Characterization of the Complete Nucleotide Sequences of IncA/C₂ Plasmids Carrying In809-Like Integrons from *Enterobacteriaceae* Isolates of Wildlife Origin

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ABSTRACT A total of 18 *Enterobacteriaceae* (17 from gulls and 1 from a clinical sample) collected from Australia, carrying IncA/C plasmids with the IMP-encoding In809-like integrons, were studied. Seven plasmids, being representatives of different origins, plasmid sizes, replicon combinations, and resistance genes, were completely sequenced. Plasmid pEc158, identified in a clinical *Escherichia coli* ST752 isolate, showed extensive similarity to type 2 IncA/C₂ plasmids. pEc158 carried none of the *bla*_{CMY-2}-like region or ARI-B and ARI-A regions, while it contained a hybrid transposon structure. The six remaining plasmids, which were of wildlife origin, were highly similar to each other and probably were fusion derivatives of type 1 and type 2 A/C₂ plasmids. The latter plasmids contained an ARI-B region and hybrid transposon structures. In all plasmids, hybrid transposon structures containing In809-like integrons were inserted 3,434 bp downstream of the *rhs2* start codon. In all cases, the one outermost 38-bp inverted repeat (IR) of the transposon was associated with the Tn1696 *tnp* module, while the other outermost 38-bp IR of the transposon was associated with either a Tn6317-like module or a Tn21 *mer* module. However, the internal structure of the transposon and the resistance genes were different in each plasmid. These findings indicated that, for the specific periods of time and settings, different IncA/C₂ plasmid types carrying In809-like elements circulated among isolates of wildlife and clinical origins. Additionally, they provided the basis for speculations regarding the reshuffling of IncA/C₂ plasmids with In809-like integrons and confirmed the rapid evolution of IncA/C₂ plasmid lineages.

KEYWORDS IMP, metallo- β -lactamases, PMLST, *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*

Plasmids of the IncA/C group are big molecules with a broad host range. These plasmids are commonly identified among agricultural and clinical bacterial isolates in the United States, Europe, and elsewhere. Among resistance genes, the IncA/C plasmids have often been found to disseminate *bla*_{CMY-2}-like cephalosporinase genes (1). However, recent reports describing the epidemic emergence of *bla*_{NDM} metallo- β -lactamase (M β L) genes in *Enterobacteriaceae*, associated with IncA/C₂ plasmids (2), revived interest in this plasmid group. Additionally, other studies have reported the emergence of IncA/C₂ plasmids carrying the *bla*_{IMP} and *bla*_{VIM} M β L genes from clinical

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isolates of Australian and Greek origin (3–5), respectively, further indicating the important role of these plasmids in the dissemination of carbapenemase-encoding genes.

In a previous study from our group, IncA/C plasmids carrying *bla*_{IMP} genes were identified in *Enterobacteriaceae* isolates recovered from cloacal samples that were collected from gull chicks at a nesting colony in New South Wales, Australia (6). These plasmids were mainly associated with the integron In809 (7), whose variable region comprised the *bla*_{IMP-4}, *qacG*, *aacA4*, and *catB3* cassettes. Therefore, in the present study, we characterized IncA/C-type plasmids carrying *bla*_{IMP} genes of representative *Escherichia coli*, *Klebsiella pneumoniae*, and *Citrobacter freundii* isolates from gulls. We also sequenced an IncA/C-type plasmid carrying In809 from a clinical isolate of *E. coli* (3) in order to examine whether the same or different plasmid with In809-like integrons is spreading in bacteria of wildlife and clinical origins.

RESULTS AND DISCUSSION

Enterobacteriaceae with IncA/C plasmids encoding IMP-like MβLs. A total of 18 *Enterobacteriaceae* isolates harboring IncA/C plasmids, which carried the IMP-encoding In809-like integrons, were included in this study. The population structure of IMP-producing isolates studied by multilocus sequence typing (MLST) is shown in Table 1.

All *E. coli* and *C. freundii* isolates carried *bla*_{IMP-4} and *bla*_{IMP-38}, respectively, while *K. pneumoniae* isolates carried either *bla*_{IMP-4} or *bla*_{IMP-26}. Characterization of the *bla*_{IMP} genetic context by PCR mapping and sequencing showed that all strains harbored In809-like integrons. The prevailing integron was the canonical In809, which was identified in 14 *E. coli* (including the isolate of human origin) and one *K. pneumoniae* (Table 1) isolate. *K. pneumoniae* ST1735 contained an In809 derivative with *bla*_{IMP-26} (In1386). Both *C. freundii* isolates had a new integron, In1385 with *bla*_{IMP-38}, differing from In809-like integrons by insertion of a group IIc intron into the *attC* site of the *bla*_{IMP} gene cassette.

