The A to Z of A/C Plasmids

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Abstract

Plasmids belonging to incompatibility groups A and C (now A/C) were among the earliest to be associated with antibiotic resistance in Gram-negative bacteria. A/C plasmids are large, conjugative plasmids with a broad host range. The prevalence of A/C plasmids in collections of clinical isolates has revealed their importance in the dissemination of extended-spectrum β-lactamases and carbapenemases. They also mobilize SGI1-type resistance islands. Revived interest in the family has yielded many complete A/C plasmid sequences, revealing that RA1, designated A/C_1, is different from the remainder, designated A/C_2. There are two distinct A/C_2 lineages. Backbones of 128-130 kb include over 120 genes or ORFs encoding proteins of at least 100 amino acids, but very few have been characterized. Genes potentially required for replication, stability and transfer have been identified, but only the replication system of RA1 and the regulation of transfer have been studied. There is enormous variety in the antibiotic resistance genes carried by A/C_2 plasmids but they are usually clustered in larger regions at various locations in the backbone. The ARI-A and ARI-B resistance islands are always at a specific location but have variable content. ARI-A is only found in type 1 A/C_2 plasmids, which disseminate bla_{CMY-2} and bla_{NDM-1} genes, whereas ARI-B, carrying the *sul2* gene, is found in both type 1 and type 2. This review summarizes current knowledge of A/C plasmids, and highlights areas of research to be considered in the future.

1. Introduction

Plasmids of the incompatibility group A/C (IncA/C) were amongst the earliest plasmids to be associated with antibiotic resistance in Gram-negative bacteria. They were first identified over four decades ago in Paris hospitals from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Witchitz and Chabbert, 1971, Chabbert et al., 1972) and have now been found in many Gram-negative species including *Klebsiella pneunomiae, Escherichia coli, Salmonella enterica, Yersinia pestis, Photobacterium damselae, Vibrio cholerae* and *Aeromonas hydrophila* (Tables 1 and 2) indicating a broad host range (Carattoli, 2009). However, until recently they were not studied in detail. As the incompatibility of most A/C plasmids studied today has not been tested, we have elected to call them A/C.

The introduction of PCR-based replicon typing (PBRT) (Carattoli et al., 2005) enabled the rapid identification of A/C plasmids in strain collections (Carattoli, 2009, Fricke et al., 2009, Welch et al., 2007, Evershed et al., 2009). A/C plasmids are now known to be strongly associated with resistance to clinically relevant carbapenems and third-generation cephalosporins. In particular, A/C plasmids carrying $bla_{\text{CMY-2}}$ and $bla_{\text{NDM-1}}$ genes are widespread (reviewed in (Carattoli, 2009, Carattoli et al., 2006, Carattoli, 2013)). This has led to renewed interest in the A/C group of plasmids. However, when the PBRT amplicons were sequenced it emerged that they were usually not identical to the *repA* of the A/C reference plasmid RA1 (Carattoli et al., 2006, Evershed et al., 2009). Hence, there appeared to be two distinct lineages of A/C plasmids that were designated A/C_1 (RA1) and A/C_2 (Carattoli et al., 2006). Based on available estimates (Ochman and Wilson, 1987, Okoro et al., 2012), the nucleotide divergence between A/C_1 (RA1) and A/C_2 plasmids represents hundreds of thousands of years of evolution and the two groups of A/C plasmids have very separate evolutionary stories.

The first complete sequences of A/C plasmids were published in 2007 (Welch et al., 2007) and many more have been sequenced since. This has helped to identify the basic biological

functions they determine and several lists of annotations for the genes found in them have been published (Del Castillo et al., 2013, Fernandez-Alarcon et al., 2011, Zhang et al., 2013, Ho et al., 2013). However, while in a number of cases the proteins they encode share significant identity with proteins of known function, for example the transfer genes encode homologs of those of F (Lawley et al., 2003), the role of only a few has been experimentally determined. Exploration of the remaining genes is needed to unravel the basic biology of A/C_2 plasmids.

The availability at the end of 2014 of sequence data for so many A/C plasmids (Tables 1 and 2) has presented an opportunity to examine the evolution and evolutionary history of these plasmids (Harmer and Hall, 2014), and provided insights into the role that they play in the carriage and dissemination of genes conferring resistance to antibiotics. As only one A/C_1 plasmid (RA1) has been sequenced to-date, much of this review will focus on the A/C_2 group.

The purpose of this review is to consolidate the current available information on A/C plasmids and serve as a resource for researchers working on this important plasmid family.

2. The A/C plasmid group

Plasmids belonging to the IncC group were first recovered in the late 1960's (Datta and Hedges, 1972, Chabbert et al., 1972). The plasmid RA1 was isolated in 1971 following the observation of transferrable tetracycline and sulphonamide resistance in isolates of the fish pathogen *Aeromonas liquafaciens* (Aoki et al., 1971). Initially, marked exclusion upon transfer of RA1 to *E. coli* K12 carrying a plasmid of group C was observed (Datta and Hedges, 1972). Subsequently, RA1 was found to be compatible with plasmids of all known incompatibility groups tested at the time. This included IncC, in addition to IncI, N, P, W, T, N, B, J and X, and RA1 was provisionally assigned to compatibility group A (Datta and Hedges, 1973). After (Datta and Hedges, 1972) further experimental work, it was suggested that plasmids in incompatibility groups A and C were very closely related, and the groups were combined as the IncA/C complex (Hedges, 1974). However, this conclusion appears to be based on entry-

105 exclusion data rather than failure of the plasmids to be stably maintained together. Hence,
106 whether RA1 is compatible with IncC plasmids such as R57b and R40a needs to be re-
107 examined using modern molecular methods.
108 A/C plasmids exhibit a relatively broad host range and are able to be stably maintained
109 in many Gram-negative bacterial species (see Tables 1 and 2) as well as *Pseudomonas*
110 (Chabbert et al., 1972). They are reported to be equivalent to the IncP3 plasmids of
111 *Pseudmonas spp* (see (Llanes et al., 1994)).
112
113 *2.1. A/C_1 and A/C_2 plasmids*
114 The *repA* amplicon of most A/C plasmids was found to differ from the *repA* sequence of
115 RA1, sharing only 93.2% nucleotide identity (Carattoli et al., 2006, Evershed et al., 2009).
116 Consequently, the two groups of A/C plasmids were designated as A/C_1 (RA1) or A/C_2
117 (Carattoli et al., 2006). When the complete sequence of RA1 was determined (Fricke et al.,
118 2009) it was found that the regions in the backbone of RA1 shared with other A/C plasmids
119 for which sequences were available at the time (Kim et al., 2008b, Welch et al., 2007) were
120 only between 84-95% identical to one another (Fricke et al., 2009). Alignment of the
121 backbone of RA1, derived by removing the region containing the resistance genes, with the
122 complete backbone of A/C_2 plasmids derived from pR148 (Del Castillo et al., 2013) is shown
123 in Figure 1, and reveals that in addition to the common regions there are regions that are
124 unique to each backbone type. The RA1 island containing the antibiotic resistance genes is
125 located in one of the unique regions. The antibiotic resistance islands (ARI), ARI-A and ARI-B,
126 found in many A/C_2 plasmids, are also in regions found only in A/C_2 plasmids.
127 Among the sequenced A/C plasmids in GenBank at the end of 2014, all but RA1 are
128 A/C_2 (Tables 1 and 2). The A/C_2 plasmids have been recovered from many different bacterial
129 species and from a number of sources including humans, cattle, pigs, fish, and poultry. In
130 addition to the complete A/C_2 plasmid sequences in the GenBank non-redundant DNA

database, there appear to be A/C_2 plasmids in over 60 draft genomes (mainly *S. enterica* and *K. pneumoniae*) in the whole genome shotgun database (WGS, November 15th, 2014).

2.2 RA1, an A/C_1 plasmid

The complete 143963 bp sequence of RA1 (GenBank accession number FJ705807 (Fricke et al., 2009)) is made up of a backbone of 130 kb and a single 13.9 kb resistance island. It contains 158 open reading frames (ORFs) of greater than 300 base pairs including genes required for initiation of replication (*repA*), conjugative transfer (*tra*) and plasmid partitioning (*stb* and *par*) (Fricke et al., 2009). Most of the Tra proteins encoded by genes in RA1 share >85% aa identity with the Tra proteins coded for in A/C_2 plasmids (see section 4.1). TraN only shares 63% aa identity. The location of the resistance island is shown in Figure 1 and the structure is described in section 8.

