Evolution of multi-resistance plasmids in Australian clinical isolates of *Escherichia coli*

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Plasmids allow the movement of genetic material, including antimicrobial resistance genes, between bacterial species and genera. They frequently mediate resistance to multiple antimicrobials and can result in the acquisition by a pathogen of resistance to all or most clinically relevant antimicrobials. Unfortunately, there are still large gaps in our understanding of how new multi-resistance plasmids evolve. Five Australian clinical institutions collaborated in this study of multi-resistance plasmids in clinical isolates of *Escherichia coli*. We characterized 72 resistance plasmids in terms of the antimicrobial resistance profile they conferred, their size and their incompatibility group. Restriction fragment length polymorphisms were used to determine the genetic relationships between the plasmids. Relationships between the host cells were determined using multi-locus enzyme electrophoresis. A lack of correlation between the evolutionary history of the host cells and their plasmids suggests that the horizontal transfer of resistance plasmids between strains of *E. coli* is common. The resistance plasmids were very diverse, with a wide range of resistance profiles and a lack of discrete evolutionary lineages. Multi-resistance plasmids did not evolve via the co-integrative capture of smaller resistance plasmids; rather, the roles of recombination and the horizontal movement of mobile genetic elements appeared to be most important.

INTRODUCTION

Since their discovery in the 1950s (Watanabe & Fukasawa, 1960), antimicrobial resistance plasmids have been increasingly associated with both Gram-positive and Gram-negative bacterial infections. Plasmid-associated resistance genes have been discovered for a majority of known antimicrobials, including the quinolones and fluoroquinolones (Hawkey, 2003; Neu, 1992), and it is not uncommon for a single plasmid to simultaneously mediate resistance to five or six antimicrobials. This ability to sequester multiple resistance genes is of particular concern to modern medicine.

Levin (1995) made two predictions regarding the evolution of multi-resistance plasmids. Where two incompatible plasmids are simultaneously selected for, he argues that new plasmids will arise by transposition and predicts that the position of resistance genes in otherwise identical plasmids will be highly variable. Where selection for the co-transfer of compatible resistance plasmids is involved, he argues that new multi-resistance plasmids will arise through co-integration and predicts the occurrence of resistance genes in plasmids with multiple replicons that can be identified as the co-integrates of other plasmids.

Both co-integration and transposition have been implicated in empirical studies of plasmid evolution (Berg *et al.*, 1998; Bradley *et al.*, 1986; Guessouss *et al.*, 1996; Mitsuhashi *et al.*, 1977; Schwarz *et al.*, 1996; Sohail & Dyke, 1995; Venkatesan *et al.*, 2001; Woodward *et al.*, 1990). However, a range of other mechanisms, including recombination and the acquisition of integron cassettes, have also been observed (Boerlin, 1999; Boyd *et al.*, 1996; Brown *et al.*, 2000; Lindler *et al.*, 1998; Prentice *et al.*, 2001; Radstrom *et al.*, 1991; Venkatesan *et al.*, 2001). While several individual plasmids have been the subject of intensive study, there is limited information available regarding plasmid populations (Blazquez *et al.*, 1996; Boyd *et al.*, 1996; Brown *et al.*, 2000; Carattoli, 2003; Carattoli *et al.*, 2001, 2002; Groves, 1979; Ling *et al.*, 1993; Petit *et al.*, 1990; Preston *et al.*, 2003; Radstrom *et al.*, 1991; Saksena & Truffaut, 1992; Tosini *et al.*, 1998). To date we know of no study that has attempted to infer the processes underlying plasmid evolution through an investigation of the genetic relationships within a large collection of single- and multiple-resistance plasmids.

Escherichia coli is a prime candidate as a species in which new multi-resistance plasmids may evolve. It is a common enteric commensal of mammals and a common cause of human infection. As such, *E. coli* strains are routinely exposed

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Abbreviation: MLEE, multi-locus enzyme electrophoresis.

to a wide range of antimicrobial agents. *E. coli* also has a very wide natural distribution (Selander *et al.*, 1987) and a propensity for plasmid carriage (Sherley *et al.*, 2003). Resistance to tetracycline, chloramphenicol or trimethoprim is relatively common in clinical pathogens in Australia, including *E. coli* (Bell & Turnidge, 1995), and is frequently plasmid-mediated (Neu, 1992). We studied a collection of single- and multi-resistance plasmids isolated from tetracycline-, chloramphenicol- and/or trimethoprim-resistant clinical isolates of *E. coli*. The plasmids were screened to determine their antibiotic resistance profiles, the genetic relatedness of both the plasmids and their host bacterial strains was determined, and an attempt was made to infer the process by which multiple resistance had evolved within this population.