IncA/C₂ plasmids carrying In809-like integrons. All IMP-encoding plasmids were transferred by conjugation from clinical strains, although with different transfer frequencies (ranging from 2×10^{-2} to 8×10^{-4} transconjugants per donor cell). Plasmid analysis revealed that the transconjugants harbored *bla*_{IMP}-positive plasmids of different sizes (~220 kb [*n* = 5], ~210 kb [*n* = 2], ~200 kb [*n* = 2], ~180 kb [*n* = 8], and ~160 kb [*n* = 1]) (Table 1).

Replicon typing and sequencing confirmed that all plasmids carrying In809-like integrons were positive for the IncA/C₂ allele, while replicon IncY was identified in two of the above-described plasmids (Table 1). Additionally, plasmid MLST (PMLST) typing assigned all plasmids to ST3, which is the most common group of IncA/C plasmids (8).

Screening for the presence of additional resistance genes showed that five of the transconjugants were positive for the *bla*_{OXA-1} gene (Table 1), while one of the above-described strains was also positive for the presence of *bla*_{DHA-1}. Two of the transconjugants, including that of human origin, were found to carry the *bla*_{TEM-1} gene. Additionally, the *sul2* and *floR* genes, conferring resistance to sulfonamides and florfenicol, were found in all IncA/C₂ plasmids except that of human origin. The *aac(3)-IId* variant was detected in 16 of the plasmids. Two of the latter plasmids, which contained A/C+Y double replicons, were also positive for the *dfrA12* gene. Finally, the plasmid-mediated quinolone resistance gene *qnrA1* was observed in the transconjugants of *E. coli* ST58 and both *C. freundii* isolates.

Structure of IncA/C₂ plasmids carrying In809-like integrons. The complete sequence of In809-like-carrying IncA/C₂ plasmid representatives of different origins, plasmid sizes, replicon combinations, and resistance genes (*n* = 7) was determined. All sequenced plasmids were closely related to each other as well as to other previously described A/C₂ plasmids (2). The backbones of the plasmids were highly conserved (>99% identity) (Fig. 1), which is in agreement with the findings of previous studies documenting that core backbones of IncA/C plasmids are highly syntenic with no genetic rearrangements (9). The IncA/C₂ backbones were composed of regions respon-

TABLE 1 Characteristics of *Enterobacteriaceae* with IncA/C₂ plasmids encoding IMP MβLs^e

