Distribution of the *ardA* family of antirestriction genes on conjugative plasmids

Paul M. Chilley and Brian M. Wilkins

Author for correspondence: Brian M. Wilkins. Tel: +44 116 252 3432/3374. Fax: +44 116 252 5101.

Department of Genetics,
University of Leicester,
Leicester LE1 7RH, UK

The *ardA* gene of I1 plasmid ColIb-P9 was previously shown to alleviate DNA restriction by type I enzymes and to promote conjugative transmission of the unmodified plasmid to a restricting host. To clarify the ecological role of *ardA*, its distribution was determined on plasmids from 23 incompatibility groups using hybridization to the coding sequence as an assay. Hybridizing sequences, shown by nucleotide sequencing to have at least 60% identity with *ardA*, were detected on plasmids belonging to the I complex (IncB, I1 and K), the F complex (IncFV) and the IncN group. The *ardA* homologues were found to specify an antirestriction phenotype which was enhanced by genetic derepression of the plasmid transfer system. *ardA* loci map in plasmid leading regions but show no consistent association with a particular type of origin-of-transfer or a leading region gene of the *ssb* (single-stranded DNA-binding protein), *psiB* (plasmid SOS inhibition) and *hok* (host killing) families. It may be significant that *ardA*⁺ plasmids are authentic enterobacterial plasmids and that type I restriction systems are associated historically with members of the Enterobacteriaceae.

Keywords: bacterial conjugation, conjugative plasmids, restriction alleviation, type I restriction enzymes

INTRODUCTION

Bacterial restriction-modification (R-M) systems consist of a restriction endonuclease plus a matching modification methyltransferase which recognize the same specificity site. Methylation of one or both strands at the recognition site protects the DNA from the cognate restriction enzyme. R-M systems can be divided into three types. Type III systems, which are the least common, cleave DNA on one side of asymmetric specificity sites. Classical type II systems, familiar through the enzymes central to recombinant DNA technology, are comprised of separate methylases and endonucleases that recognize short specificity sites with an axis of rotational symmetry. Type I systems are more complex; the main enzyme is a multisubunit protein active in restriction and modification, the specificity sites are characteristically asymmetric, and DNA cleavage occurs at variable and often considerable distances from the recognition sites (Wilson & Murray, 1991; Bickle & Krüger, 1993; Barcus & Murray, 1995).

Restriction enzymes destroy unmodified DNA that is transferred between bacteria, irrespective of whether transfer is by bacteriophage infection, transformation or plasmid-mediated conjugation (Boyer, 1971). The enzymes are prevalent and diverse, being found in approximately one quarter of bacterial isolates and recognizing more than 200 specificities (Roberts & Macelis, 1994; Barcus & Murray, 1995). Hence, restriction is likely to be a frequently encountered barrier to the flow of DNA between bacterial strains in nature. Not surprisingly, phages and plasmids have evolved strategies for evading restriction (Bickle & Krüger, 1993; Barcus & Murray, 1995; Wilkins, 1995). One such strategy involves the activity of antirestriction genes.

The first plasmid-borne antirestriction gene to be described was *ardA*. The locus was identified on conjugative plasmid ColIb-P9 of the IncI1 group and shown to act specifically against type I restriction enzymes (Delper *et al.*, 1991; Read *et al.*, 1992). Carriage of *ardA* was found to protect unmodified ColIb transferred by bacterial conjugation, with protection requiring expression of the gene in the transconjugant cell. Such a mode of action is consistent with the position of *ardA* in the plasmid leading region, defined as the first portion of the plasmid to enter the recipient cell (Read *et al.*, 1992). An *ardA* homologue has also been found on the leading region of

Abbreviation: R-M, restriction-modification.

The EMBL accession numbers for the sequences reported in this paper are Z34467 and Z34468.

IncN plasmid pKM101, which contains an additional second antirestriction gene called *ardB* (Belogurov *et al.*, 1992, 1993). Detection of conserved *ardA* genes in the leading regions of IncI1 and IncN plasmids is remarkable, since these plasmids differ extensively in backbone portions involved in replication and transfer (Rees *et al.*, 1987; Couturier *et al.*, 1988; Pohlman *et al.*, 1994; Lanka & Wilkins, 1995).

The objective of the work described here was to determine the distribution and arrangement of *ardA* antirestriction genes on plasmids with the aim of elucidating the ecological role of the gene family. To this end, we examined representatives of 23 incompatibility (Inc) groups of enterobacterial conjugative plasmid for the presence of a conserved *ardA* gene. We have also determined whether *ardA* genes are consistently linked to a particular type of origin of transfer (*oriT*) site or leading region gene. Well-characterized leading-region genes include plasmid *ssb* loci, determining classic single-stranded DNA-binding proteins (Chase *et al.*, 1983; Golub & Low, 1985; Howland *et al.*, 1989), *psiB*, specifying a protein that interferes with the bacterial SOS response (Bagdasarian *et al.*, 1986; Golub *et al.*, 1988; Jones *et al.*, 1992), and *hok* (*flm*) (Gerdes *et al.*, 1986; Loh *et al.*, 1988), which encodes an inducible toxin that kills cells from within (Sakikawa *et al.*, 1989; Gerdes *et al.*, 1990).