METHODS

Source of bacterial strains and plasmid isolation. All bacterial isolates were supplied by the following Australian institutions: The Alfred Hospital (Melbourne), The Canberra Hospital (Canberra), Institute of Medical and Veterinary Science (Adelaide), Royal North Shore Hospital (Sydney) and Royal Perth Hospital (Perth). These institutions were asked to supply isolates of *E. coli* resistant to any combination of tetracycline, chloramphenicol and/or trimethoprim. The isolates were obtained from a range of body sites. However, multiple isolates from the same patient were excluded from analysis. Pure cultures were stored at $-70\,^{\circ}$C in 6·25 % (v/v) glycerol. Samples were restreaked fresh from frozen storage for each experiment to minimize time in culture and hence the possibility of plasmid loss. All isolates were confirmed as *E. coli* on the basis of their biochemical profiles. The resistance or sensitivity of each strain to tetracycline (20 µg ml^{-1}), chloramphenicol (25 µg ml^{-1}) and trimethoprim (10 µg ml^{-1}) was determined by overnight growth following toothpicking onto supplemented Miller's LB agar (Bacto) plates.

Resistance plasmids were transferred from the resistant isolates to a plasmid-free, rifampicin (200 µg ml^{-1})-resistant, laboratory strain of *E. coli* K-12 (J53) by mating (conjugation) using rifampicin+tetracycline, rifampicin+chloramphenicol or rifampicin+trimethoprim as selective agents as appropriate. All matings were carried out both in broth culture (Miller's LB broth; Bacto) without shaking and on solid agar plates (tetrazolium-lactose agar; Levin *et al.*, 1979). Putative transconjugants were restreaked onto fresh selective plates and their identity was confirmed on the basis of biochemical profiles. Pure cultures were stored at $-70\,^{\circ}$C in 6·25 % (v/v) glycerol. The plasmid content of the transconjugants was electrophoretically compared with that of the donors using a modified in-well lysis technique (de Souza *et al.*, 1998). All transconjugants were also subjected to alkaline-lysis plasmid isolation (Sambrook *et al.*, 1989) and transconjugants containing multiple plasmids were excluded from the study.

Plasmid characterization. To determine the resistance profiles conferred by the plasmids, the transconjugant cells were screened for sensitivity to 10 antimicrobials [chloramphenicol, 30 µg ml^{-1}; tetracycline, 30 µg ml^{-1}; trimethoprim, 5 µg ml^{-1}; neomycin, 30 µg ml^{-1}; kanamycin, 30 µg ml^{-1}; streptomycin, 10 µg ml^{-1}; spectinomycin, 100 µg ml^{-1}; gentamicin, 10 µg ml^{-1}; sulfisoxazole, 0·25 µg ml^{-1}; ampicillin, 10 µg ml^{-1} (Bacto)] using a soft-agar disc diffusion method. Briefly, 100 µl of a fresh overnight culture in Miller's LB broth was inoculated into 3 ml soft agar (0·6 %, w/v, in Miller's LB broth), vortexed and decanted onto the surface of a Miller's LB agar plate. Antimicrobial-impregnated filter discs (Bacto) were then deposited onto the surface of the plate, which was incubated overnight. The diameter of the zones of inhibition surrounding the antimicrobial discs was measured. Isolates were deemed resistant only when the zone of inhibition was less than or equal to the resistance breakpoint recommended by the manufacturer (intermediate and sensitive strains were scored as sensitive). The plasmid-free host strain was included as a sensitive control.