Isolate	ST	Size of <i>bla</i> _{IMP} plasmid ^b (kb)	Replicon(s)	Integron with <i>bla</i> _{IMP} genes	β-Lactamase content	Conjugal transfer frequency ^c	PMLST/ cgPMLST ^d	Additional resistance markers	Regions of IncA/C ₂ used for PCR screening					
									<i>atpA</i>	<i>traO</i>	<i>orf39</i>	<i>ald2</i>	<i>merP</i>	<i>xerD</i>
<i>E. coli</i>														
Ec23	58	~200	A/C ₂	In809	IMP-4, OXA-1	2 × 10 ⁻⁴	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>qnrA1</i>	+	+	+	—	—	+
Ec19	167	~180 (188.855)	A/C₂	In809	IMP-4	4 × 10⁻³	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>aac(3)-IId</i>	+	—	+	—	—	+
Ec90	167	~180	A/C ₂	In809	IMP-4	5 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	—	+	+	—	+
Ec93	167	~180	A/C ₂	In809	IMP-4	5 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	—	+	—	—	+
Ec94	167	~180	A/C ₂	In809	IMP-4	4 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	—	+	—	—	+
Ec98	167	~180	A/C ₂	In809	IMP-4	5 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	—	—	+	—	—	+
Ec109	167	~180	A/C ₂	In809	IMP-4	4 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	—	+	—	—	+
Ec31	189	~220	A/C ₂	In809	IMP-4	1 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	—	—	—	+	—	+
Ec78	189	~220 (233.057)	A/C₂	In809	IMP-4	1 × 10⁻³	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>aac(3)-IId</i>	—	—	+	+	—	+
Ec117	189	~220	A/C ₂	In809	IMP-4	8 × 10 ⁻⁴	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	—	+	—	—	+
Ec33	1114	~180	A/C ₂	In809	IMP-4	2 × 10 ⁻³	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	+	+	—	+	+
Ec34	1114	~180	A/C ₂	In809	IMP-4	5 × 10 ⁻⁴	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>aac(3)-IId</i>	+	+	+	—	—	+
Ec9	1178	~220 (228.517)	A/C₂+Y	In809	IMP-4, OXA-1	1 × 10⁻³	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>arr-3</i>, <i>dfrA12</i>, <i>aphA1</i>, <i>aac(3)-IId</i>	+	—	+	+	—	+
Ec158^a	752	~160 (165.169)	A/C₂	In809	IMP-4, TEM-1	2 × 10⁻²	ST3/3.24	<i>dfr</i>-like, <i>aac(3)-IId</i>	+	+	+	—	—	—
<i>K. pneumoniae</i>														
Kp57	1734	~220	A/C ₂ +Y	In809	IMP-4, OXA-1	1 × 10 ⁻⁴	ST3/NA	<i>sul2</i> , <i>floR</i> , <i>dfrA12</i> , <i>aac(3)-IId</i>	+	—	+	+	—	+
Kp55	1735	~210 (215.528)	A/C₂	In1386	IMP-26, OXA-1, DHA-1	1 × 10⁻⁴	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>arr-3</i>, <i>aac(3)-IId</i>	+	+	+	+	+	+
<i>C. freundii</i>														
Cf52	138	~210 (219.342)	A/C₂	In1385	IMP-38, OXA-1, TEM-1	8 × 10⁻⁴	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>arr-3</i>, <i>dfrA27</i>, <i>aadA16</i>, <i>aadA2</i>, <i>aac(3)-IId</i>, <i>qnrA1</i>	+	+	+	+	—	+
Cf53	140	~200 (200.459)	A/C₂	In1385	IMP-38, OXA-1	8 × 10⁻⁴	ST3/3.23	<i>sul2</i>, <i>floR</i>, <i>arr-3</i>, <i>dfrA27</i>, <i>aadA16</i>, <i>qnrA1</i>	+	+	+	+	—	+

^aIMP-4-producing *E. coli* Ec158 was recovered from clinical material.^bSizes of all plasmids were deduced by S1 nuclease assay. The sizes of fully sequenced plasmids are reported in parentheses.^cTransfer frequencies are expressed as transconjugants per donor cell.^dNA, not applicable.^eStrains from which IMP-encoding plasmids were completely sequenced and characterized in this study are shown in boldface.

FIG 1 Comparison of linear maps of the IMP-encoding IncA/C₂ plasmids pEc9, pEc19, pCf52, pCf53, pKp55, pEc78, pEc158, and pIMP-PH114 (7). Plasmid regions are represented by blocks and are classified by function into different groups. Unique sequences in each plasmid were not colored. Homologous segments are indicated by light gray shading, representing $\geq 99\%$ sequence identity.

sible for replication (*repA* gene), conjugative transfer (Tra1 and Tra2 regions), and plasmid maintenance (*higBA* and *parAB* operons and *xerD*- and *kfrA*-like genes).

Analysis of sequenced plasmids by the core gene PMLST (cgPMLST) scheme (8) indicated that all IncA/C₂ plasmids of wildlife origin belonged to a novel cgST, designated cgST3.23, being a single-locus variant (SLV) of cgST3.4. cgST3.23 differed from cgST3.4 by having allele A059-8 instead of A059-2. On the other hand, cgPMLST analysis of the plasmid of human origin indicated that pEc158 lost allele A157. The absence of A157 was denoted the 0 allele (A157-0), and pEc158 was assigned to the novel cgST3.24 (an SLV of cgST3.3). cgST3.23 and cgST3.24 differed by four alleles. In agreement with the conclusions of a previous study (8), these results underlined the higher discrimination power of the cgPMLST scheme than PMLST.