METHODS

Plasmids and bacterial strains. Conjugative plasmids were obtained from the collections of B. M. Wilkins, P. H. Williams (University of Leicester) and the National Collection of Type Cultures, London. IncFV plasmids and pBF105 (Finlay *et al.*, 1986) were provided by L. Frost. pLG221 is ColIb*drd-1 cib::Tn5* (Howland *et al.*, 1989). R387*drd-86* was isolated as described for R16*drd-86* (Bradley, 1984). pRS1078 is a pACYC184-derivative carrying 10.2 kb of the F plasmid leading region (Ray & Skurray, 1983). Vectors used were pUC18 (Yanisch-Perron *et al.*, 1988) and the lower copy-number plasmid pHG165 (Stewart *et al.*, 1986). Recombinant plasmids constructed include: pLG2051 [pUC18 Ω (R16 *ardA*⁺ *Pst*I 2.2 kb)]; pLG2052 [pUC18 Ω (R16 *ardA*⁺ 0.62 kb)]; pLG2053 [pHG165 Ω (F₀*lac* *ardA*⁺ *Eco*RI 11.5 kb)], Fig. 4; pLG2054 [pUC18 Ω (F₀*lac*⁺ *ardA* *Sal*I-*Sph*I 3.3 kb)], Fig. 4. pLG2055, equivalent to pLG290 (Read *et al.*, 1992), is pUC19 containing ColIb *ardA*⁺ on a 2.8 kb *Sal*I-*Pst*I fragment from pLG2012 (Rees *et al.*, 1987). Sets of unidirectional deletions were produced by the method of Henikoff (1984). Bacterial strains included nalidixic acid-resistant derivatives (designated by the suffix N) of C600 ($r_K^+m_K^+$) and 5K ($r_K^-m_K^+$; see Read *et al.*, 1992), J53 (see Bradley, 1984) and BW85 (Rees *et al.*, 1987).

Dotblot and Southern hybridizations. Genomic DNA was isolated by the method of Ausubel *et al.* (1991). Bacteria grown overnight in broth were lysed with SDS and proteins were removed by digestion with proteinase K. Cell wall debris, polysaccharides and remaining proteins were removed by selective precipitation with hexadecyltrimethyl ammonium bromide-NaCl solution. DNA was recovered from the supernatant by isopropanol precipitation. Dotblots contained 10 ng heat-denatured genomic DNA per spot. Hybridization was carried out at 65 °C in the presence of 0.5 M phosphate, pH 7.2, containing 7% SDS and 1 mM EDTA (Church & Gilbert, 1984). Post-hybridization washes were at 65 °C in

0.1 × SSC + 0.1% SDS (high stringency), 0.5 × SSC + 0.1% SDS (medium stringency) or 3 × SSC + 0.1% SDS (low stringency). Other methods, including radiolabelling of probes, were as previously described by Howland *et al.* (1989).

Probes. PCR-derived probes were synthesized using *Taq* DNA polymerase. Primers for PCR were, for ColIb *ardA* (Read *et al.*, 1992), 5'-GGCATATGTCTGTTGTTGCACCTG-3' and 5'-CCGGATCCTCACCGACGGAACACAT-3' and, for pKM101 *ardA* (Belogurov *et al.*, 1992), 5'-GGGGATCCATGACTGATATTACGACC-3' and 5'-CCAAGCTTTCAGCAAGTCATGTTAAA-3'. Identities of ColIb and pKM101 PCR products were verified by sizing fragments generated by *Sma*I and *Acl*I, respectively. The *pnd* and *hok* probes extended from residue 140 to 562 in the alignments of Gerdes *et al.* (1990), and were synthesized using 18-mer primers; pAN-1 (Nielsen *et al.*, 1991) and pPR311 (Rasmussen *et al.*, 1987) were templates. Other probes included a 1.55 kb *Pst*I fragment for ColIb *oriT* (Rees *et al.*, 1987), a 0.53 kb *Eco*RV fragment of R6-5 for *psiB* (see Jones *et al.*, 1992), and a 0.7 kb *Ava*II fragment of F for *ssb* (see Howland *et al.*, 1989).

Nucleotide sequencing. Sequencing was performed on denatured double-stranded plasmid DNA using either the Sequenase version 2.0 kit (USB) or the Cyclist Exo⁻ *Pfu* sequencing kit (Stratagene). Primers included M13 universal and reverse primers. 18-mers complementary to F₀ *lac* sequences were also used on pLG2053 templates. Data were analysed using the GCG package (version 7).

Genetic tests. Phage λ assays were as previously described (Read *et al.*, 1992). Mobilization frequencies of *oriT* recombinants were determined as before (Rees *et al.*, 1987), with conjugation occurring on the surface of a nitrocellulose disk for 60 min. The *oriT* recombinant was pLG2009, except for tests with an Ap^r conjugative plasmid when pLG2008 was used. The assay for the Pnd⁺ phenotype involved incubation of broth-growing bacteria in the presence of 200 μ g rifampicin ml⁻¹ for 40 min at 37 °C. Cell suspensions were then plated on nutrient agar to determine loss of colony-forming ability.