A PCR-based method was used to assign incompatibility groupings to the plasmids by determining the presence or absence of incA/C, incFII, incN, incP and incW replicons, as described previously (Sherley *et al.*, 2003). Plasmid sizes were determined by electrophoretic comparison of native plasmid DNA isolated using alkaline lysis (Sambrook *et al.*, 1989) with plasmids of known size (R388, 31·8 kbp; R136, 62·1 kbp; pIP40a, 145·5 kbp; MIP233, 227·3 kbp).
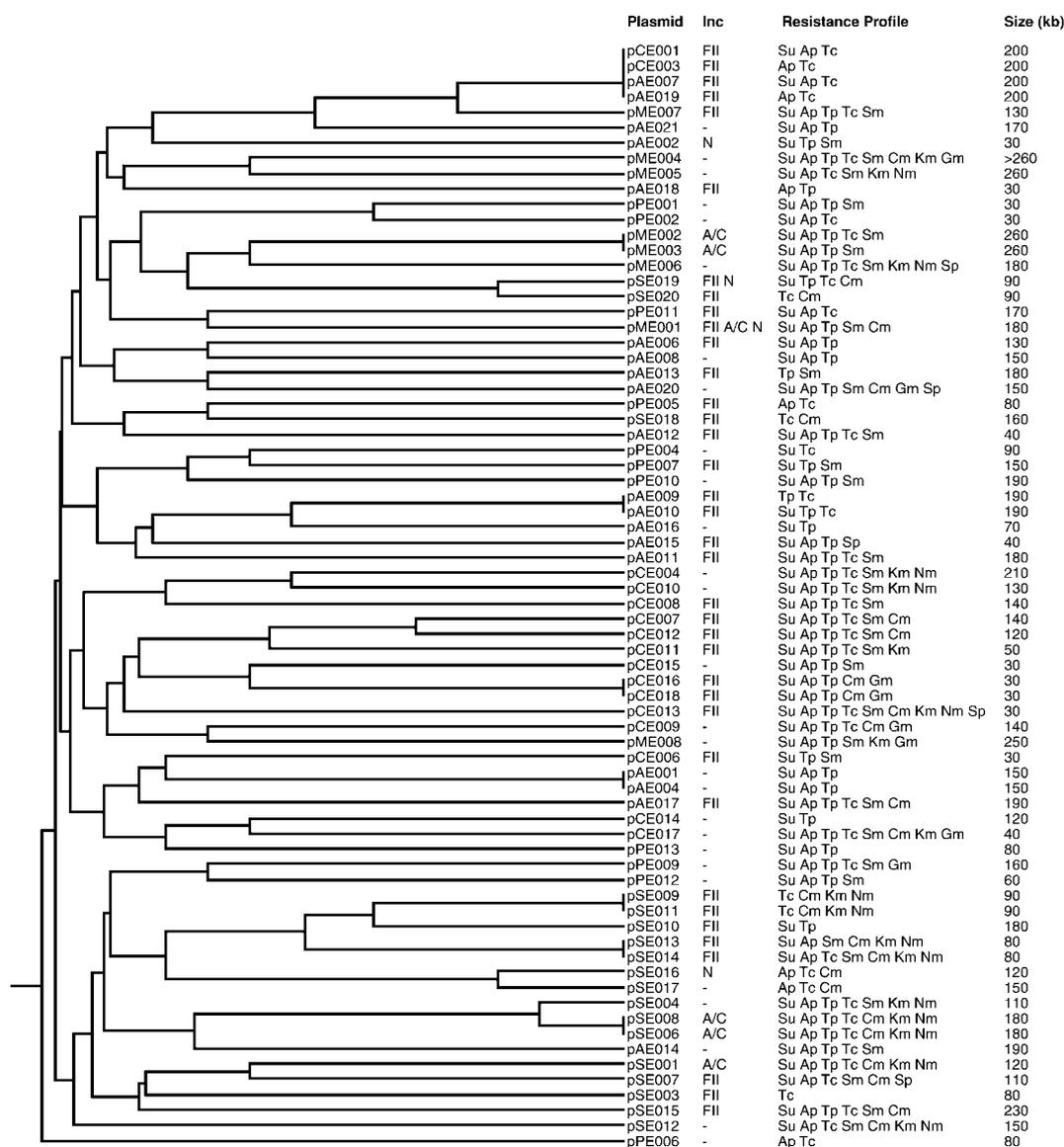
To determine the degree of genetic similarity between resistance plasmids, they were isolated from the transconjugant cells by alkaline lysis, then subjected to restriction fragment length polymorphism (RFLP) analysis using *Bam*H1, *Eco*RI, *Hin*dIII, *Pst*I and *Sac*I (Roche). Relatedness was determined by pair-wise comparison of banding patterns following restriction digestion and electrophoresis. Given the large degree of variability observed, we chose to score plasmid pairs as identical, highly similar, similar or dissimilar. For each restriction enzyme, identical plasmid pairs were scored as 0, plasmid pairs that were non-identical but shared >50 % of digest bands were scored as 1, plasmid pairs that shared <50 % of digest bands were scored as 2, and plasmid pairs with no shared bands were scored as 3. The scores for each enzyme were then pooled to give an overall relatedness score, ranging from 0 to 15 for every plasmid pair. These data were graphically represented using Unweighted Pair Group Matching Analysis (Rohlf, 1993). A pair-wise comparison of the observed antimicrobial resistance profiles was also determined for each plasmid pair (Jacard scores).

Strain characterization. The genetic relationships between the donor strains of *E. coli* were determined by multi-locus enzyme electrophoresis (MLEE) as described previously (Gordon & Lee, 1999). Only strains from which plasmids were successfully isolated were included in this analysis. Thirteen donor strains from The Canberra Hospital perished during storage and were also excluded.

Statistical analyses. The correlations between the RFLP data, resistance profile data and MLEE data were determined from their respective Euclidean/Jacard distance matrices using a Mantel test (Mantel, 1967). The relationship between individual antimicrobial resistances and the plasmid RFLP data was also determined from their respective distance matrices using a Mantel test. The correlation between pairs of antimicrobial resistances was determined using a Spearman correlation (Sokal & Rohlf, 1969).