IMP-encoding plasmid pEc158 of human origin. Plasmid pEc158 (165,169 bp), of human origin, appeared to be a derivative of type 2 A/C₂ plasmids, like pIP1202 from *Yersinia pestis* IP275 from Madagascar (10). pEc158 contained the i1 (428 bp between 21956 and 21957 in pRMH760) and i2 (462 bp between 38820 and 38821 in pRMH760) insertions (11), which are usually found only in type 2 A/C₂ plasmids (2). Additionally, it contained the type 2 version of the R2 (*orf1847*) region (2). Also, an additional 7,697-bp segment (nucleotide [nt] 26028 to 33724) was found in the pEc158 backbone, inserted in exactly the same position as that in the type 2 A/C₂ plasmid pIP1202 (10). This segment consisted of a 4,437-bp fragment (nt 26028 to 30464) encoding IncN conjugative transfer proteins (12), an IS26 element, and a gene encoding a resolvase. Insertion of the latter segment probably deleted a 4,477-bp sequence of the IncA/C plasmid backbone (nt 25590 to 30066 in pRMH760 [11]).

Like other type 2 A/C₂ plasmids, pEc158 carried none of the *bla*_{CMY-2}-like, ARI-B, and ARI-A regions. However, it had another resistance island, a hybrid transposon structure inserted into the *rhs2* gene (3,434 bp downstream of the *rhs2* start codon [Fig. 2]). Interestingly, this insertion point has not been previously identified for resistance

FIG 2 Detailed comparison of class II transposon structures found in the IncA/C₂ plasmids pEc9, pEc19, pCf52, pCf53, pKp55, pEc78, pEc158, and pIMP-PH114 (7). Open reading frames (ORFs) are shown as rectangles (arrows within rectangles indicate the direction of transcription). Intact insertion sequences (IS) are represented by arrows, while truncated IS elements appear as rectangles. Resistance genes, IS elements, and transposases are shown in red, yellow, and green, respectively. Orange rectangles indicate integrases. Gray rectangles indicate plasmid scaffold regions; the group II intron is shown in blue. The IncY-like replicon of plasmid pEc9 is indicated as a purple rectangle. The remaining genes are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading.

islands of other type 2 A/C₂ plasmids (2). The resistance island of pEc158 contained In809, encoding IMP-4 (7). The IRI of In809 was located between *resI* and *resII* sites of the Tn1696 module in precisely the same position as In4 in Tn1696. The 3' conserved segment (3'CS) of In809 was truncated 174 bp after the start codon of *orf5*. Immediately downstream of Δ *orf5*, a Tn501-like sequence including the 38-bp inverted repeat (IR) of the transposon, a *chrA*-like gene encoding a chromate ion transporter, IS6100, a macrolide resistance operon [*mph*(A)], and IS26 were found. A similar structure has also been observed in plasmid pLEW517 (13). Adjacent to Tn501-like sequence, a 6,928-bp segment (nt 129155 to 136082), comprising the *aac*(3)-*Ild* resistance gene, three open reading frames (ORFs) similar to *orf39-41* from pCTX-M3 (14), an *ISCFr1* element, and a 1,582-bp fragment of *ISCR1* was found. Downstream of Δ *ISCR1*, there were a putative *dfr*-like gene and a copy of integron 3'CS. Furthermore, a Tn21 fragment consisting of a 452-bp fragment of *tniB* Δ 1, *tniA*, and a truncated mercury resistance operon (*mer*) was found next to the 3'CS, 108 bp downstream of *orf5*, as in In2-like integrons. A Tn2 transposon, encoding TEM-1 β -lactamase, was found next to Δ *merA* and at the bound-

FIG 3 Detailed comparison of ARI-B regions found in the IncA/C₂ plasmids pEc9, pEc19, pCf52, pCf53, pKp55, pEc78, pEc158, and pIMP-PH114 (7). Open reading frames (ORFs) are shown as rectangles (arrows within rectangles indicate the direction of transcription). Intact insertion sequences (IS) are represented by arrows, while truncated IS elements appear as rectangles. Resistance genes and IS elements are shown in red and yellow, respectively. Gray rectangles indicate plasmid scaffold regions; the remaining genes are shown in white. Homologous segments (representing $\geq 99\%$ sequence identity) are indicated by light gray shading.

ary of the plasmid backbone. Insertion of Tn2 probably deleted the remaining part of the *mer* operon and a 5,525-bp segment (nt 131832 to 113356 in pEc-Gr4873 [5]) of the plasmid backbone. Two copies of an IS4321 element, which was shown previously to target the IRs of Tn21-like transposons (15), disrupted the IR of the Tn1696 *tnp* module and the IR of the Tn501-like *chrA* module.