2.3. A/C_2 plasmids – two distinct types

The precise content of the backbone of A/C_2 plasmids was initially obscured due to the presence of a *sul2*-containing resistance island ARI-B (see section 9.1). As *sul2* was present in all of the early sequenced A/C_2 plasmids, it was believed that *sul2* was part of the plasmid backbone (Welch et al., 2007, Fernandez-Alarcon et al., 2011). However, the subsequent availability of sequences for plasmids that do not contain this island, pRMH760 (GenBank accession number KF976462 (Harmer and Hall, 2014)) and pR148 (Genbank accession number JX141473 (Del Castillo et al., 2013)), has allowed the precise definition of the original backbone (Harmer and Hall, 2014). It also revealed that in most instances the ARI-B resistance island that carries *sul2* is associated with deletions adjacent to one end. The A/C_2 backbone is 127.8 kb for type 1 A/C_2 or 129.2 kb for type 2 A/C_2 and contains over 120 open reading frames encoding over 100 amino acids (aa), including genes required for replication,

156 conjugative transfer, DNA metabolism, partitioning and stability, toxin/anti-toxin, and many
157 genes of unknown function (Figure 2A). These are described in more detail in sections 3-6.
158 A recent analysis of the backbones of complete A/C_2 plasmid sequences identified two
159 distinct types, type 1 and type 2, that diverged a long time ago (Harmer and Hall, 2014). Each
160 type has accumulated single nucleotide polymorphisms (SNP), with the backbones differing
161 by approximately 1%. Based on current estimates for accumulation of SNPs (Okoro et al.,
162 2012), the divergence of the two lineages is likely to have occurred at least 5000 years ago
163 and therefore before they began to accumulate antibiotic resistance genes.
164 The two types also differ in two regions (R1 and R2) where part of a large gene has
165 been replaced (see Figure 2 in Harmer and Hall, 2014). The replacements give rise to two
166 versions of the *rhs* gene (*rhs1* and *rhs2* in type 1 and type 2, respectively) and of the open
167 reading frame between *traA* and *dsbC* that predict proteins of 1832 aa (orf1832) in type 1 and
168 1847 aa (orf1847) in type 2. In addition, two short regions, i1 and i2 (428 bp and 462 bp,
169 respectively), are present in type 2 but not in type 1 (Figure 2A). This highlights the
170 importance of performing detailed examinations of the plasmid backbone, particularly in
171 epidemiological studies where essentially no single base differences should be observed if a
172 close relationship is to be inferred.
173
174 *2.3.1. Type 1 A/C_2 plasmids*
175 At the end of 2014, there were roughly twice as many sequenced type 1 A/C_2 plasmids
176 (Table 1) as there were type 2 (Table 2). However, this is due to a focus on sequencing
177 plasmids carrying genes coding for extended spectrum β-lactamases, particularly *bla*$_{CMY}$ and
178 more recently *bla*$_{NDM}$, all of which fall within type 1. Thirty of 35 sequenced type 1 A/C_2
179 plasmids carry at least one copy of *bla*$_{CMY-2}$ or a variant of it (Table 3). The *bla*$_{CMY-2}$ gene is
180 associated with the mobile element ISEcp1 in an island that is always in the same location,
181 between *traA* and orf1832 (Figure 2A). This indicates that this island inserted once and,

though further rearrangements have occurred subsequently (Fernandez-Alarcon et al., 2011, Partridge, 2011), all *bla*$_{CMY-2}$-carrying A/C$_2$ plasmids are derived from a common ancestor. Moreover, fifteen are from *S. enterica* or *E. coli* from turkeys, chickens and cows in the US, creating a further sampling bias.

Type 1 A/C$_2$ plasmids, with one exception (pCFSAN001921), also carry an antibiotic resistance island, ARI-A, in a specific location in the backbone (see section 9.2). Eight of the sequenced type 1 A/C$_2$ plasmids carry both *bla*$_{CMY-2}$ and the carbapenem resistance gene *bla*$_{NDM-1}$ within ARI-A (see section 9.2). Only the *bla*$_{NDM-1}$-carrying plasmids, and three further type 1 A/C$_2$ plasmids have been recovered from humans (Table 1), and this bias needs to be addressed.

pRMH760 (Harmer and Hall, 2014) and pR148 (Del Castillo et al., 2013), both of which lack ARI-B, represent the precursor of A/C$_2$ plasmids carrying *bla*$_{CMY}$ or both *bla*$_{CMY}$ and *bla*$_{NDM}$ (Harmer and Hall, 2014). However, they each confer resistance to several antibiotics. This highlights the importance of tracking resistance to older antibiotics that are not the current first line, as the vehicles that carry those resistance genes are likely to be the ones that acquire further resistance genes.

2.3.2. Type 2 A/C$_2$ plasmids

The A/C$_2$ type 2 plasmids are representative of a broader set of origins. Nine of the 16 type 2 plasmids were recovered from humans or the hospital environment, with the remainder recovered from fish, turkeys, pigs and cows (Table 2). However, a set of three plasmids that carry the *bla*$_{KPC-2}$ gene encoding the KPC-2 carbapenemase were recovered from a patient and the environment in a single hospital and are closely related (Conlan et al., 2014). Though it had previously been claimed that in A/C$_2$ plasmids the clusters of resistance genes additional to the ARI-B island are all in the same location (Doublet et al., 2012, Johnson and Lang, 2012), it is now clear that is not the case (Harmer and Hall, 2014). Unlike type 1

208 A/C_2 plasmids, type 2 A/C_2 plasmids have acquired resistance islands (see section 9) on many
209 occasions and carry them in multiple different locations (Table 4 and Figure 2B) clustering
210 within or around the *rhs2* gene (Harmer and Hall, 2014).
211
212 *2.3.3. Hybrid A/C_2 plasmids*
213 The level of nucleotide identity in the A/C_1 and type 1 and type 2 A/C_2 backbone DNA
214 is very high and homologous recombination between them is possible, though the
215 opportunities for recombination are likely to be limited due to entry exclusion and
216 incompatibility.
217 Among the A/C_2 plasmids examined previously (Harmer and Hall, 2014), only pYR1
218 (GenBank accession number CP000602) contained features usually found only in type 1 or
219 type 2 A/C_2 plasmids. It contains the i1 and i2 insertions characteristic of type 2, plus the type
220 2 version of R1, orf1847. However, it also contains *rhs1,* the type 1 version of R2. In addition,
221 the ARI-B island is found in the same position in A/C_2 plasmids of both type 1 and type 2, and
222 may have been shared via homologous recombination.
223 Despite this evidence for hybrid formation, these plasmid lineages should still be
224 separately considered in analyses that aim to deduce evolutionary steps or to create
225 networks.
226
227 **3. Replication**
228 The A/C backbone contains a number of genes and ORFs that may be involved in
229 replication or DNA synthesis during transfer (Figure 2A; see section 6.4), however only *repA*
230 has been characterized.
231
232 *3.1. Replication of RA1*

The RA1 replicon was confined to a 2.79 kb XbaI-SmaI fragment that was sufficient for replication in both *Enterobacteriaceae* and in *Pseudomonas* spp (Llanes et al., 1994). A 289 aa RepA protein was identified (Llanes et al., 1994). However, the complete sequence of RA1 (GenBank accession number FJ705807) revealed two nucleotides are missing from the original *repA* sequence (GenBank accession number X73674). Hence, the *repA* gene is 1101 bp encoding a 367 aa RepA protein (Figure 3A). Thirteen direct repeats (iterons) of 19 bp were also identified downstream of *repA* (Llanes et al., 1994). However, two of these do not conform to the consensus (yaTRTGGG---gCTGCACG) and were not included in Figure 3B. The location of the iterons downstream, rather than upstream, of the *repA* gene appears to be a feature characteristic of A/C and IncP plasmids (Llanes et al., 1996). A DnaA box on which the chromosomal initiator protein DnaA may act was found next to the iterons. The origin of replication was mapped to a 600 bp fragment that was sufficient to initiate replication when RepA was supplied *in trans* (Llanes et al., 1996). This fragment contains nine of the eleven iterons and the DnaA box. The 2.79 kb fragment also contains two imperfect inverted repeats located upstream of *repA* (Llanes et al., 1994, Llanes et al., 1996) but a role for these has not been established.

3.2. Replication of A/C$_2$ plasmids

Our analysis revealed that the A/C$_2$ replicon has features similar to A/C$_1$ (Figure 3A). A/C$_2$ plasmids encode a 367 aa RepA that is 98 % identical to the A/C$_1$ RepA. Fourteen iterons of 19 bp (yaTRTGGG--cgcTGCACG) are located downstream of *repA.* The region also includes a DnaA box downstream of the iterons and imperfect inverted repeats upstream of *repA*. There is high conservation of the iteron sequences between A/C$_1$ and A/C$_2$ plasmids (Figure 3B).

4. Conjugative transfer

In contrast to other plasmid families such as IncF, little work has been done to examine the conjugative machinery of A/C plasmids despite the clear importance of A/C plasmids in resistance transmission. Indeed, whether A/C plasmids, including those that have been sequenced, can transfer is often not reported. In an early study, RA1 transferred at a frequency of approximately 10^{-2} transconjugants per donor on solid media and approximately 10^{-4} in broth (Bradley, 1989). In contrast, the frequency of transfer of other A/C plasmids tested was 10^{-4} to 10^{-7} on solid media and in broth. More recently, for A/C_2 plasmids for which information is available, the transfer efficiency has ranged from as high as 6 x 10^{-2} to as low as 10^{-6} transconjugants per donor (Harmer and Hall, 2014, Poole et al., 2009, Fricke et al., 2009, Welch et al., 2007, Carraro et al., 2014a). A large number of A/C plasmids found in *Salmonella* strains were able to transfer into a new host (Welch et al., 2007). However, conjugative transfer could not be detected for several A/C_2 plasmids found in *S. enterica* serovar Newport (Welch et al., 2007), or in *S. enterica* serovars Senftenberg and Ohio isolates from Australian pigs (Evershed et al., 2009).

Further experimental work is required to answer some key questions regarding the conjugative transfer functions of A/C plasmids, including determining the functions of the specific *tra* genes and determining the genetic differences behind variable transfer frequencies of closely related A/C plasmids.

4.1 Transfer genes

Conjugative transfer genes in A/C plasmids have been identified by the homology of their predicted proteins with characterized Tra proteins, and equivalent genes are present in RA1 and the A/C_2 group. RA1 has been shown to produce numerous 9 nm thick, flexible pili resembling those of F (Bradley, 1980). The role of the Tra proteins has not been confirmed experimentally and further proteins may be involved, e.g. *topB* (section 6.4).