RESULTS

Overall, 72 plasmids mediating resistance to combinations of tetracycline, trimethoprim and chloramphenicol were isolated. These resistance plasmids were generally very diverse at the DNA level (Fig. 1), although eight plasmids (identified on the basis of their RFLP profiles) were isolated from multiple bacterial strains. Of these, seven plasmids were isolated twice each from within the same institution, and one plasmid was isolated four times in isolates from two different institutions. The bacterial strains harbouring identical plasmids were not themselves identical based on MLEE.

Plasmid	Inc	Resistance Profile	Size (kb)
pCE001	FII	Su Ap Tc	200
pCE003	FII	Ap Tc	200
pAE007	FII	Su Ap Tc	200
pAE019	FII	Ap Tc	200
pME007	FII	Su Ap Tp Tc Sm	130
pAE021	-	Su Ap Tp	170
pAE002	N	Su Tp Sm	30
pME004	-	Su Ap Tp Tc Sm Cm Km Gm	>260
pME005	-	Su Ap Tc Sm Km Nm	260
pAE018	FII	Ap Tp	30
pPE001	-	Su Ap Tp Sm	30
pPE002	-	Su Ap Tc	30
pME002	A/C	Su Ap Tp Tc Sm	260
pME003	A/C	Su Ap Tp Sm	260
pME006	-	Su Ap Tp Tc Sm Km Nm Sp	180
pSE019	FII N	Su Tp Tc Cm	90
pSE020	FII	Tc Cm	90
pPE011	FII	Su Ap Tc	170
pME001	FII A/C N	Su Ap Tp Sm Cm	180
pAE006	FII	Su Ap Tp	130
pAE008	-	Su Ap Tp	150
pAE013	FII	Tp Sm	180
pAE020	-	Su Ap Tp Sm Cm Gm Sp	150
pPE005	FII	Ap Tc	80
pSE018	FII	Tc Cm	160
pAE012	FII	Su Ap Tp Tc Sm	40
pPE004	-	Su Tc	90
pPE007	FII	Su Tp Sm	150
pPE010	-	Su Ap Tp Sm	190
pAE009	FII	Tp Tc	190
pAE010	FII	Su Tp Tc	190
pAE016	-	Su Tp	70
pAE015	FII	Su Ap Tp Sp	40
pAE011	FII	Su Ap Tp Tc Sm	180
pCE004	-	Su Ap Tp Tc Sm Km Nm	210
pCE010	-	Su Ap Tp Tc Sm Km Nm	130
pCE008	FII	Su Ap Tp Tc Sm	140
pCE007	FII	Su Ap Tp Tc Sm Cm	140
pCE012	FII	Su Ap Tp Tc Sm Cm	120
pCE011	FII	Su Ap Tp Tc Sm Km	50
pCE015	-	Su Ap Tp Sm	30
pCE016	FII	Su Ap Tp Cm Gm	30
pCE018	FII	Su Ap Tp Cm Gm	30
pCE013	FII	Su Ap Tp Tc Sm Cm Km Nm Sp	30
pCE009	-	Su Ap Tp Tc Cm Gm	140
pME008	-	Su Ap Tp Sm Km Gm	250
pCE006	FII	Su Tp Sm	30
pAE001	-	Su Ap Tp	150
pAE004	-	Su Ap Tp	150
pAE017	FII	Su Ap Tp Tc Sm Cm	190
pCE014	-	Su Tp	120
pCE017	-	Su Ap Tp Tc Sm Cm Km Gm	40
pPE013	-	Su Ap Tp	80
pPE009	-	Su Ap Tp Tc Sm Gm	160
pPE012	-	Su Ap Tp Sm	60
pSE009	FII	Tc Cm Km Nm	90
pSE011	FII	Tc Cm Km Nm	90
pSE010	FII	Su Tp	180
pSE013	FII	Su Ap Sm Cm Km Nm	80
pSE014	FII	Su Ap Tc Sm Cm Km Nm	80
pSE016	N	Ap Tc Cm	120
pSE017	-	Ap Tc Cm	150
pSE004	-	Su Ap Tp Tc Sm Km Nm	110
pSE008	A/C	Su Ap Tp Tc Cm Km Nm	180
pSE006	A/C	Su Ap Tp Tc Cm Km Nm	180
pAE014	-	Su Ap Tp Tc Sm	190
pSE001	A/C	Su Ap Tp Tc Cm Km Nm	120
pSE007	FII	Su Ap Tc Sm Cm Sp	110
pSE003	FII	Tc	80
pSE015	FII	Su Ap Tp Tc Sm Cm	230
pSE012	-	Su Ap Tc Sm Cm Km Nm	150
pPE006	-	Ap Tc	80