RESULTS

Distribution of *ard* genes on enterobacterial conjugative plasmids

The initial screen was for homologues detectable by hybridization to a probe of ColIb *ardA*. The gene was identified previously as an ORF encoding a predicted 19 kDa polypeptide of 166 amino acid residues (Delver *et al.*, 1991; Read *et al.*, 1992). Probe was prepared from PCR-amplified *ardA* coding sequence and tested for hybridization to DNA samples representing 23 Inc groups of plasmid characterized in *E. coli* and other enteric bacteria. The standard method involved dotblots of total genomic DNA tested at the lowest stringency sufficient to eliminate non-specific-binding of the probe to the plasmid-free hosts BW85, C600 and J53. These strains represent different *E. coli* K12 pedigrees. Table 1 shows that *ardA*-hybridizing sequences were detected on plasmids representing IncB, FV, I1, K and N groups. Fig. 1 illustrates the intensities of representative signals. No further group of plasmids was identified as hybridization positive when the test was repeated using PCR amplified pKM101 *ardA* as probe.

In addition to the IncFI, FII, FIV, I2 and X plasmids listed in Table 1, the following groups were found to lack

Table 1. Carriage of *ardA*, *ssb*, *psiB* and *hok*-family genes by some enterobacterial plasmids

Presence/absence of a sequence hybridizing to the appropriate probe is indicated by $\pm$.

Inc group	Plasmid(s)	<i>ardA</i> *	<i>ssb-psiB</i> †	<i>hok</i> family	
				<i>hok</i>	<i>pnd</i>
F complex					
FI	F, R386	—	+	+	—
FII	R1 <i>drd-16</i> , R100 <i>drd-1</i>	—	+	+	—
FIV	R124	—	+	+	—
FV	F ₀ <i>lac</i>	+	+	+	—
I complex					
B	R16, R621a	+	+	—	+
I1	Collb-P9, R64	+	+	—	+
I2	R721, TP114	—	—	+	—
K	R387	+	+	—	+
Others					
N	pKM101, N3	+	—	—	—
X	R6K	—	—	—	—

* Plasmids classified as negative for *ardA* hybridization were *Ard*[−] in the functional test.

† The single + indicates carriage of the genes as a pair; with the exception of F₀/*lac* (this work), *ssb* and *psiB* data are from Golub *et al.* (1988) and refer to the Inc group rather than the named plasmids.

Fig. 1. Dot blot hybridizations at low stringency to the ColIb *ardA* probe. Genomic DNA of bacteria harbouring a plasmid of the indicated Inc group was used. Plasmids were ColIb-P9 (I1), pKM101 (N), R16 (B), R711b (D), R387 (K), RA3 (U), R27 (HI1), pS-a (W), R446b (L/M), R391 (J), TP114 (I2) and F₀/*lac* (FV). DNA of plasmid-free C600 and J53 were the negative (−ve) controls. At high stringency, a positive signal was obtained only for I1, B and K plasmids. Weak signals produced by D, HI1, J and W plasmids at low stringency were not detected at medium stringency. Prolonged exposure was required to detect signals from FV and N plasmids at medium stringency.

an *ardA*-hybridizing sequence as judged by tests with bacteria harbouring the indicated plasmids: A/C (RA1, R40a), Com9 (R71a), D (R711b), HI1 (R27), HI2 (R478,

pSD114), HII (pHH1508a), J (R391), L/M (R446b, R471), P (RP4, R751), T (Rts-1), U (RA3), W (pS-a, R388) and Y (P1 Cm). It was noted that a probe consisting of a ColIb restriction fragment containing *ardA* plus flanking sequences of ~ 360 bp hybridized at high stringency with the representative Com9, IncFI, FII, FIV and HI2 plasmids. The positive signals presumably reflect sequence similarities between regions that border the *ardA* locus and emphasize the need to use a probe limited to the coding sequence.

IncFV plasmid F₀/*lac* stands out among F complex plasmids in containing an *ardA*-homologue. To check that other F-like plasmids lack such a sequence, restriction digests of plasmids F, R1*drd-16*, R100*drd-1* and R124 were tested following Southern blotting for hybridization to the ColIb *ardA* probe. The test was also applied to pRS1078, which is a recombinant carrying the F leading region (Ray & Skurray, 1983). No hybridization was detected at low stringency to any F-type sequence with the exception of F₀/*lac*.

Equivalent hybridization tests were carried out with a probe consisting of PCR product to the pKM101 *ardB* coding sequence. Hybridization was found only to pKM101 and N3 of the IncN group.

Plasmids with *ardA*-hybridizing sequences specify an antirestriction phenotype

The functional assay for *ardA* measures alleviation of restriction of unmodified *λ*vir following infection of a strain (C600N) expressing the *Eco*KI R-M system (Read *et*

Table 2. Alleviation of *Eco*KI restriction of unmodified *λ*vir in plasmid-containing cells

Plasmid in strain C600	Restriction-alleviation index*		
	Native plasmid	<i>drd</i> mutant of plasmid	Multicopy recombinant
ColIb (IncI1)	26	164	7000
R16 (IncB)	27	180	7100
R387 (IncK)	24	127	NT
F ₀ <i>lac</i> (IncFV)	22	150	4300
pKM101 (IncN)	23	NA	NT

NT, Not tested.

NA, Not available.

