77–101.
- Wellington, E. M. H., Boxall, A. B. A., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M. & other authors (2013).** The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect Dis* **13**, 155–165.
- Wood, D. W., Setubal, J. C., Kaul, R., Monks, D. E., Kitajima, J. P., Okura, V. K., Zhou, Y., Chen, L., Wood, G. E. & other authors (2001).** The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**, 2317–2323.
- Wu, C. I. & Ting, C. T. (2004).** Genes and speciation. *Nat Rev Genet* **5**, 114–122.
- Yakobson, E. & Guiney, G. (1983).** Homology in the transfer origins of broad host range IncP plasmids: definition of two subgroups of P plasmids. *Mol Gen Genet* **192**, 436–438.
- Yano, H., Miyakoshi, M., Ohshima, K., Tabata, M., Nagata, Y., Hattori, M. & Tsuda, M. (2010).** Complete nucleotide sequence of TOL plasmid pDK1 provides evidence for evolutionary history of IncP-7 catabolic plasmids. *J Bacteriol* **192**, 4337–4347.
- Yano, H., Deckert, G. E., Rogers, L. M. & Top, E. M. (2012).** Roles of long and short replication initiation proteins in the fate of IncP-1 plasmids. *J Bacteriol* **194**, 1533–1543.

Edited by: M. Holden