Fig. 1. Graphical representation of the similarities between plasmid restriction profiles (RFLP analysis). All plasmids were initially isolated on the basis of resistance to chloramphenicol, tetracycline and/or trimethoprim. RFLP analysis was based on restriction by *Sac*I, *Pst*I, *Hin*dIII, *Eco*RI and *Bam*HI. RFLP data were scored as a distance matrix and represented visually using the unweighted pair-group method (Rohlf, 1993). Resistance profiles were determined using the disc-diffusion method. Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Su, sulfisoxazole; Tc, tetracycline; Tp, trimethoprim. Plasmid incompatibility grouping was determined using a PCR-based method (Sherley *et al.*, 2003). Plasmid sizes were determined by electrophoretic comparison with plasmids of known size and are shown rounded to the nearest 10 kbp.

Plasmid size

The 72 plasmids varied in size from approximately 32 kbp to considerably larger than 250 kbp (Fig. 1), with a majority (approx. 50 %) falling within a 128–256 kbp size range. This result contrasts with the bimodal distribution of plasmid sizes observed in environmental isolates of *E. coli* (Sherley *et al.*, 2003), where the majority of plasmids >32 kbp fall into the 64–128 kbp size range. There was no correlation

between the number of antimicrobials that a plasmid mediated resistance to and its size.

Plasmid incompatibility

PCR-based screening for conserved regions of the incA/C, FII, N, P and W replicons successfully identified 61 % of the plasmids as belonging to one or more of these incompatibility groups. The vast majority (51 % of all plasmids

isolated) belonged to incFII, with only a handful of isolates belonging to incN or A/C, and no plasmids belonging to incP or W (Fig. 1). Two clinical resistance plasmids were identified as having multiple replicons: pME001 from Melbourne was identified as incFII, incN and incA/C, while pSE019 from Sydney was identified as incFII and incN.

It is possible that some of the plasmid incompatibility screening results were false negatives, as described by Gotz *et al.* (1996). We optimized the conditions for each primer pair so that there were no false-negative and no false-positive reactions detected for either of the following two control sets: (i) the set of 20 incompatibility replicon typing probes described by Couturier *et al.* (1988); and (ii) a set of 18 wildtype plasmids, including R805a (IncBI2), pIP40a (IncC), R1 (IncFII), R1drd19 (IncFII), R136 (IncFII), R16 (IncFII), R6 (IncFII), R27 (HI1), R478 (HI2), MIP233 (IncHI3), TP114 (IncI2), R1215 (IncM), R390 (IncN), R1010 (IncN), R751 (IncP), R934 (IncP), pHH1307 (IncW) and R388 (IncW).

The distribution of antimicrobial resistance genes

Plasmid-mediated resistance was observed for all ten of the antimicrobials tested (ampicillin, chloramphenicol, gentamicin, kanamycin, neomycin, streptomycin, spectinomycin, sulfisoxazole, tetracycline and trimethoprim). Resistance was more common to some antimicrobials than others, while the number of antimicrobials that a given plasmid mediated resistance to ranged from one to nine, with the majority of plasmids determining resistance to either three or six antimicrobials (Fig. 2).

A total of 35 different resistance profiles were observed (Fig. 1) and the combinations of resistance genes observed were not random (Table 1). Resistance patterns fell broadly into two groups: resistance to combinations of kanamycin,

Table 1. Significant correlations in antimicrobial resistance mediated by plasmids isolated from clinical *E. coli*

Correlations were determined for all pairwise combinations of antimicrobial resistance; only correlations with a significance of $P<0.05$ are shown. Plasmids were originally isolated on the basis of resistance to chloramphenicol, tetracycline and/or trimethoprim. Any correlations between these three antimicrobials have been deliberately excluded from this table as associations may have reflected sampling bias.

Antimicrobial pair	Correlation	Significance
Neomycin and kanamycin	0·8510	$P<0.00005$
Kanamycin and chloramphenicol	0·3005	$P<0.05$
Tetracycline and kanamycin	0·3005	$P<0.05$
Tetracycline and neomycin	0·2963	$P<0.05$
Neomycin and chloramphenicol	0·2882	$P<0.05$
Gentamicin and chloramphenicol	0·2863	$P<0.05$
Trimethoprim and sulfisoxazole	0·5340	$P<0.00005$
Sulfisoxazole and streptomycin	0·4211	$P<0.0005$
Trimethoprim and streptomycin	0·3361	$P<0.005$
Sulfisoxazole and ampicillin	0·3283	$P<0.005$
Streptomycin and ampicillin	0·2673	$P<0.05$
Streptomycin and kanamycin	0·2566	$P<0.05$

neomycin, chloramphenicol and tetracycline was common, and resistance to combinations of sulfisoxazole, trimethoprim, streptomycin and ampicillin also tended to occur.