* Defined as the efficiency of plating (e.o.p.) of *λ*vir.0 on C600N harbouring the plasmid divided by e.o.p. on plasmid-free C600N. The value for the latter was 1.4×10^{-4} . An index of 7100 indicates complete restriction alleviation. Appropriate *drd* mutants were pLG221 (ColIb-P9), R16*drd-86*, R387*drd-86* and pED208 (F₀*lac*). Multicopy recombinants were pLG2055 (ColIb), pLG2051 (R16) and pLG2053 (F₀*lac*).

al., 1992). The assay showed that restriction was alleviated by about 25-fold when C600N harboured a representative of each of the five Inc groups of plasmid with *ardA* homology (Table 2). Yet higher levels of restriction alleviation were detected when wild-type ColIb, F₀*lac*, R16 and R387 were replaced with plasmids with a *drd* mutation that derepresses expression of the transfer genes. The restriction-alleviation index of C600N carrying F, R1*drd-16*, R100*drd-1* or pRS1708 was 1.0, showing that standard IncFI and IncFII plasmids and the recombinant with the F leading region lack a gene that can function in vegetative cells to alleviate type I restriction systems. Similarly, the FIV, I2 and X plasmids failed to alleviate restriction in the functional test (Table 1).

Linkage of *ardA* to leading region genes on B and K plasmids

ColIb, R16 and R387 belong to the I1-B-K subset of the I complex of incompatibility groups (Bradley, 1984). Subset members have homologous replicons (Couturier *et*

al., 1988), encode similar conjugative pili (Bradley, 1984), and carry conserved *ssb* and *psiB* genes (Golub *et al.*, 1988). Moreover, the subset determines functionally similar DNA transfer systems as demonstrated by their ability to mobilize small ColIb *oriT* recombinants with high to intermediate frequencies (data of A. T. Thomas & B. M. Wilkins). Mobilization abilities, defined as the frequency of transfer of the *oriT* recombinant relative to the conjugative plasmid, were 0.94 for ColIb*drd-1*(I1), 2.5×10^{-2} for R387 (K) and 3.0×10^{-4} for R16*drd-86* (B). Values in negative controls were 5.2×10^{-7} for TP114*drp-1* (I2) and 7.0×10^{-7} for pUB307 (P).

The similarities were exploited to determine the sector of R16 and R387 carrying the *ardA* sequence. To this end, R16 and R387 were cleaved with restriction enzymes that cut infrequently and fragments were tested following Southern transfer for hybridization to probes containing ColIb *oriT*, F *ssb* and R6-5 *psiB*. Each probe hybridized at high stringency to a 25 kb *Bam*HI fragment of R16 and to a 23 kb *Eco*RI fragment of R387. The same fragments were recognized by the ColIb *ardA* PCR product, indicating that the *ardA* homologue is located in a sector of R16 and R4387 that includes *oriT* and leading region genes.

Nucleotide sequence of *ardA* on an IncB plasmid

The R16 *ardA* homologue is located on a 2.2 kb *Pst*I fragment, which, when cloned in the multicopy pUC18 vector plasmid, caused complete alleviation of *Eco*KI restriction (pLG2051, Table 2). A set of nested deletions was produced from one end of the cloned *Pst*I fragment and deletion plasmids were tested for retention of the *ardA*-hybridizing sequence. One of the smallest hybridizing plasmids, pLG2052, contained 620 bp of R16 DNA. In the functional test, pLG2052 completely alleviated restriction of *λ*.0 infecting C600N, showing the plasmid to be Ard⁺. Direct sequencing of pLG2052 from the forward and reverse M13/pUC universal primers allowed the sequence of both strands of the cloned fragment to be determined. The results showed that the R16 fragment includes an ORF with 94% nucleotide

		30		60		90
I1	MS--VVP	AVYVGTWHKY	NCGSIAGRN	FDLTTFFD	DERDFFAAC	RALHQDEADPE
B	..--					
FV	..TTTT	Y.....	F.K.....E..	GRE..YE..Q..	A..W.A.F....	W..I.SQFV...S.E
N	..TDITT	..S.....Y.....	A.L...D..SSEE	YER..E..AN...	F....W..I.SD.....	DFIN..K...E..N
		120		150		
I1	EAYRLW	VEDTGETDFD	ITFRDANW	GEADSEEA	FAVEFASD	TGGLLADVPET
B	.G.....	P.....	K.....	V.....		
FV	A.FIA.A.Y...	C.Y.A.D..YR...	E...D..R.MVE	N...NE...PLRS...	F..W...SSGYV	H..Y..SN--
N	A.FVAF.DLFNS	...L....YM..	KD..T..E.YLN	S...NEI..S..R...	IV.....IGD	SLH....NMT

Fig. 2. Comparison of the predicted amino acid sequences of ArdA polypeptides specified by IncI1 (ColIb-P9), B (R16), FV (F₀*lac*) and N (pKM101) plasmids. Points indicate identity with the ColIb sequence. Hyphens indicate gaps introduced to maximize alignments. The ColIb and pKM101 sequences were derived elsewhere (Read *et al.*, 1992; Belogurov *et al.*, 1992).

Fig. 3. Leading region of IncFV plasmid F_0/lac . Kilobase coordinates are those of Finlay *et al.* (1986). Line 2 shows restriction sites mapped on the 23 kb *HindIII* fragment. The location of *oriT*, *traM*, *traJ* and the promoter-proximal portion of *traY* were derived by Di Laurenzio *et al.* (1991). Dotted lines indicate the approximate position of *ssb* and *psiB*. pLG2054Δ1 and pLG2054Δ2 represent linear plasmid deletants produced by unidirectional digestion of pLG2054 from the *Bam*HI site in the polylinker. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I.

sequence identity to ColIb *ardA*. The nucleotide sequence is available as accession number Z34468 in the EMBL database. The predicted amino acid sequence of the product is given in Fig. 2.