The majority of *tra* genes are contained within two separate regions (Figure 2A). The first, a 28 kb region, contains three groups of genes. *traI*, *traD* are separated from *traL*, *traE*, *traK*, *traB*, *traV*, *traA* by seven short reading frames of unknown function, and *traC*, *trhF*, *traW*, *traU*, *traN* are further separated by orf1832/1847 and *dsbC*. The remaining transfer genes, *traF*, *traH* and *traG,* are contained within a separate 6.1 kb region (Figure 2A). The likely roles of the various Tra proteins can be inferred by analogy with those of plasmid F (Lawley et al., 2003, Arutyunov and Frost, 2013). These genes encode proteins involved in conjugative processes including a relaxase (*traI*) belonging to the MOB_H group (Garcillan-Barcia et al., 2009), a coupling protein (*traD*), and mating pair stabilization (*traNG*) proteins. The genes involved in type IV secretion system (T4SS) assembly (*traLEKBVACWUFHG*) code for proteins that belong to the MPF_F group (Guglielmini et al., 2013).

The closest homologs to the Tra proteins encoded by A/C plasmids are those produced by the *Vibrio cholera* SXT integrative conjugative element (ICE) (GenBank Accession Number AY055428) and the R391 ICE from *Providencia rettgeri* (GenBank Accession Number AY090559) (Kim et al., 2008b). The proteins produced by *tra* genes of A/C plasmids and SXT/R391 share 33% to 78% aa identity (Figure 4), and the genes that have been shown to be required for transfer of SXT (Beaber et al., 2002) all have homologs in A/C plasmids.

In the DNA processing system of SXT/R391, an additional gene, *mobI,* is required for nicking at the origin of transfer (Ceccarelli et al., 2008). The MobI protein is proposed to be an auxiliary component of the relaxosome that is required for *oriT* processing to initiate conjugative transfer (Ceccarelli et al., 2008). Recently, a gene also designated *mobI*, that codes for a distant homolog of the SXT MobI, has been shown to be essential for the conjugative transfer of A/C_2 plasmids (Carraro et al., 2014b). The A/C_2 *mobI* is located upstream of *repA* (Figure 2A). Though a possible origin of transfer (*oriT*) was found adjacent to *mobI* in A/C_2 plasmids (Carraro et al., 2014b), deletion of this region resulted in only a 10-fold reduction in the transfer frequency from 10^{-3} to 10^{-4} transconjugants per recipient. As transfer was not

completely abolished, it is unlikely to be the sole *oriT* and an alternative *oriT* locus remains to be identified.

4.2. Entry exclusion

Despite the observation of entry-exclusion in early studies (Datta and Hedges, 1973), a homolog of TraS from F (Lawley et al., 2003, Arutyunov and Frost, 2013) or Eex from SXT (Marrero and Waldor, 2007) have not been found. This phenomenon deserves further investigation in order to identify the genes involved.

4.3. Regulation of transfer

It is still unclear which traits separate those plasmids that can transfer at high or low frequencies from those that cannot transfer at all. Though it has been proposed that the loss of conjugative function in many of the A/C_2 plasmids carrying the ISEcp1-*bla*$_{CMY-2}$ island could be due to the location of this island within the *tra* region (Figure 2A) (Poole et al., 2009), this is unlikely as this island lies within an intergenic region and some plasmids from this group have retained the ability to transfer (Call et al., 2010, Sekizuka et al., 2011). However, rearrangements in this region have led to loss of *tra* genes in some ISEcp1-*bla*$_{CMY-2}$ plasmids (Fernandez-Alarcon et al., 2011), and this may correlate with the transfer deficiency.

Recent elegant work has identified and characterized the master regulator of *tra* gene expression and plasmid transfer (Carraro et al., 2014a). The transcriptional activator complex AcaCD is essential for conjugative transfer, which could not be detected for Δ*acaC*, Δ*acaD*, and Δ*acaCD* mutants. Transcription of *tra* genes was positively regulated by AcaCD, and AcaCD binding sites were identified upstream of *traI*, *traL*, *traV*, *traN* and *traF*. In addition, AcaCD positively regulates the expression of fourteen other regions in the A/C_2 backbone and putative AcaCD binding sites have been identified in each of these positions (Carraro et al., 2014a). These genes will provide a rich source for future experimentation.

Production of AcaCD is regulated by a repressor encoded by *acr1* upstream of *acaCD,* and deletion of the *acr1* gene caused the frequency of transfer to increase 100-fold from ~10^{-3} transconjugants per recipient for the wild-type plasmid to 10^{-1} in the Δ*acr1* mutant (Carraro et al., 2014a). The genes, *acr1* and *acaCD,* for the master regulator system are located between *traG* and *stbA* together with a further gene, *acr2,* that encodes a repressor (Figure 2A). The *acr2* gene was previously reported to encode an H-NS-like protein (Fernandez-Alarcon et al., 2011). Though deletion of *acr2* led to a 5-6 fold increase in conjugation frequency, its precise role remains to be established (Carraro et al., 2014a).

Carraro et al. (2014a) detected insertions, deletions or substitutions within *acaC* and/or *acaD* in a number of sequenced plasmids, and these should be transfer defective. Information on transfer proficiency was available for only two of them and both were transfer defective (Carraro et al., 2014a). However, *acaC* and *acaD* are both intact (Harmer and Hall, unpublished) in the recently reported pSRC119-A/C, which does not transfer (Harmer et al., 2015), indicating that further factors remain to be identified. The high transfer frequency of pRMH760 (6 x 10^{-2} transconjugants per recipient (Harmer and Hall, 2014)), which is equivalent to that of the pVCR94delta *acr1* deletion derivative described above, also suggests a more complex story as the *acr1-acaD-acaC* region is identical in the two plasmids.

5. Plasmid stability

There has been no experimental work to determine the features required for stable maintenance of A/C plasmids. However, three regions potentially involved have been identified (green genes in Figure 2A).

5.1 parA-parB

A Pfam search of the protein encoded by *parA* revealed the presence of a conserved CbiA domain (Pfam PF01656). This domain is present in plasmid partitioning proteins of the

362 MinD/ParA family, first characterized in the IncP plasmids RK2 (Motallebi-Veshareh et al.,
363 1990). The adjacent *parB* gene encodes a protein containing both ParB (PF02195) and KorB
364 (PF08535) domains. KorB is a major regulatory element in the replication and maintenance of
365 RK2 (Kornacki et al., 1987). Interestingly, the A/C ParA shares 96.1% aa identity with the
366 chromosomal ParA found in a number of bacterial species including *Xenorhabdus*
367 *nematophila*, however the importance of this observation is not yet clear.
368
369 *5.2 stbA*
370 The *stbA* gene located upstream of *repA* encodes a protein, StbA (NCBI Reference
371 Sequence WP_000077457), sharing 30.5% aa identity with the ParM partitioning protein
372 (NCBI Conserved Domain accession number cd10227) from the IncFII plasmid NR1 and from
373 the *Staphylococcus aureus* plasmid pSK41. ParM is essential for the accurate segregation of the
374 low-copy-number plasmid NR1 (R100) via polymerization into double helical protofilaments
375 similar to filamentous actin (van den Ent et al., 2002). Whilst ParM in pSK41 is homologous to
376 ParM of NR1, the polymerization kinetics and the regions required for plasmid segregation via
377 filament formation are different (Popp et al., 2010).
378
379 *5.3 A toxin-antitoxin system?*
380 Toxin-antitoxin systems are known to be involved in the postsegregational killing of
381 plasmid-free daughter cells (Van Melderen, 2010), which contributes to plasmid maintenance.
382 Transcriptome mapping of an A/C_2 plasmid, pAR060302, identified two genes (orfs 19 and 20
383 in Figure 2A) transcribed at levels 47- and 20-fold higher than *repA* (Lang et al., 2012). The
384 products of orf 19 and 20 were similar to the *S. enterica* serovar Paratyphi B toxin-antitoxin
385 system (Lang et al., 2012). Though the A/C system is incompletely characterized, one of the
386 genes (NCBI reference sequence WP_000124640) encodes a putative XRE-like transcriptional
387 regulator predicted to function as the antitoxin component, while the adjacent gene (NCBI

388 reference sequence WP_000270043) encodes the putative toxin component of the system.
389 Deletion of the antitoxin gene was lethal to the cell (Lang et al., 2012, Carraro et al., 2014a).
390
391 **6. Other plasmid-encoded functions**
392 A number of other genes have been annotated in sequenced A/C$_2$ plasmids (Del
393 Castillo et al., 2013, Fernandez-Alarcon et al., 2011, Zhang et al., 2013, Ho et al., 2013). Here,
394 these annotations have been extended and enhanced for all predicted proteins of greater than
395 100 aa using BLAST and Pfam searches. Over fifteen more encode proteins of 90-99aa, but
396 only those with identified functions are shown in Figure 2A.
397
398 *6.1. rhs*
399 An *rhs* gene is found in A/C$_2$ plasmids but is not present in RA1 (Figure 1). Genes in
400 this family are a core component of Enterobacterial genomes (Jackson et al., 2009) and were
401 first identified as sites that promote recombination in *Escherichia coli* (Lin et al., 1984). They
402 are an ancient family of genes, comprising six distinct lineages. Whilst the primary structures
403 of Rhs proteins are well conserved, the C-terminal regions (also known as the tip) are highly
404 variable, leading to diversification of the family through C-terminal displacement (Jackson et
405 al., 2009). The replacement of part of the C-terminus of Rhs is one of the key features that
406 distinguish between type 1 and type 2 A/C$_2$ plasmids (see Figure 2 in Harmer and Hall, 2014).
407 Whilst genes sharing homology with *rhs* have been associated with bacteriocin production
408 (Sisto et al., 2010), mediation of intercellular competition (Koskiniemi et al., 2013) and
409 inhibition of protein synthesis (Aggarwal and Lee, 2011), the biological function of *rhs* in A/C$_2$
410 plasmids remains unknown.
411 In A/C$_2$ plasmids there appears to be a strong tendency for resistance genes to be
412 incorporated into the *rhs* gene. Six out of the eight resistance island locations found in type 2
413 A/C$_2$ plasmids are within *rhs* (Figure 2B). In the case of p1643_10 two independent insertions

have both occurred in *rhs* (Table 4). Additionally, in eight type 1 A/C_2 plasmids a fragment of *rhs* has been deleted, either via a deletion internal to the *rhs* gene (pRMH760) or as part of a larger deletion originating from within ARI-A and extending 3478 bp (pNDM-KN, pNDM10469, pNDM10505, pNDM102337, pNDM-US and pKP1-NDM-1) or 6953 bp (pMR0211) into the backbone (see (Harmer and Hall, 2014)). This is intriguing, but the reasons for this trend remain to be determined.