The correlation between antimicrobial resistance profiles and RFLP data

There was a significant correlation between the plasmid relatedness data derived from RFLP analysis and those
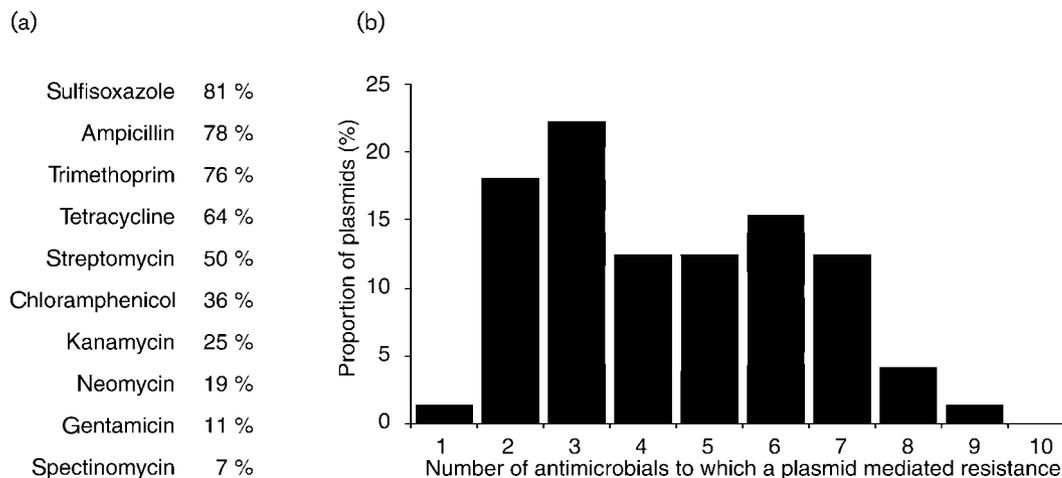
Fig. 2. The distribution of antimicrobial resistance in clinical plasmids mediating resistance to tetracycline, trimethoprim or chloramphenicol. Plasmids were initially isolated on the basis of resistance to chloramphenicol, tetracycline and/or trimethoprim. (a) The proportion of plasmids mediating resistance to each of 10 antimicrobials. (b) The proportion of plasmids mediating resistance to a given number of antimicrobials. $n = 72$.

derived from resistance profiles (Mantel test; $R_{xy} = 0.150$, $P < 0.001$). Which is to say that plasmids with a similar genetic backbone also shared broadly similar resistance profiles. Some of the variation between these two sets of data is due to the relatively low level of variation in resistance profile compared to RFLP analysis. Nevertheless, in many cases plasmids with identical resistance profiles shared little or no resemblance in their plasmid backbones. For example, pCE006 and pAE002 shared an identical resistance profile (sulfisoxazole, trimethoprim and streptomycin resistance) but had totally dissimilar RFLP banding patterns. In cases such as this it is possible that dissimilar plasmid backbones have horizontally acquired the same resistance genes from mobile genetic elements (this is discussed further below). However, the possibility that the observed genetic differences may reflect the presence of unrelated resistance genes must also be considered.

Some discrepancies between the RFLP and resistance data reflect differences in resistance profile that were not observed at the DNA level. In some cases plasmids with identical RFLP profiles differed by the loss or gain of resistance to a single antibiotic. For example, while pCE001 and pCE003 appeared identical from their restriction profiles, pCE003 was not sulfisoxazole-resistant and pCE001 was. Similarly, pSE013 and pSE014 differed only by the presence or absence of tetracycline resistance (Fig. 1).

The relationship between individual antimicrobial resistances and the RFLP data

The relationships between individual antimicrobial resistance markers and the RFLP data were determined using Mantel analysis. Ampicillin, chloramphenicol, neomycin, kanamycin, spectinomycin and tetracycline resistance all correlated significantly with the RFLP data ($P < 0.05$; Fig. 3). In contrast, gentamicin, streptomycin, sulfisoxazole and trimethoprim resistance did not correlate significantly with the RFLP data, suggesting more frequent horizontal movement of these resistance genes. This division between antimicrobial resistances agrees well with the above finding that tetracycline, chloramphenicol, kanamycin and neomycin resistance correlated significantly with each other, as did sulfisoxazole, trimethoprim and streptomycin resistance.