Location and nucleotide sequence of *ardA* on an IncFV plasmid

Plasmid pED208 is a mutant of F_0/lac that is derepressed for transfer. Southern hybridization showed the *ardA*-related sequence to be on the ~23 kb *Hind*III fragment which contains *oriT* and the pED208 leading region and is present in recombinant pBF105 (Finlay *et al.*, 1986). The *ardA* hybridizing sequence was delimited further to a 11.5 kb *Eco*RI fragment, carried by pLG2053, and then to a 3.3 kb *Sph*I–*Sal*I fragment, carried by pLG2054 (Fig. 3). Although pLG2053 was *Ard*⁺ (Table 2), pLG2054 failed to alleviate *Eco*KI restriction indicating that it lacks a functional *ardA* gene.

Direct sequencing of pLG2054 from the forward M13/pUC universal primers showed that the *Sph*I site is located ~120 bp downstream of the 5'-end of an *ardA*-type sequence. Although the 5'-terminal third portion of ColIb *ardA* is inessential for conferring an *Ard*⁺ phenotype on vegetative cells (Read *et al.*, 1992), the partial *ardA* gene in pLG2054 is in the promoterless orientation. The finding that the *Sph*I site is towards the 5'-end of *ardA* defines the direction of transcription of the gene on pED208 as being towards *oriT*. Data obtained from pLG2054 were used to design primers for sequencing both strands of *ardA*⁺ on pLG2053. The nucleotide sequence has the EMBL accession number Z34467. The derived amino acid sequence is given in Fig. 2.

F_0/lac *ardA* is linked to *ssb*, *psiB* and *hok* leading region genes

Southern hybridizations showed pED208 to contain *ssb*, *psiB* and *hok* sequences, as is the case for plasmid F. Tests

Fig. 4. E15 fragment of ColIb-P9 specifies the *Hok*⁺ phenotype. Values show colony-forming ability of plasmid-containing cells following incubation in the presence of rifampicin for the indicated times. □, BW85(pHG165); ○, BW85(Collbdrd-1); ●, BW85(pMC3).

with pLG2053 fragments showed the *psiB* probe to recognize the *Eco*RI insert and to anneal with approximately equal intensities to the 1.7 kb *Pvu*II–*Eco*RI and the 2.0 kb *Pvu*II–*Sph*I fragments (Fig. 3). Hence, pED208 *psiB* maps at coordinate 40.2 ± 0.5 kb, the latter value being the size of a *psiB* locus. The *ssb* probe hybridized to the *Eco*RI insert in pLG2053 and to the 3.3 kb *Sph*I–*Sal*I fragment present in pLG2054. The hybridizing region was mapped further by testing for its retention on pLG2054-derivatives progressively deleted from one end of the cloned fragment (Fig. 3). Molecules of the size of pLG2054Δ1 but not Δ2 retained the *ssb*-hybridizing region. Hence, pED208 *ssb* maps close to coordinate 44.0 ± 0.5 kb, the latter value approximating to the size of a plasmid *ssb* gene (Chase *et al.*, 1983; Howland *et al.*, 1989). The pED208 leading region also contains a *flm* homologue, shown by hybridization of the *hok* probe to a 2.2 kb *Eco*RI fragment that flanks *oriT*.

ColIb-P9 carries a *hok*-family gene outside the leading region

Plasmid-borne members of the *hok* family include the *hok* (*flm*) and *pnd* subfamilies (Sakikawa *et al.*, 1989; Gerdes *et al.*, 1990). The *hok* probe failed to recognize ColIb but the *pnd* probe hybridized to the 16 kb *Sal*I (S3) fragment and the 1.9 kb *Eco*RI (E15) fragment therein (for map, see Rees *et al.*, 1987). To confirm that the *pnd*-type sequence is a functional killer gene, the 1.9 kb *Eco*RI fragment was inserted in vector pHG165 to give pMC3. This recombinant specified a pronounced *Hok*⁺ phenotype, as indicated by loss of viability of host cells incubated with rifampicin (Fig. 4). The drug induces killing by preventing synthesis of the short-lived RNA molecules that regulate synthesis of the toxin (Sakikawa *et al.*, 1989; Nielsen *et al.*, 1991). The combined data indicate that the ColIb trailing region, defined as the last portion of the plasmid to enter the recipient cell, contains a *pnd* gene

located some 5.4–7.3 kb from *oriT*. IncB and IncK plasmids also carry *pnd*. The gene maps outside the leading region, since the probe hybridized to 6.6 kb *Bam*HI fragment of R16 and to a 2.8 kb *Eco*RI fragment of R387. These fragments differ from those identified above as carrying *oriT*, *ssb*, *psiB* and *ardA*.