6.2. Methyltransferases

The A/C_2 backbone contains three genes, *dcm1*, *dcm2*, and *dcm3* (NCBI Reference Sequences WP_015059976, WP_00936897 and WP_000201432, respectively) predicted to encode Gammaproteobacteria DNA cytosine-5-methyltransferases from two different families (Pfams PF01555 for *dcm1* and PF00145 for *dcm2* and *dcm3*). Cytosine methylation is an integral step in bacterial restriction modification processes, protecting the host sequence from cleavage by sequence-specific restriction enzymes designed to degrade non-methylated (foreign) DNA (Wilson and Murray, 1991). This allows bacteria to protect themselves from infection by bacteriophage (Wilson and Murray, 1991). Hence, the carriage of *dcm* genes on A/C_2 plasmids may provide protection against degradation by host nucleases. Though no experimental work has been performed on the *dcm* genes in A/C_2 plasmids, a number have lost one of the genes, *dcm1*, as the result of a deletion associated with ARI-B, apparently without deleterious effect. The presence of three genes encoding DNA cytosine-5-methyltransferases may represent a type of protective redundancy.

6.3 ssb-bet-exo

The Exo (Pfam 09588; YqaJ), Bet (Pfam 03837; RecT) and Gam (Pfam 06064) proteins encoded by bacteriophage lambda function together to facilitate RecA-independent recombination between short regions of identity and this has been harnessed as the lambda

Red system (Hillyar, 2012). Homologs of the lambda Bet and Exo recombination proteins encoded by A/C plasmids and SXT/R391 have been identified (Garriss et al., 2013). However, a homolog of the lambda Gam protein was not found. The A/C *bet* gene encodes a protein sharing only 25% aa identity with the lambda Bet (RecT) single-strand annealing protein (NCBI conserved domain accession number TIGR01913) and 61% identity with the SXT/R391 Bet. The A/C *exo* gene encodes a protein (NCBI reference sequence WP_000706875) sharing 24% aa identity with the lambda Exo and 64% identity with the SXT/R391 Exo.

In lambda, *gam-bet-exo* are adjacent to one another. However, a gene encoding a putative single-stranded DNA-binding protein, *ssb*, is found upstream of *bet-exo* in A/C_2 plasmids and SXT/R391 ICE (Garriss et al., 2013, Chen et al., 2011). The *ssb* gene of A/C_2 plasmids encodes a 176 aa protein, Ssb (NCBI reference sequence WP_000987165), sharing 40% aa identity with Ssb from SXT and 46% aa identity with the *E. coli* Ssb (NCBI conserved domain accession number cd04496, Pfam PF00436). Ssb in *E. coli* binds to and protects single-stranded DNA intermediates during DNA replication, repair and recombination (Shereda et al., 2008, Raghunathan et al., 2000, Meyer and Laine, 1990). Interestingly, whilst *bet* and *exo* are also present in RA1 (A/C_1), *ssb* is not.

6.4. Other DNA metabolism genes

6.4.1 nuc

All A/C_2 plasmids contain a gene previously annotated as *ybaA* encoding a 201 aa 5'-nucleotidase (NCBI reference sequence WP_000071870) with a HD metal dependent phosphohydrolase domain (Pfam 13023). This gene is designated *nuc* in Figure 2A. 5'-nucleotidases with dephosphorylation activity play roles in nucleic acid repair, signal transduction and nucleic acid metabolism (Hunsucker et al., 2005, Aravind and Koonin, 1998).

6.4.2 Sulfate modification of DNA?

A 2454 bp orf (NCBI reference sequence WP_001187969; orf 102 in Figure 2) upstream of *nuc* encodes a protein sharing 30% aa identity with a conserved phosphoadenosine phosphosulfate reductase family protein (NCBI Conserved Domain cl00292). In *Streptomyces lividians*, a related protein has been experimentally shown to modify DNA by addition of a sulfur-containing compound, possibly sulfate. This modification sensitizes the DNA to degradation during electrophoresis (Zhou et al., 2005). The role for this protein in A/C plasmids is not known.

6.4.3 topB

The *topB* gene encodes a DNA topoisomerase III protein (NCBI reference sequence WP_000366823) sharing 38% aa identity with the well-characterised TraE (NCBI conserved domain accession number TIGR010156) from plasmid RP4 (Li et al., 1997). DNA topoisomerases play a functional role in the regulation of the number of topological links between DNA strands by catalyzing transient single- or double-stranded breaks, crossing the strands through one another, before resealing the breaks (Roca, 1995). The regulation of topological links is also important in removing supercoils during transcription and DNA replication, and for strand breakage during recombination (Wang, 2002). In RP4, the TraE topoisomerase is involved in the resolution of DNA replication intermediates formed during conjugative transfer (Li et al., 1997), indicating that *topB* may also be an A/C transfer gene.

6.4.4 kfrA

The *kfrA* gene encodes the 346 aa protein KfrA (NCBI reference sequence WP_000101568). This protein contains a domain sharing 32% amino with the KfrA_N plasmid replication region DNA-binding domain (Pfam 11740). KfrA has been well-characterized in the IncP plasmid RK2 and is a DNA binding protein with a long, alpha-helical tail (Jagura-Burdzy and Thomas, 1992). In IncP plasmids, *kfrA* is part of a tricistronic operon required for

the stable maintenance of the plasmid. Homologs of the two other genes in the operon, *kfrB* and *kfrC*, have not been identified in A/C plasmids. Inactivation of *kfrA* resulted in the long-term accumulation of plasmid-free cells, whereas the wild-type RK2 plasmid was able to persist without selection (Adamczyk et al., 2006). The role for *kfrA* in A/C plasmids needs investigation.

6.4.5 uvrD

The *uvrD* gene encodes a UvrD/REP helicase (NCBI reference sequence WP_000811656), containing both N-terminal and C-terminal domains (Pfam 00580 and 13361, respectively). The REP helicases catalyse ATP dependent unwinding of double stranded DNA to single stranded DNA (Korolev et al., 1997).

6.4.6 ter

The *ter* gene encodes a protein, Ter (NCBI reference sequence WP_001097010), with a conserved DNA replication terminus binding domain (Pfam 05472). This gene has also previously been annotated as *tus* in some A/C_2 plasmids. Proteins in the Ter family have been shown to specifically bind to DNA replication terminus sites on plasmids and the chromosome, blocking progress of the DNA replication fork (Hidaka et al., 1989).

6.4.7 int

The *int* gene encodes a putative XerD tyrosine recombinase (NCBI reference sequence WP_000543934) with a conserved integrase domain (Pfam 00589). It shares 34% aa identity with the well-characterized XerD tyrosine recombinase of *E. coli* which together with XerC frees intertwined DNA molecules after termination of replication (Hallet et al., 1999).

6.4.8 pri

518 The *pri* gene encodes a putative primase-helicase (NCBI reference sequence
519 WP_000900352) with both a primase-helicase zinc binding domain (Pfam 08273) and a
520 topoisomerase-primase catalytic domain (Pfam 13362). Helicases and primases play a critical
521 role in DNA polymerization. The primase synthesizes short RNA molecules in a template-
522 dependent manner and provides the DNA polymerase with a 3'-hydroxyl group to continue
523 chain elongation (Mendelman, 1995).
524
525 *6.5. Protein export and folding*
526 The SppA protein (NCBI reference sequence WP_001348528), encoded by *sppA*, shares
527 30% aa identity with the *E. coli* S49 signal peptide peptidase A (NCBI conserved domain
528 accession number cd07023). The crystal structure of *E. coli* SppA revealed that in addition to
529 its role in signal peptide hydrolysis, it may also have a role in the quality assurance of
530 periplasmic- and membrane-bound proteins (Kim et al., 2008a).
531 The *dsbA* and *dsbC* genes code for the DsbA and DsbC proteins (NCBI reference
532 sequences WP_000139696 and WP_001259346, respectively) that are members of the
533 disulphide bond formation system (Collet and Bardwell, 2002). DsbA shares 34% aa identity
534 with the thioredoxin domain-containing protein DsbA from *E. coli* (Pfam PF01323 and NCBI
535 conserved domain accession number cd02972). DsbA forms intra-chain disulphide bonds as
536 peptides emerge into the cell's periplasm (Kadokura and Beckwith, 2009), thereby stabilizing
537 the secreted proteins (Collet and Bardwell, 2002). The location of *dsbA* adjacent to *sppA* may
538 indicate that they act in concert.
539 DsbC of A/C plasmids shares 28% aa identity with *E. coli* DsbC and other members of
540 this subfamily of proteins (NCBI conserved domain accession number cd03020). DsbC has
541 been shown to act in the bacterial periplasm to correct non-native disulfide bonds formed by
542 DsbA and to prevent the aggregation of incorrectly folded proteins (Collet and Bardwell,
543 2002). Both DsbA and DsbC could be involved in ensuring the correct folding of components

of the type IV secretion system. However, the location of *dsbC* upstream of *traC* is common to F-type transfer systems and DsbC may be particularly important for folding of proteins encoded in this operon.