The relationship between host-cell relatedness data and plasmid relatedness data

MLEE indicated that the donor bacterial strains were highly variable. There was a significant correlation between the bacterial MLEE data and the plasmid RFLP data (Mantel test; $P < 0.05$), but it was very small ($R_{xy} = 0.075$), only half the size of the correlation between antimicrobial resistance profiles and the RFLP-based plasmid phylogeny. In other words, plasmids did not generally share the same relationships as their host cells. There was no significant correlation between the MLEE data and plasmid resistance profiles.
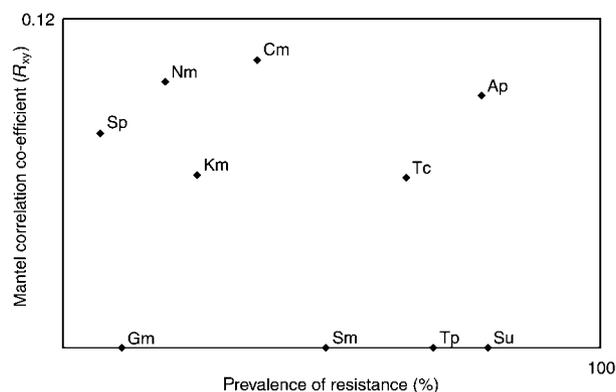
Fig. 3. The correlation between individual antimicrobial resistances and the plasmid backbone. Distance matrices were created to describe the resistance/sensitivity of the plasmids for each of 10 antimicrobials. These matrices were compared with the RFLP similarity matrix using a Mantel test (Mantel, 1967). The prevalence of resistance to each antimicrobial was calculated as the proportion (%) of plasmids mediating resistance to that antimicrobial ($n = 72$). Gentamicin, streptomycin, trimethoprim and sulfisoxazole resistance failed to correlate significantly with the RFLP data and are displayed as having a correlation of zero. All plasmids were initially isolated on the basis of resistance to chloramphenicol, tetracycline and/or trimethoprim. Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Su, sulfisoxazole; Tc, tetracycline; Tp, trimethoprim.

DISCUSSION

Our results indicate that the tetracycline, trimethoprim and chloramphenicol resistance plasmids carried by Australian clinical isolates of *E. coli* lack discernable evolutionary lineages, instead demonstrating the distribution of similar resistance profiles in diverse genetic backgrounds. Furthermore, the assembly of resistance genes in these plasmids is non-random. While it could be argued that transposition events explain the non-random association of resistance genes, they cannot explain the high level of plasmid diversity. Where evolution has occurred via the insertion or loss of a transposon, the newly evolved plasmids should differ only marginally in their restriction patterns; most of these plasmids shared less than 50% of their restriction digest bands with one another. The best explanation for this high level of diversity in the plasmid backbone is frequent recombination. Because plasmids with identical resistance profiles are generally no more closely related than average, the implication is that antimicrobial resistance genes and gene clusters are exploiting pre-existing plasmids and that the overall rate of plasmid recombination in *E. coli* is high. The lack of correlation between RFLP and MLEE data suggests that the horizontal movement of plasmids between cells is also frequent, further exacerbating the flow of genetic information by increasing the opportunity for recombination between novel plasmid pairs.

These results are in strong agreement with a previous study of conjugative plasmids in environmental isolates of *E. coli*. A sequence-based analysis of incompatibility group FIA, FIB and FII plasmids carried by strains from the ECOR reference collection (Boyd *et al.*, 1996) found that recombination played a major role in the evolution of these plasmids. Furthermore, the phylogenetic relationships between the plasmids (based on multiple gene sequences) differed from those of the host cells, implying a high rate of plasmid transfer even between the major ECOR groups. Other authors have also highlighted the importance of recombination in the evolution of plasmids from various members of the *Enterobacteriaceae* (Blazquez *et al.*, 1996; Boerlin, 1999; Petit *et al.*, 1990; Preston *et al.*, 2003). However, some authors have found that transposons and integron cassette systems are major mediators of resistance plasmid evolution in the *Enterobacteriaceae*, rather than recombination (Brown *et al.*, 2000; Carattoli *et al.*, 2002; Guessouss *et al.*, 1996; Radstrom *et al.*, 1991; Tosini *et al.*, 1998).

In our plasmid population, tetracycline, chloramphenicol, kanamycin and neomycin resistance correlated significantly with one another and with the plasmid RFLP data. This suggests that the plasmids carrying these markers have a shared genetic history. That is to say that tetracycline, chloramphenicol, kanamycin and neomycin resistance appear primarily to be transferred vertically or via large-scale recombination events. Resistance to trimethoprim, sulfisoxazole and streptomycin also tended to co-occur. However, resistance to these antimicrobials did not correlate significantly with the plasmid genetic backbone, suggesting more frequent horizontal movement of the genes conferring resistance to these agents.

The finding that trimethoprim, sulfisoxazole and strepto-mycin resistance are horizontally mobile and also co-occur would suggest association with transposable genetic elements. Furthermore, the association between the *sulI* sul-phonamide resistance gene and class I integrons in the *Enterobacteriaceae* (Radstrom *et al.*, 1991), and the frequent association of streptomycin and trimethoprim resistance gene cassettes with these integrons (Radstrom *et al.*, 1991; White *et al.*, 2001) strongly suggest that the correlation between these resistances may reflect resistance-cassette arrays within integrons. It is worth noting that it has previously been argued that integrons and their associated cassettes are more likely to move as a group than as independent units (Martinez-Freijo *et al.*, 1999).