DISCUSSION

Hybridization tests showed that sequences homologous to the ColIb *ardA* antirestriction gene are present on plasmids belonging to the B, FV, I1, K and N incompatibility groups. The homologue on the IncN plasmids is assumed to be the antirestriction gene described by Belogurov *et al.* (1992) and was not examined further. The hybridizing regions on the B and FV plasmids were shown to contain an ORF related to ColIb *ardA*. Nucleotide positional identities for the *ardA* genes are ColIb/R16, 95%; ColIb/*F₀lac*, 63%; ColIb/pKM101, 62%; pKM101/*F₀lac*, 64%. Hence, the hybridization assay readily detected genes diverged by about 40%. The predicted protein products have 166 (ColIb and R16), 168 (*F₀lac*) and 169 (pKM101) amino acid residues and are highly acidic, with net charges ranging from –22 for R16 ArdA, to –29 for *F₀lac* ArdA. The *ardA* homologues on R16 and *F₀lac* are inferred to be functional antirestriction genes. The evidence is that the R16 homologue was delimited to a 620 bp fragment that determines the restriction-alleviation phenotype, while disruption of the *F₀lac* *ardA* sequence by subcloning destroyed the antirestriction phenotype conferred by a larger fragment carrying the intact gene. Less attention was given to *ardA* on IncK plasmids in view of their close relationship to IncB and I1 plasmids.

While B, I1 and K plasmids are classified with I2 plasmids into the I complex of incompatibility groups (Bradley, 1984), I2 plasmids lack a conserved *ardA* gene. This is unsurprising in view of the extensive differences between the B-I1-K subset and I2 plasmids, which are manifest by replicon typing (Couturier *et al.*, 1988), properties of conjugative pili (Bradley, 1984), ability to mobilize ColIb *oriT* recombinants (see above), and carriage of *ssb*, *psiB* and *pnd* genes (Table 1). A more intriguing finding is that FV plasmid *F₀lac* is unique among the F-type plasmids examined in carrying a conserved *ardA* gene. This is remarkable because F-like groups have homologous transfer systems, *oriTs* and leading regions (Ippen-Ihler & Skurray, 1993; Lanka & Wilkins, 1995; Table 1). No evidence was obtained that F or standard FII plasmids specify an antirestriction function active against the classic type I restriction enzyme *Eco*KI. Conjugative transposon Tn916 from *Enterococcus faecalis* has a sequence (ORF18) predicted to specify a 166 amino acid product with 26% amino acid sequence identity to ArdA (Flannagan *et al.*, 1994). The function of ORF18 is unknown but it maps in the portion of Tn916 which is necessary for conjugation.

Plasmids can carry other genes active against type I restriction systems, as shown by the presence of *ardB* of pKM101. The gene is non-homologous to *ardA* but maps in the leading region (Belogurov *et al.*, 1993; Fig. 5). Conserved *ardB* genes appear to have a limited distribution, since PCR-amplified *ardB* hybridized only to the two IncN plasmids (pKM101 and N3) in our sample of plasmids (unpublished data). It is unknown what ecological advantage the carriage of two different *ard* genes confers on IncN plasmids. A sequence (*kbcA*) sharing 39% nucleotide sequence identity with pKM101 *ardB* and predicted to encode an acidic 146 amino acid polypeptide has been identified on IncPα plasmids. However, *kbcA* differs from *ardB* in being located outside the leading region and in failing to alleviate *Eco*KI restriction, even when carried by a high-copy-number vector (Larsen & Figurski, 1994). It is unlikely that *kbcA* contributes to an antirestriction system that is activated during conjugation, since transconjugant production by unmodified RP4 is ~100-fold more sensitive to *Eco*KI restriction than is the case for unmodified ColIb*drd-1* (data of A. T. Thomas & B. M. Wilkins). Both plasmids are thought to have similar numbers of *Eco*KI sites: there are nine sites in the nucleotide sequence of RP4 (Pansegrau *et al.*, 1994), and an estimated seven on ColIb. The latter value is derived from the finding of four *Eco*KI sites in the 54 kb nucleotide sequence of the Tra region on Inc11 plasmid R64 (T. Komano, personal communication).

A unifying feature of *ard* genes is their location in plasmid leading regions. Here the genes are found in heterogeneous assemblies with members of other gene families, namely *ssb*, *psiB* and *hok* (Fig. 5). It is stressed that there is no consistent association between *ardA* and any other known leading region gene or type of *oriT*. Transfer regions can be typed according to the sequence of the nick region, defined as the *oriT* subregion recognized by the plasmid-encoded relaxase for the cleavage and joining of

Fig. 5. Content and arrangement of genes on the leading region of representative I1 (ColIbP-9), FV (*F₀lac*), FI (F) and N (pKM101) plasmids. Arrows under genes indicate known directions of transcription. Map limits for *flm*, *psiB* and *ssb* on *F₀lac* are described in the text. Data for the I1, FI and N plasmids are from Read *et al.* (1992), Bagdasarian *et al.* (1992) and Belogurov *et al.* (1993), respectively. *oriT* shading indicates sequence relationships at the nick region (Lanka & Wilkins, 1995).

DNA strands during conjugation. Three types have been identified: the nick region of FI, FII, FV and N plasmids constitute one type, whereas the I1 nick region belongs to a second type exemplified by IncP plasmids (Lanka & Wilkins, 1995). Taken together, the evidence suggests that *ardA* genes are recent accretions to leading regions. This conclusion is consistent with the different position of *ardA* relative to *oriT* and *psiB* on the I1 and FV plasmids. Despite these organizational variations, it is impressive that the transcriptional orientation of *ard*, *ssb* and *psiB* is the same in each case examined (Fig. 5). The arrangement, which causes the transcribed strand to be the same as the transferred strand, may be important for the expression of the genes during conjugation (Jones *et al.*, 1992; Read *et al.*, 1992).