6.6 Other genes

An open reading frame of 90 aa located between ORF 17 and 18 (Figure 2A) was previously identified as a HU-like DNA binding protein (Fernandez-Alarcon et al., 2011).

The *yacC* gene encodes a putative exonuclease (NCBI reference sequence WP_000997323) with an RNase T exonuclease domain (Pfam 00929). In *E. coli*, members of the RNase T family are responsible for the end-turnover of tRNA and for processing the 3' end of tRNA precursors (Deutscher and Marlor, 1985).

7. Mobilization of *Salmonella* genomic island 1

Members of the *Salmonella* genomic island 1 (SGI1) family are integrative mobilizable elements that contain various combinations of antibiotic resistance genes in a complex class 1 integron (Hall, 2010, Mulvey et al., 2006). SGI1 in both *Salmonella enterica* and *Proteus mirablis* is almost always found integrated into the bacterial chromosome within the last 18 bp of the *trmE* (originally known as *thdF*) gene. It was discovered that, in the presence of the conjugative helper A/C_2 plasmid R55, SGI1 could be excised from the chromosome and transferred from *S. enterica* donor strains to *S. enterica* and *E. coli* recipient strains that lack SGI1 (Doublet et al., 2005). The transfer frequency was 10^{-5} – 10^{-6} transconjugants per donor.

A subsequent study confirmed that SGI1 could be mobilized by other A/C plasmids including RA1 at frequencies ranging between 1.9 x 10^{-2} to 3.1 x 10^{-7} transconjugants per donor (Douard et al., 2010). SGI1 could not be mobilized by conjugative FI, FII, HI2, I1, L/M, N or P plasmids, indicating that the presence of conjugative transfer functions alone is not sufficient for mobilization of SGI1 and suggesting a specific relationship between the A/C

570 plasmid family and the ability to mobilize SGI1 (Douard et al., 2010). RA1 was not transferred
571 into the transconjugants tested, confirming that SGI1 was mobilized *in trans* by the A/C
572 plasmid rather than being incorporated into the plasmid and then transferred. In a later
573 study, pVCR94 and SGI1 were rarely transferred together, suggesting that each prevents the
574 transfer of the other (Carraro et al., 2014a). SGI1 (Boyd et al., 2001), and the closely related
575 SGI2 (Hamidian et al., 2015), both include genes encoding TraN, TraH and TraG that are most
576 related to the corresponding proteins of A/C plasmids and SXT/R391, and their role in this
577 phenomenon needs to be examined experimentally.
578 Recent work has shown that the A/C transcriptional activator complex AcaCD (see
579 section 4.3) regulates the high frequency excision and mobilization of SGI1, and putative
580 AcaCD-binding motifs were found upstream of the SGI1 genes *xis, rep*, *traN* and *traH/traG*
581 (Carraro et al., 2014a). This may explain the specific relationship between SGI1 and A/C
582 plasmids.
583 The mobilization of SGI1 by A/C plasmids provides another mechanism by which
584 bacteria are able to spread a multiply drug resistant phenotype.
585
586 **8. The resistance island in RA1**
587 The structure of the RA1 resistance island, RI-RA1, is shown in Figure 5. The *tet*(D)
588 tetracycline resistance determinant is flanked by directly-orientated copies of IS*26* in the 5.2
589 kb transposon-like structure Tn*tet*(D). The *sul2* sulphonamide resistance gene and the small
590 mobile element CR2 are within a 4.4 kb segment that appears to be derived from the recently
591 described genomic island GI*sul2* (Nigro and Hall, 2011). RAx (52637 bp), a deletion derivative
592 of RA1 that has lost 91326 bp, has also been sequenced (GenBank accession number
593 FJ705806). Relative to the RAx resistance island, RI-RA1 appears to have suffered a 2366 bp
594 IS*26*-mediated deletion that has removed the *resG* gene and 736 bp of the small mobile
595 element CR2 (Figure 5). In addition, RAx appears to have acquired a 2.9 kb insertion, not

found in the RA1 sequence. This insertion contains the kanamycin and neomycin resistance determinant *aphA1* bounded by 24 bp perfect inverted repeats (IR) and flanked by 9 bp direct repeats (DR).

9. Resistance islands in A/C$_2$ plasmids

A detailed analysis of the structure of insertions in the A/C$_2$ backbone that include antibiotic resistance genes is beyond the scope of this review. However, the location of the insertions that bring the resistance genes into these plasmids can define sub-lineages and serve as powerful epidemiological markers. After a resistance region has been acquired further evolution occurs *in situ*, namely within a scaffold that remains in a single location. Evolution of resistance gene clusters *in situ* has also been described for IncHI1 plasmids (Cain and Hall, 2012) and IncW plasmids (Revilla et al., 2008), and is likely to be far more common than currently acknowledged.

ARI-B is the only resistance island that can be found in both type 1 and type 2 plasmids (Tables 3 and 4) and the *sul2* gene included within it was originally believed to be part of the backbone. However, ARI-B is found in only 35 of the 51 sequenced A/C$_2$ plasmids listed in Tables 1 and 2. As the ARI-B island is always in the same location, it appears to have been acquired only once and has subsequently evolved *in situ* in several ways, often gaining additional antibiotic resistance genes. This occurred early as R55, the oldest A/C plasmid sequenced to date, includes ARI-B. Its various forms (Table 5) are described in section 9.1.

With the exception of one plasmid (GenBank Accession Number CP006050), all sequenced type 1 A/C$_2$ plasmids include a resistance island, ARI-A, in the same position (Figure 2B), indicating that this island was also acquired once (Harmer and Hall, 2014). When this may have occurred is unknown, as no sequences are available for older plasmids of this type. Most of the variation in resistance gene carriage is due to the gain and/or loss of resistance genes carried within ARI-A, and this represents another case of evolution *in situ*. As

described below (section 9.2), the island containing the *bla*$_{CMY-2}$ gene is always in the same location (Figure 2A), again indicating a common ancestor. This island includes a DNA segment associated with ISEcp1. In some plasmids *bla*$_{CMY-2}$ has been duplicated and is associated with both complete and partial copies of ISEcp1, and these structures are described in detail elsewhere (Partridge, 2011, Fernandez-Alarcon et al., 2011).

In contrast to type 1, type 2 A/C$_2$ plasmids include resistance islands in different locations within a 13 kb backbone region in or surrounding the *rhs2* gene (Figure 2B). In the 16 type 2 A/C$_2$ plasmids sequenced at the end of 2014, there are eight different resistance island locations (Figure 2B), only six of which were identified a year earlier (Harmer and Hall, 2014). The resistance gene content of each island is shown in Table 4 and evolution *in situ* is again evident for locations 3 and 4. The structure of a few has been reported in detail (Doublet et al., 2012, Zhang et al., 2013, Drieux et al., 2013). In one case, namely the related plasmids pKEC-dc3, pKEC-a3c and pKEC-39c, an additional insertion carrying the *bla*$_{FOX-1}$ (FOX-1 is an ESBL) and *dfrA14* resistance genes has inserted within orf 29 in Figure 2A.

9.1. ARI-B

The antibiotic resistance island ARI-B includes the sulphonamide resistance gene *sul2* and all or part of the small mobile element CR2. ARI-B plays an important role in multiple antibiotic resistance because, in addition to *sul2*, various configurations of ARI-B that carry additional resistance genes have been observed (Table 5).

The sequence of the backbone uninterrupted by ARI-B (Figure 6) was recently defined (Harmer and Hall, 2014), and further analysis (unpublished observations) has revealed that when ARI-B is present the complete backbone is present only in pYR1, pEA1509, R55 and pCFSAN001921. In three of these plasmids, R55 (isolated in France in 1969), pEA1509 (isolated in France in 2001) and pCFSAN001921 (isolated in the USA in 2011), ARI-B is bounded by the two outer ends of a recently described 15 kb genomic island GI*sul2* (Nigro

and Hall, 2011), but the central region has been lost (Figure 6). It appears therefore that the island was originally formed via the integration of GI*sul2*. ARI-B in R55 has gained a central *floR*-containing segment, presumably via homologous recombination between the two partial CR2s. ARI-B in pCFSAN001921 has gained a 15 kb segment containing the *tetA*(A) tetracycline resistance determinant, but has retained only the first 61 bp of the *int* end of GI*sul2* and the last 2065 bp of the *sul2* end. ARI-B in pIMP-PH114 is a deletion derivative of the island seen in R55. A single IS*26* has replaced 1967 bp of the *int* end of GI*sul2* and 11646 bp of the A/C_2 backbone. pYR1 contains a different ARI-B configuration (Figure 6) with a fragment of GI*sul2* that extends from the *sul2* end to the *ter* end of CR2 defined previously (Yau et al., 2010). However, it has lost 413 bp usually found between the *ter* end of CR2 and *sul2*.

In the remaining ARI-B forms, further events have occurred leading to the structures seen in modern day A/C_2 plasmids. In all cases, the right-hand boundary between GI*sul2* and the A/C_2 backbone is preserved, but the left-hand end of the island (*int* end) has been lost. This is associated with backbone deletions of various sizes (Table 5), mostly mediated by IS*26*.