Despite the fact that our results indicate a role for both recombination and transposition events, neither of Levin's predictions for the mechanisms of resistance plasmid evolution (Levin, 1995) fit these data well. We found a large bias in terms of plasmid incompatibility grouping in this plasmid set, but did not find a correspondingly high degree of genetic similarity between the plasmids. Nor did we find evidence that resistance genes had primarily been moving horizontally within the plasmid population. On the contrary, a significant correlation between some antimicrobial resistances and the plasmid backbone (as represented by RFLP data) argues strongly for vertical transmission of the corresponding resistance genes. We did observe some plasmids with multiple replicons, as would be expected where plasmid co-integration had occurred. However, if multi-resistance plasmids in *E. coli* had been evolving through the co-integrative capture of smaller resistance plasmids we would expect a strong correlation between the size of a plasmid and the number of antimicrobials that it mediated resistance to. No such relationship existed, nor was it evident from the plasmid restriction profiles that any plasmids had co-integrated other members of this plasmid population.

In the case of the plasmid pSE019 (incFII and incN) a highly related plasmid, pSE020 (incFII), that carried two fewer resistance markers (for sulfisoxazole and trimethoprim resistance) was isolated from the same host cell and is almost certainly a breakdown product of pSE019. The question then is whether pSE020 arose through the loss of a co-integrated incN plasmid bearing sulfisoxazole and trimethoprim resistance genes, or through some other means. The most serious argument against pSE019 being a co-integrate is that both pSE019 and pSE020 were very similar in size ($\sim$90 kbp). They most certainly did not differ by the 20–30 kbp that might be expected as a minimum for the size of a second conjugative plasmid. Some mobilizable plasmids transfer by co-integration into conjugative plasmids (Riemmann & Haas, 1993), so there is the possibility that co-integration of a very low weight mobilizable plasmid is responsible for these results, a solution that would also explain the absence of any similar conjugative incN plasmids.

The stable co-integration of a small mobilizable plasmid and a conjugative plasmid has previously been described in a member of the *Enterobacteriaceae* (Mitsuhashi *et al.*, 1977), as has the co-integration of relatively small conjugative plasmids resulting in co-integrates no larger than 140 kbp (Chu *et al.*, 2001). However, co-integration is rarely reported in the *Enterobacteriaceae* and in at least two cases large plasmid co-integrates have been observed to be unstable (Bradley *et al.*, 1986; Woodward *et al.*, 1990). Co-integrate plasmids may be unstable for various reasons. Some incompatibility groups have temperature-dependent transfer systems, so a co-integrate containing one replicon that is temperature-sensitive and a second that is not may be unstable outside of a fixed temperature range (Bradley *et al.*, 1986). Bacterial species differ significantly in the number and size-range of the plasmids they carry (Sherley *et al.*, 2003), suggesting that cellular factors in some way limit plasmid size. The presence of multiple replication regions on a single plasmid may also be directly destabilizing in a host species with a tendency to carry multiple plasmids as incompatibility between a plasmid with multiple replicons and a second plasmid can result in the loss of genetic material, including the incompatible replicon, from the hybrid plasmid (Coetzee *et al.*, 1975).

Whatever the means by which plasmids are evolving at a genetic level, the unequal distribution of plasmid incompatibility groups has important implications. Evolution via the

co-selection of incompatible plasmids (Condit & Levin, 1990) requires only two selective pressures, and these need not both be antimicrobial resistance genes. Several factors such as virulence determinants, adhesins, heavy metal resistance determinants and genes involved in the metabolism of unusual substrates are also found on plasmids. Furthermore, according to the local optimization model (Eberhard, 1990) all plasmids are actively selected for within their own environmental niche. As a result, treatment with a single antimicrobial may be sufficient to select for the movement of genes mediating resistance to that antimicrobial onto other actively selected plasmids. Moreover, selection for horizontally mobile genes such as sulfonamide, streptomycin and trimethoprim resistance genes, will increase the opportunity for associated mobile genetic elements to become involved in the evolution of multi-resistance plasmids, hence increasing the overall rate of horizontal gene movement and particularly the movement of clusters of genes.

In conclusion, these clinically derived plasmids do not belong to distinct plasmid lineages. They exhibit evidence of broad-scale inter-plasmid gene transfer, probably involving a range of mechanisms, including recombination, transposition and integration. The lack of correlation between plasmids and their hosts would suggest that horizontal plasmid transfer is common in clinical *E. coli* strains, while the substantial bias towards incFII plasmids increases the opportunity for plasmid evolution to occur via exposure to a minimum number of selective forces.

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