It is unclear why some plasmids carry *ardA* genes but others do not. The *ardA*⁺ elements identified here are judged to be authentic enterobacterial plasmids, since they were isolated from organisms closely related to *E. coli* (Lanka & Wilkins, 1995). Furthermore, F complex, I complex and IncN plasmids constitute part of the defined plasmid population of a set of *Escherichia*, *Shigella*, *Salmonella* and *Klebsiella* strains collected by E. D. G. Murray in the period between 1917 and 1954 (Datta & Hughes, 1983). Type I R-M systems have historically been associated with a similar cluster of enterobacterial organisms. It is tempting therefore to conclude that *ardA* has the ecological role of allowing plasmids to evade a particular type of restriction system prevalent in their natural hosts. However, this conclusion does not accommodate the finding that some 'Murray' plasmids lack a conserved *ardA* gene (see FII, I2 and X plasmids; Table 1). Moreover, type I R-M systems are now recognized to be more widely distributed than previously considered (Barcus & Murray, 1995), as shown by the recent discovery of such a system in *Mycoplasma pulmonis* (Dybvig & Yu, 1994) and in *Bacillus subtilis* (T. A. Trautner, personal communication).

ACKNOWLEDGEMENTS

We thank Laura Frost for the gift of F₀*lac*, pED208 and pBF105, Noreen Murray and Lise Raleigh for perspectives on the distribution and detection of type I restriction systems, and Angela Thomas for unpublished data. P.M.C. was a research student funded by the Medical Research Council. The work was supported by MRC grant G9321196MB.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1990). *Current Protocols in Molecular Biology*, vol. 1, pp. 2.4.1–2. New York: John Wiley & Sons.

Bagdasarian, M., Bailone, A., Bagdasarian, M. M., Manning, P. A., Lurz, R., Timmis, K. N. & Devoret, R. (1986). An inhibitor of SOS induction, specified by a plasmid locus in *Escherichia coli*. *Proc Natl Acad Sci USA* **83**, 5723–5726.

Bagdasarian, M., Bailone, A., Angulo, J. F., Scholz, P., Bagdasarian, M. & Devoret, R. (1992). PsiB, an anti-SOS protein, is transiently expressed by the F sex factor during its transmission to an *Escherichia coli* K-12 recipient. *Mol Microbiol* **6**, 885–893.

Barcus, V. A. & Murray, N. E. (1995). Barriers to recombination: restriction. In *Population Genetics of Bacteria*, Society for General Microbiology Symposium **52**, pp. 31–58. Edited by S. Baumberg, J. P. W. Young, E. M. H. Wellington & J. R. Saunders. Cambridge: Cambridge University Press.

Belogurov, A. A., Delver, E. P. & Rodzevich, O. V. (1992). IncN plasmid pKM101 and IncI1 plasmid ColIb-P9 encode homologous antirestriction proteins in their leading regions. *J Bacteriol* **174**, 5079–5085.

Belogurov, A. A., Delver, E. P. & Rodzevich, O. V. (1993). Plasmid pKM101 encodes two nonhomologous antirestriction proteins (ArdA and ArdB) whose expression is controlled by homologous regulatory sequences. *J Bacteriol* **175**, 4843–4850.

Bickle, T. A. & Krüger, D. H. (1993). Biology of DNA restriction. *Microbiol Rev* **57**, 434–450.

Boyer, H. W. (1971). DNA restriction and modification mechanisms in bacteria. *Annu Rev Microbiol* **25**, 153–176.

Bradley, D. E. (1984). Characteristics and function of thick and thin conjugative pili determined by transfer-derepressed plasmids of incompatibility groups I₁, I₂, I₃, B, K and Z. *J Gen Microbiol* **130**, 1489–1502.

Chase, J. W., Merrill, B. M. & Williams, K. R. (1983). F sex factor encodes a single-stranded DNA binding protein (SSB) with extensive sequence homology to *Escherichia coli* SSB. *Proc Natl Acad Sci USA* **80**, 5480–5484.

Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991–1995.

Couturier, M., Bex, F., Bergquist, P. L. & Maas, W. K. (1988). Identification and classification of bacterial plasmids. *Microbiol Rev* **52**, 375–395.

Datta, N. & Hughes, V. M. (1983). Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics. *Nature* **306**, 616–617.

Delver, E. P., Kotova, V. U., Zaviigelsky, G. B. & Belogurov, A. A. (1991). Nucleotide sequence of the gene (*ard*) encoding the antirestriction protein of plasmid ColIb-P9. *J Bacteriol* **173**, 5887–5892.

Di Laurenzio, L., Frost, L. S., Finlay, B. B. & Paranchych, W. (1991). Characterization of the *oriT* region of the IncFV plasmid pED208. *Mol Microbiol* **5**, 1779–1790.

Dybvig, K. & Yu, H. (1994). Regulation of a restriction and modification system via DNA inversion in *Mycoplasma pulmonis*. *Mol Microbiol* **12**, 547–560.

Finlay, B. B., Frost, L. S. & Paranchych, W. (1986). Nucleotide sequence of the *traYALE* region from IncFV plasmid pED208. *J Bacteriol* **168**, 990–998.