The most commonly seen configuration of ARI-B (Figure 7A) is associated with a 10984 bp IS*26*-mediated deletion of the backbone. This deletion is found in 25 of the 35 sequenced plasmids containing ARI-B, mostly in type 1 but also in a few type 2 A/C_2 plasmids (Table 5). However, 19 of the type 1 plasmids were isolated in the USA from *E. coli* or *S. enterica* derived from animals, mainly food-producing animals, and may represent a single plasmid spreading. This ARI-B configuration contains the *floR* (florfenicol and chloramphenicol), *strAB* (streptomycin), *sul2* (sulphonamides) and *tet*(A) (tetracycline) resistance determinants. The *strA* and *strB* genes are carried on a 1778 bp segment derived from Tn*5393*, whilst *tet*(A) is carried on a 2401 bp segment derived from RP1 (Figure 7). In 16 of the plasmids, the configuration of the island is identical (Figure 7A), as is the island in pCFSAN007405 which has a longer adjacent deletion of 11039 bp. A further three differ only

674 by inversion of the short segment between the two IS*26* (Figure 7B). Four more have lost this
675 entire segment together with one copy of IS*26* (Figure 7C) or both (Figure 7E), or part of the
676 internal segment (Figure 7D).

677 Two further members of the group with the 10984 bp deletion have gained additional
678 resistance genes (Table 5) associated with mobile genetic elements. ARI-B in pPGO10208 is
679 identical to the configuration in Figure 7A, but has gained a translocatable unit (see (Harmer
680 et al., 2014) for definition) consisting of an IS*26* and the macrolide resistance genes *mel* and
681 *mph* (Figure 7F). ARI-B in IncA/C-LS6 is identical to the configuration in Figure 7C but has
682 gained the amikacin, kanamycin and neomycin resistance gene *aphA6* together with ISPa14, a
683 partial copy of *strB* and a complete copy of *strA* (Figure 7G).

684 Members of an additional group of ARI-B forms contain a 4.4 kb segment sharing
685 98.9% nucleotide identity with the IncN plasmid R46 associated with a 4477 bp backbone
686 deletion (Table 5). The ARI-B in this group have a variable set of resistance genes that can
687 include *aphA1*, *catA1*, *strAB*, *erm*(42), *sul2* and *tet*(D). These forms have been described
688 previously (Kim et al., 2008b) and compared recently (see Figure 1C in Harmer et al. (2015)).
689 The ARI-B in pP91278, which includes *tet*(D) in addition to *sul2*, is associated with a 10407 bp
690 backbone deletion, and has been described previously (Kim et al., 2008b).

691

692 *9.2. ARI-A*

693 The structure of ARI-A in the A/C_2 plasmid pRMH760 was the first to be described in
694 detail (Partridge and Hall, 2003a, Partridge and Hall, 2004). The island has a complex mosaic
695 structure composed of a class 1 integron and multiple transposons included within a larger
696 class II transposon structure that is flanked by a 5 bp duplication of the target (Partridge and
697 Hall, 2003a, Partridge and Hall, 2004). The two outermost 38 bp inverted repeats associated
698 with the Tn*1696 tnp* module and the pDU *mer* module are interrupted by either IS*4321* or

699 IS*5075* (Partridge and Hall, 2003b) (Figure 8). The interruption of the IR effectively "locks"
700 this island in place.
701 Though for the majority of type 1 A/C_2 plasmids the island in this position has been
702 described as "flanked" by IS*4321*/IS*5075* or InsD (e.g. (Wasyl et al., 2014, Sekizuka et al.,
703 2011)), closer examination reveals that in the thirty-four sequenced type 1 A/C_2 plasmids
704 that include ARI-A, it is always in the same location as ARI-A in pRMH760, namely 1711 bp
705 upstream of the *rhs* gene (Figure 2B). Hence, the extremities of the island are highly
706 conserved and the true boundaries are shown in Figure 8. Twenty-seven sequenced plasmids
707 have both ends of ARI-A intact, whilst seven have deletions originating from within the island
708 which have removed part of ARI-A together with either 3478 bp or 6953 bp from the
709 backbone to the right of ARI-A (Table 3). However, the internal structure of the island and the
710 antibiotic and mercuric ion resistance genes present varies considerably (Table 3). Evolution
711 of ARI-A in type 1 A/C_2 plasmids is characterized by the acquisition or loss of additional
712 mobile genetic elements and deletion events associated with elements such as IS*26* (Harmer
713 and Hall, 2014, Fricke et al., 2009, Kim et al., 2008b). In some cases, no antibiotic resistance
714 genes remain (pAM04528, pCVM22513, pCVM21538, pCVM21550, pCVM22425,
715 pCFSAN000934_02, pCFSAN000941_02 and pCFSAN007426).
716
717 **10. Other insertions in A/C_2 plasmids**
718 Insertion sequences have been found in a number of A/C plasmids, for example IS*1294*
719 in pAR060302 and pSCEC2. A large transposon distantly related to Tn*7* was recently
720 identified in some *bla*$_{NDM-1}$ carrying plasmids and named Tn*6170* (Harmer and Hall, 2014). Its
721 location is shown in Figure 2B. Tn*6170* includes an *hsdR* operon that had been identified
722 previously (Carattoli et al., 2012).
723 pYR1 also carries a putative 6.8 kb transposon inserted in an ORF with no known
724 function (orf 91 in Figure 2A). This transposon contains a gene annotated as "*dhfr1*" (Welch et

al., 2007). However, it is not *dfrA1* and its product does not share identity with any known dihydrofolate reductase. Furthermore, there is no experimental evidence to suggest it confers resistance to trimethoprim.

11. Unraveling the evolutionary history of A/C plasmids

A number of earlier studies conducted comparative analyses of sequenced A/C plasmids by performing a direct comparison of the whole plasmid sequence, including the resistance regions (Fricke et al., 2009, Del Castillo et al., 2013, Zhang et al., 2013, Welch et al., 2007, Fernandez-Alarcon et al., 2011). However, the variability in the continually evolving resistance regions, the fact that resistance islands can be in different locations, and the presence of deletions adjacent to some of them leads to complications. Consequently, this approach fails to accurately identify the highly conserved backbone. More recently, meta-analyses have been performed (Meinersmann et al., 2013, Zhou et al., 2012) and these also failed to detect subtle backbone variations, which represent thousands of years of evolution. Analyzing the backbone and the resistance islands separately provides a clearer picture of the evolution of A/C plasmids in general, and resistance islands in particular (Harmer and Hall, 2014). The precise positions of resistance islands within the plasmid backbone also provides important additional information in determining relationships but this factor has rarely been considered to date, even in epidemiological studies where it is critical (see e.g. (Hazen et al., 2014)).

11. Conclusions

The availability of extensive sequence data for many A/C$_2$ plasmids has provided a fascinating insight into their evolution and evolutionary history. However, much remains to be determined for this important plasmid group. Experimental studies on A/C plasmids are few and generally the biological functions they encode have been inferred based on homology

to systems in other well-studied plasmids. Further work addressing the role of the various genes identified is now needed to build a sound foundation for understanding the basic biological processes encoded by these plasmids. Importantly, whether A/C_1 and A/C_2 plasmids are compatible also needs to be re-examined using known members of each group using known members of each group and modern molecular methods. Further examination of the host range and determination of the genes required for entry-exclusion is also warranted.

From an evolutionary perspective, the sequences of older A/C plasmids would be very informative. In particular, genomes for more type 1 A/C_2 plasmids that were isolated prior to the emergence of CMY-2 are needed. Type 1 plasmids from a wider range of sources and bacterial species are also needed to correct the sampling bias that currently exists in this group.

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Table 1. Sequenced type 1 A/C_2 plasmids

Plasmid	Size (bp)	Organism	Source	County	Year[a]	GenBank Accession no.	Reference
pRMH760	170613	*K. pneumoniae*	Human	Australia	1997	KF976462	(Harmer and Hall, 2014)
pR148	165906	*A. hydrophila*	Fish	Thailand	2007	JX141473	(Del Castillo et al., 2013)
pIMP-PH114	151885	*K. pneumoniae*	Human	France	2010	KF250428	(Ho et al., 2013)
pCVM21538	80098	*S. enterica*	Chicken	USA	-	CP009563	(Cao et al., 2012)
p199061_160[b]	161081	*E. coli*	Turkey	USA	1995	HQ023863	(Fernandez-Alarcon et al., 2011)
pUMNK88_161	165073	*E. coli*	Pig	USA	2007	HQ023862	(Fernandez-Alarcon et al., 2011)
pAR060302	166530	*E. coli*	Cow	USA	2002	FJ621588	(Fernandez-Alarcon et al., 2011)
peH4H	148105	*E. coli*	Cow	USA	2002	FJ621586	(Call et al., 2010)
pSN254	176473	*S. enterica*	-	USA	2000	CP000604	(Welch et al., 2007)
pSD_174	173673	*S. enterica*	Cow	USA	-	JF267651	(Han et al., 2012a)
pAM04528	158213	*S. enterica*	Human	USA	1998	FJ621587	(Call et al., 2010)
pSH111_166	165791	*S. enterica*	Cow	USA	2001	JN983043	(Han et al., 2012b)
pSH696_135[c]	135423	*S. enterica*	Turkey	USA	2000	JN983048	(Han et al., 2012b)
pSH163_135[c]	135168	*S. enterica*	Turkey	USA	2002	JN983045	(Han et al., 2012b)
pCVM22425	158195	*S. enterica*	Cow	USA	2003	CP009560	(Cao et al., 2012)
pCVM21550	120340	*S. enterica*	Pig	USA	-	CP009564	(Cao et al., 2012)
pCVM22513	120346	*S. enterica*	Cow	USA	2003	CP009562	(Cao et al., 2012)
pCFSAN000405	190894	*S. enterica*	Turkey	USA	2004	CP009409	-
pCFSAN007425	166432	*S. enterica*	Turkey	USA	2002	CP009411	-
pCFSAN007428	164898	*S. enterica*	Chicken	USA	2006	CP009414	-
pCFSAN007427	175497	*S. enterica*	Turkey	USA	2009	CP009413	-
pCFSAN007426	103575	*S. enterica*	Turkey	USA	2008	CP009412	-
pCFSAN000934_02	158521	*S. enterica*	Dog	USA	2003	CP009567	-
pCFSAN000941_02	118585	*S. enterica*	Horse	USA	2000	CP009570	-
pCFSAN007405	190894	*S. enterica*	Turkey	USA	2004	CP009409	-
IncA/C-LS6	171925	*K. pneumoniae*	Human	Italy	2011	JX442976	(Villa et al., 2013)
pMR0211[d]	178277	*P. stuartii*	Human	Afghanistan	2011	JN687470	(McGann et al., 2012)
pNDM-1_Dok01[d]	195560	*E. coli*	Human	Japan	2009	AP012208	(Sekizuka et al., 2011)
pNDM-KN[d]	162746	*K. pneumoniae*	Human	Kenya	2009	JN157804	(Carattoli et al., 2012)
pNDM10469[d]	137813	*K. pneumoniae*	Human	Canada	2010	JN861072	(Mulvey et al., 2011)
pNDM10505[d]	166744	*E. coli*	Human	Canada	2010	JF503991	(Mulvey et al., 2011)
pNDM102337[d]	165974	*E. coli*	Human	Canada	2008	JF714412	(Mulvey et al., 2011)
pNDM-US[d]	140825	*K. pneumoniae*	Human	USA	2010	CP006661	(Hudson et al., 2014)
pKP1-NDM-1[d]	137538	*K. pneumoniae*	Human	Australia	2010	KF992018	-
pCFSAN001921[e]	221009	*S. enterica*	Chicken	USA	2011	CP006050	(Hoffmann et al., 2013)

[a] Year of isolation. – denotes year not provided.
[b] Named pAPEC1990_61 in GenBank.
[c] Contain complex rearrangements adjacent to ARI-A. Possible assembly issues.
[d] Contains *bla*$_{NDM-1}$ in ARI-A.
[e] Unnamed in GenBank accession number CP006050.

Table 2. Sequenced type 2 A/C_2 plasmids

Plasmid	Size (bp)	Organism	Source	County	Year[a]	GenBank Accession no.	Reference
pIP1202	182913	*Y. pestis*	Human	Madagascar	1995	NC_009141	(Welch et al., 2007)
pYR1[b]	158038	*Y. ruckeri*	Fish	USA	-	CP000602	(Welch et al., 2007)
R55	170810	*K. pneumoniae*	Human	France	1969	JQ010984	(Doublet et al., 2012)
pEA1509	162202	*E. aerogenes*	Human	France	2001	FO203354	(Diene et al., 2013)
pKOX-86d	193725	*K. oxytoca*	Human	USA	2012	CP008790	-
pTC2	180184	*P. stuartii*	Human	Greece	2012	JQ824049	(Drieux et al., 2013)
p1643_10	167779	*S. enterica*	Turkey	Poland	2010	KF056330	(Wasyl et al., 2014)
pSCEC2	135615	*E. coli*	Pig	China	2010	KF152885	(Zhang et al., 2013)
pSRC119-A/C	174068	*S. enterica*	Pig	Australia	2000	KM670336	(Harmer et al., 2015)
pKEC-39c	319976	*E. cloacae*	Environment	USA	2012	CP008824	(Conlan et al., 2014)
pKEC-dc3	268334	*K. pneumoniae*	Human	USA	2012	CP007732	(Conlan et al., 2014)
pKEC-a3c	272297	*C. freundii*	Environment	USA	2012	CP007558	(Conlan et al., 2014)
pVCR94deltaX[c]	120572	*V. cholerae*	Human	Rwanda	1994	KF551948	(Carraro et al., 2014b)
pPG010208	135803	*E. coli*	Cow	Chile	2004	NC_019065	(Fernandez-Alarcon et al., 2011)
pP99-018	150157	*P. damselae*	Fish	Japan	1999	NC_008612	(Kim et al., 2008b)
pP91278	131520	*P. damselae*	Fish	USA	1991	NC_008613	(Kim et al., 2008b)

[a] Year of isolation. – denotes year not provided.
[b] Has *rhs1,* i.e. hybrid type 2/1 plasmid.
[c] A deletion derivative of a larger naturally occurring plasmid.

Table 3. Resistance islands in type 1 A/C_2 plasmids.

Plasmid	Antibiotic resistance genes in ARI-A	ARI-B[a]	ISEcp1-*bla*$_{CMY-2}$[b]
pRMH760	*aadB, aphA1,* *bla*$_{TEM-1}$, *catA1, dfrA10, sul1*	-	-
pCFSAN007428	*aadB, aphA1,* *bla*$_{TEM-1}$, *cmlA1*	+	+
pSH696_135	*aadB,* *bla*$_{TEM-1}$, *cmlA1, sul1*	+	+[c]
pSH163_135	*aadB,* *bla*$_{TEM-1}$, *cmlA1, sul1*	+	+[c]
pSD_174	*aadA1, sul1*	+	+
IncA/C-LS6	*aadA1, arr2, cmlA7, dfrA14, oxa10, qnrA6, sul1*	+	+
pR148	*aadA1, catA2, oxa10, sul1, tet*(A)	-	-
pCFSAN007405	*aadA1, aadB, aphA1,* *bla*$_{TEM-1}$, *cmlA1, sul1*	+	+[c]
pUMNK88_161	*aadA1, aadA2, cmlA1, sul1*	+	+
pCFSAN007425	*aadA2, dfrA12, sul1*	+	+
pSN254	*aac(3)-VIa, aadA2/1, sul1*	+	+[c]
p199061_160	*aac(3)-VIa, aadA2/1, sul1*	+	+[d]
pAR060302	*aac(3)-VIa, aadA2/1, sul1*	+	+
pCFSAN007427	*aac(3)-VIa, aadA2/1, sul1*	+	+
pEH4H	*aac(3)-VIa, aadA2/1, aphA1, sul1*	+	+[c]
pCFSAN000405	*aac(3)-VIa, aadA2/1, aadB, aphA1,* *bla*$_{TEM-1}$, *cmlA1, sul1, tet*(A)	+	+
pNDM-1_Dok01[e, f]	*aadA2, armA,* *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *dfrA12, mel, mph2, sul1*	-	+
pNDM-KN[e, f, g]	*aadA1, arr3,* *bla*$_{NDM-1}$, *ereA3, cmlA7, rmtC, sul1*	-	+
pNDM-US[e, g]	*aacA4, aphA6,* *bla*$_{NDM-1}$, *rmtC, sul1*	-	+
pKP1-NDM-1[e, g]	*aacA4, aphA6,* *bla*$_{NDM-1}$, *rmtC, sul1*	-	+
pNDM10469[e, g]	*aacA4,* *bla*$_{NDM-1}$, *oxa1, rmtC, sul1*	-	+
pNDM10505[e, f, g]	*aacA4, aacC3, aphA6,* *bla*$_{NDM-1}$, *oxa1, rmtC, sul1*	-	+
pNDM102337[e, f, g]	*aacA4, aacC3, aphA6,* *bla*$_{NDM-1}$, *oxa1, rmtC, sul1*	-	+
pMR0211[e, h]	*aacA4, aadA1, aadB, aphA6, armA,* *bla*$_{NDM-1}$, *cmlA1, mel, mph2, oxa10, qnrA1, sul1*	+	+
pIMP-PH114	*aacC4, aphA6,* *bla*$_{DHA-1}$, *bla*$_{IMP-26}$, *catB3, sul1*	+	-
pSH111_166	*dfrA1*	+	+
pAM04528	None[i]	+	+[c]
pCVM22513	None[i]	+	+
pCVM21538	None[i]	+	-
pCVM21550	None[i]	+	+[c]
pCVM22425	None[i]	+	+[c]
pCFSAN000934_02	None[i]	+	+[c]
pCFSAN000941_02	None[i]	+	+[c]
pCFSAN007426	None[i]	+	+
pCFSAN001921	Not present	+	-

[a] Gene content is available in Table 5.
[b] *bla*$_{CMY-2}$ or variants of it.
[c] Posses a second copy of ISEcp1-*bla*$_{CMY-2}$
[d] This strain has a 3759 bp deletion to the left of the *bla*$_{CMY-2}$ island.
[e] Contains *bla*$_{NDM-1}$ in ARI-A.
[f] Contain Tn*6170* 556 bp downstream of *rhs1*.
[g] Have lost 3478 bp from the backbone adjacent to ARI-A.
[h] Has lost 6953 bp from the backbone adjacent to ARI-A.
[i] Contains remnants of ARI-A, though no resistance genes are present.