Flannagan, S. E., Zitzow, L. A., Su, Y. A. & Clewell, D. B. (1994). Nucleotide sequence of the 18-kb conjugative transposon Tn916 from *Enterococcus faecalis*. *Plasmid* **32**, 350–354.

Gerdess, K., Rasmussen, P. B. & Molin, S. (1986). Unique *tra* of plasmid maintenance: postsegregation killing of plasmid-free cells. *Proc Natl Acad Sci USA* **83**, 3116–3120.

Gerdess, K., Poulsen, L. K., Thisted, T., Nielsen, A. K., Martinussen, J. & Andreassen, P. H. (1990). The *bok* killer gene family in Gram-negative bacteria. *New Biol* **2**, 946–956.

Golub, E. I. & Low, K. B. (1985). Conjugative plasmids of enteric bacteria from many different incompatibility groups have similar genes for single-stranded DNA-binding proteins. *J Bacteriol* **162**, 235–241.

Golub, E. I., Bailone, A. & Devoret, R. (1988). A gene encoding an

- SOS inhibitor is present in different conjugative plasmids. *J Bacteriol* **170**, 4392–4394.
- Henikoff, S. (1984).** Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.
- Howland, C. J., Rees, C. E. D., Barth, P. T. & Wilkins, B. M. (1989).** The *ssb* gene of plasmid ColIb-P9. *J Bacteriol* **171**, 2466–2473.
- Ippen-Ihler, K. & Skurray, R. A. (1993).** Genetic organization of transfer-related determinants on the sex factor F and related plasmids. In *Bacterial Conjugation*, pp. 23–52. Edited by D. B. Clewell. New York: Plenum Press.
- Jones, A. L., Barth, P. T. & Wilkins, B. M. (1992).** Zygotic induction of plasmid *ssb* and *psiB* genes following conjugative transfer of IncI1 plasmid ColIb-P9. *Mol Microbiol* **6**, 605–613.
- Lanka, E. & Wilkins, B. M. (1995).** DNA processing reactions in bacterial conjugation. *Annu Rev Biochem* **64**, 141–169.
- Larsen, M. H. & Figurski, D. H. (1994).** Structure, expression, and regulation of the *kilC* operon of promiscuous IncPα plasmids. *J Bacteriol* **176**, 5022–5032.
- Loh, S. M., Cram, D. S. & Skurray, R. A. (1988).** Nucleotide sequence and transcriptional analysis of a third function (Flm) involved in F-plasmid maintenance. *Gene* **66**, 259–268.
- Nielsen, A. K., Thorsted, P., Thisted, T., Wagner, E. G. H. & Gerdes, K. (1991).** The rifampicin-inducible genes *srnB* from F and *pndA* from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. *Mol Microbiol* **5**, 1961–1973.
- Pansegrau, W., Lanka, E., Barth, P. T., Figurski, D. H., Guiney, D. G., Haas, D., Helinski, D. R., Schwab, H., Stanisich, V. A. & Thomas, C. M. (1994).** Complete nucleotide sequence of Birmingham IncPα plasmids. *J Mol Biol* **239**, 623–663.
- Pohlman, R. F., Genetti, H. D. & Winans, S. C. (1994).** Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. *Mol Microbiol* **14**, 655–668.
- Rasmussen, P. B., Gerdes, K. & Molin, S. (1987).** Genetic analysis of the *parB*⁺ locus of plasmid R1. *Mol & Gen Genet* **209**, 122–128.
- Ray, A. & Skurray, R. (1983).** Cloning and polypeptide analysis of the leading region in F plasmid DNA transfer. *Plasmid* **9**, 262–272.
- Read, T. D., Thomas, A. T. & Wilkins, B. M. (1992).** Evasion of type I and type II DNA restriction systems by IncI1 plasmid ColIb-P9 during transfer by bacterial conjugation. *Mol Microbiol* **6**, 1933–1941.
- Rees, C. E. D., Bradley, D. E. & Wilkins, B. M. (1987).** Organization and regulation of the conjugation genes of IncI1 plasmid ColIb-P9. *Plasmid* **18**, 223–236.
- Roberts, R. J. & Macelis, D. (1994).** REBASE – restriction enzymes and methylases. *Nucleic Acids Res* **22**, 3628–3639.
- Sakikawa, T., Akimoto, S. & Ohnishi, Y. (1989).** The *pnd* gene in *E. coli* plasmid R16: nucleotide sequence and gene expression leading to cell Mg²⁺ release and stable RNA degradation. *Biochim Biophys Acta* **1007**, 158–166.
- Stewart, G. S. A. B., Lubinsky-Mink, S., Jackson, C. G., Cassel, A. & Kuhn, J. (1986).** pHG165: a pBR328 copy number derivative of pUC8 for cloning and expression. *Plasmid* **15**, 172–181.
- Wilkins, B. M. (1995).** Gene transfer by bacterial conjugation: diversity of systems and functional specializations. In *Population Genetics of Bacteria*, Society for General Microbiology Symposium **52**, pp. 59–88. Edited by S. Baumberg, J. P. W. Young, E. M. H. Wellington & J. R. Saunders. Cambridge: Cambridge University Press.
- Wilson, G. G. & Murray, N. E. (1991).** Restriction and modification systems. *Annu Rev Genet* **25**, 585–627.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Received 10 April 1995; revised 22 May 1995; accepted 31 May 1995.