1144 Table 4. Resistance islands in type 2 plasmids.

Plasmid	RI position[a]	Antibiotic resistance genes in RI	ARI-B[b]
pIP1202	1	*aadA2*, *bla*$_{SHV-1}$, *sul1*, *tet*(D)	+
pYR1	2	*strA*, *strB*, *tet*(B)	+
R55	3	*aadB*, *bla*$_{OXA-21}$, *catA1*, *sul1*	+
pEA1509	3	*aacA4*, *aadA1*, *bla*$_{TEM-24}$, *dfrA1*, *sul1*	+
pKOX-86d	4	*aadA2*, *aadB*, *bla*$_{CARB-2}$, *catB3*, *cmlA1*, *dfrA18*, *mph*(E), *msr*(E), *qnrA1*, *sul1*[c]	-
pTC2	4	*aadA1*, *aadA2*, *aphA1*, *bla*$_{SVH-129}$, *bla*$_{VIM-1}$, *dfrA1*, *dfrA12*, *mph*(A), *sul1*	-
p1643_10	4, 5	*aacA4*, *aadB*, *aphA1*, *bla*$_{OXA-21}$, *strA*, *strB*, *sul1*; *bla*$_{CTX-M-25}$[d]	-
pSCEC2	6	*cfr*	+
pSRC119-A/C	7	*aacC4*, *aadA2*, *hph*, *sul1*	+
pKEC-39c[e]	8	*aacA4*, *aadB*, *bla*$_{KPC-2}$, *bla*$_{SHV-129}$, *dfrA14*, *mph*(E), *msr*(E), *qnrA1*, *sul1*[f]	-
pKEC-dc3[e]	8	*aacA4*, *aadB*, *bla*$_{KPC-2}$, *bla*$_{SHV-129}$, *dfrA14*, *mph*(E), *msr*(E), *sul1*[g]	-
pKEC-a3c[e]	8	*aacA4*, *aadB*, *bla*$_{KPC-2}$, *bla*$_{SHV-129}$, *dfrA14*, *mph*(E), *msr*(E), *qnrA1*, *sul1*[f]	-
pVCR94deltaX	-	-	+
pPGO10208	-	-	+
pP99-018	-	-	+
pP91278	-	-	+

1145
1146 [a] Resistance island other than ARI-B, as shown in Figure 2B.
1147 [b] Gene content listed in Table 5.
1148 [c] Contains two copies of *sul1*.
1149 [d] *bla*$_{CTX-M-25}$ is located within RI 5.
1150 [e] Contain a second 41 kb insertion elsewhere in the backbone, bounded by 6 bp direct repeats, containing β- lactamase
1151 (*bla*$_{FOX-5}$) and trimethoprim (*dfrA14*) resistance determinants, plus putative bleomycin and aminoglycoside
1152 phosphotransferase resistance proteins.
1153 [f] Contains four copies of *sul1*.
1154 [g] Contains three copies of *sul1*.
1155
1156

1157 Table 5. Antibiotic resistance genes in ARI-B
1158

Plasmid	A/C$_2$ type	Antibiotic resistance genes in ARI-B	Variant[a]	Backbone Δ (bp)
R55	2	*floR*, *sul2*	-	-
pYR1	2	*sul2*	-	-
pEA1509	2	*sul2*	-	-
pCFSAN001921	1	*merRTPCA*, *sul2*, *tet*(A)	-	-
pIP1202	2	*aphA1*, *catA1*, *strAB*, *sul2*	-	4477
pP99-018	2	*aphA1*, *catA1*, *sul2*, *tet*(D)	-	4477
pSRC119-A/C	2	*aphA1*, *erm*(42), *sul2*, *tet*(D)	-	4477
pP91278	2	*sul2*, *tet*(D)	-	10407
pSN254	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
P199061_160	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pUMNK88_161	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pAR060302	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pSD_174	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pMR0211	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCFSAN000405	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCFSAN007425	1	*floR*, *strA*, *strB*, *sul2*, tet(A)	A	10984
pCFSAN007428	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCFSAN007427	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	D	10984
pCFSAN007426	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	B	10984
pCFSAN000934_02	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCFSAN000941_02	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pAM04528	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pEH4H	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pSH111_166	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	B	10984
pSH696_135	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	C	10984
pSH163_135	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	C	10984
pCVM21538	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCVM22425	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	B	10984
pCVM21550	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pCVM22513	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	A	10984
pSCEC2	2	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	G	10984
IncA/C-LS6	1	*aphA6*, *floR*, *strA*, *strB*, *sul2*, *tet*(A)	F	10984
pPGO10208	2	*floR*, *mph*(E), *msr*(E), *strA*, *strB*, *sul2*, *tet(A)*	E	10984
pCFSAN007405	1	*floR*, *strA*, *strB*, *sul2*, *tet*(A)	-	11039
pIMP-PH114	1	*floR*, *sul2*	-	11646

1159 [a] As shown in Figure 7.

Figure legends

Fig. 1. Comparisons between A/C_1 and A/C_2 plasmid backbones. The backbones are drawn to scale from GenBank accession numbers JX141473 (pR148) and FJ705807 (RA1) after removing the antibiotic resistance islands and opening the sequence 1139 bp upstream of *repA*. Regions containing genes involved in plasmid replication (*rep*), partitioning (*parAB*), conjugative transfer (*tra*) and the *rhs* gene are indicated by horizontal arrows. Vertical arrows show the location of resistance islands. Regions with shared nucleotide identity are indicated by shading according to the key below.

Fig. 2. Genetic organization of A/C_2 plasmids. **A**. A/C_2 plasmid backbone. The A/C_2 backbone sequence of pR148 (GenBank accession number JX141473) defined in Harmer and Hall (2014) was linearized 1139 bp upstream of *repA* and is drawn to scale. Horizontal arrows indicate the location, size and orientation of ORFs, and every tenth reading frame is numbered below the arrows. The positions of the i1 and i2 insertions found in type 2 A/C_2 plasmids are indicated by vertical arrows, and the two regions of replacement between type 1 and type 2 A/C_2 plasmids, orf1832/orf1847 and *rhs1*/*rhs2,* are indicated by dual names above and below. ORFs coding for hypothetical proteins greater than 100 aa and *acr1* are shown. Genes coding for proteins with a known function are named above and colored according to the key. Locations of resistance islands are indicated by vertical arrows (ARI-A and ARI-B) or below (ISEcp1-*bla*$_{CMY-2}$). **B.** Antibiotic resistance island locations in sequenced type 2 A/C_2 plasmids. Horizontal arrows indicate the direction and extent of the *rhs2*, *yacC, ter* and *kfrA* genes. Horizontal arrows with no name indicate open reading frames with no known function. 0 marks the *rhs* start codon. Numbered vertical arrows indicate the location of the RIs found in various type 2 A/C_2 plasmids (see Table 5 for detail). ARI-A marks the location of the resistance island found in type 1 A/C_2 plasmids.

Fig. 3. A/C_1 and A/C_2 replicons. **A.** Replication region in A/C plasmids. Replication regions are drawn to scale from GenBank accession numbers FJ705807 (A/C_1) and KF976462 (A/C_2). The large open box indicated the extent of the *repA* gene and a horizontal arrow indicates the direction. Small open boxess mark the locations of conserved inverted repeats (IR1 and IR2) and 19 bp iterons. A vertical line indicates the conserved DnaA box. The extent of the fragment containing the origin is indicated above. Bent arrows mark the location of PBRT primers for A/C plasmids. **B.** Iteron sequences in A/C_1 and A/C_2 plasmids. The iterons and consensus sequence for A/C_1 are as described by Llanes et. al. (1994), except that iterons 1 and 10 for A/C_1 were omitted. In the alignment, white lettering with a black background denotes a base that differs from the majority base at that position. The consensus is defined as follows: capital letters represent conserved bases and small letters represent the presence of that nucleotide in at least 10 of the iterons. R denotes a purine base (either A or G) and Y denotes a pyrimidine base (either C or T).

Fig. 4. Comparion of transfer regions in A/C_2 plasmids and SXT. **A.** Region 1, *traI* to *traN* (Bases 43782-71893 in GenBank accession number KF976462 and 45176 to 67147 in GenBank accession number AY055428). **B.** Region 2, *traF* to *mobI* (Bases 159773 to 170613 and 1 to 862 in GenBank accession number KF976762, and 88747 to 99483 and 1 to 4241 in GenBank accession number AY055428). Transfer regions are drawn to scale. Horizontal arrows mark the location, orientation and extent of genes which are named above. Shading indicates shared genes, with the percent aa identity shown.

Fig. 5. Antibiotic resistance islands in RA1 and RAx. The islands are drawn to scale from GenBank accession numbers FJ705807 (bases 113251 to 126345) and FJ705806 (bases 31882 to 50224). Insertion sequences and the small mobile element CR2 are shown as open boxes with IS numbers or names indicated inside. A vertical bar marked “o” indicates the ori

and “t” the ter end of CR2, respectively. Genes and ORFs are shown below the line as named arrows indicating the direction of transcription. Segments derived from GI*sul2* and the extent of Tn*tet*(D) are indicated below. Open flags denote direct repeats. A extent of a deletion in RA1 is indicated and an insertion in RAx are shown above.

Fig. 6. Structure of ARI-B in pEA1509, R55 and pYR1. Surrounding A/C_2 backbone is drawn to scale from GenBank accession number KF976462, and resistance islands are drawn to scale from GenBank accession numbers FO203354 and CP000602.

Fig. 7. Structures of ARI-B associated with the 10984 bp backbone deletion. **A.** The most common ARI-B configuration in sequenced A/C_2 plasmids. Drawn to scale from GenBank accession number FJ621586. **B.** Configuration present in pCFSAN007426, pSH111_166 and pCVM21550. **C.** Configuration present in pSH696_136 and pSH163_135. **D.** Configuration present in pCFSAN007427. Δ denotes a deletion of 282 bp adjacent to the right-hand IS*26*. **E.** Configuration present in pPG010208. **F.** Configuration present in IncA/C-LS6. **G.** Configuration in pSCEC2. Insertion sequences and the small mobile element CR2 are shown as open boxes. “o” and “t” indicate the ori or ter end of CR2, respectively. IS numbers or names are indicated. Genes and ORFs are shown above or below the line as named arrows indicating the direction of transcription. Segments derived from GI*sul2*, RP1 or Tn*5393* are indicated below. Thick vertical lines indicate terminal inverted repeats.

Fig. 8. Boundaries of ARI-A. A/C backbone sequence is denoted by lower case letters. The 5 bp duplication of the target sequence generate upon insertion of the transposon is underlined. The 38-bp inverted repeats or IR_{tnp} and IR_{mer} are boxed. The base in the IR adjacent to which the IS (either IS*4321* or IS*5075*) may insert is marked by vertical arrows.