IMP-encoding plasmids of wildlife origin. On the other hand, all In809-like-carrying plasmids (pEc9, pEc19, pCf5, pCf53, pKp55, and pEc78) of wildlife origin were hybrid A/C₂ plasmids. They contained the characteristic type 2 i1 insertion (except plasmid pEc78) and the type 1 versions of R1 and R2 regions. These findings indicated that these plasmids have evolved through recombination between homologous regions in type 1 and type 2 A/C₂ backbones. Apart from the backbone, all plasmids of wildlife origin carried the ARI-B resistance island, while they lacked the *bla*_{CMY-2}-like-containing and ARI-A region. Additionally, they harbored another resistance island, a hybrid transposon structure. Interestingly, these two resistance islands served as hotspots for further plasmid evolution by *in situ* acquisition of different genetic units.

The ARI-B resistance island occurred upstream of Tra1. This island consisted of IS26, an ISCR2 element, and the *floR* and *sul2* genes (Fig. 3), and it differed from the respective region of type 1 A/C₂ plasmids. Plasmids pCf52, pCf53, and pKp55 carried additional genetic units upstream of the ARI-B region. In all three plasmids, an IncN-related fragment (4,447 bp) was found in precisely the same position as that previously described in pEc158. Adjacent to the IncN-related fragment, plasmids pCf52 and pCf53 included a 5,233-bp sequence (nt 30894 to 36126 in pCf52) comprising IS26, an *mphA* operon, and IS6100. Upstream from IS6100, a 4,500-bp sequence (nt 36127 to 40626 in pCf52), exhibiting 99% identity with a contiguous region described in the chromosomes of *K. pneumoniae* isolates, like *K. pneumoniae* ATCC 35657 (GenBank accession no. CP015134), was identified. This sequence, which has not been previously found in plasmids, contained four ORFs and was followed by an ISCR1 element. An array of gene

cassettes similar to the class 1 integron In1086 (GenBank accession no. [KP870112](#)), whose variable region comprised the *aacA4*, *bla*_{OXA-1}, *catB3*, *arr-3*, *dfrA27*, and *aadA16* cassettes, was located upstream of the *ISCR1* element. The 3'CS of In1086 was disrupted by the *ISCR1*. In1086 also lacked the entire 5'CS due to insertion of IS26 bounding the ARI-B region. In plasmid pKp55, an integron array similar to that of In37 from pHS2 (16) was identified next to the ARI-B region. In37 shares the first four cassettes with the In1086 integron, suggesting evolution from a common ancestor. Similar to In1086 in pCf52 and pCf53, In37 lacked the entire 5'CS. The 3'CS of In37 was also truncated by a Tn3-like transposon, which was disrupted by IS26 bounding the IncN-related fragment. Similar to pEc158, a 4,477-bp segment of the plasmid backbone (nt 25590 to 30066 in pRMH760 [11]) was lost from pCf52, pCf53, and pKp55 due to a deletion adjacent to the ARI-B island. However, deletions of different sizes were observed in the plasmid backbones of pEc9, pEc19 (nt 22933 to 30066 in pRMH760), and pEc78 (nt 15717 to 30066 in pMH760) (Fig. 1) next to the ARI-B.

Furthermore, all plasmids of wildlife origin included a hybrid transposon structure, containing In809-like integrons, inserted 3,434 bp downstream of the *rhs2* start codon, as found in pEc158. The internal structure of the transposon and the resistance genes were different in each plasmid (Fig. 2). However, in all cases, the one outermost 38-bp IR of the transposon was associated with the Tn1696 *tnp* module. In three of the plasmids (pEc19, pCf52, and pCf53), the IR of the Tn1696 *tnp* module was disrupted by IS5075, while in the remaining plasmids it was intact. The transposons contained In809-like integrons, located in the Tn1696 module, in the same configuration as that previously described for In4. Similar to In809 in pEc158, the 3'CS of In809-like integrons was disrupted by a Tn501-like sequence that, in almost all cases (except pCf53), was close to a pCTX-M3-like sequence (5,346 bp; nt 136457 to 141802 in pKp55) consisting of the *aac(3)-IId* gene, three ORFs (*orf39* to *orf41*), and an *ISCR1*. The other outermost 38-bp IR of the transposon, which was associated with either a Tn6317-like module (pEc9, pEc19, and pEc78) (17) or a Tn21 module (pKp55, pCf52, and pCf53) (18), was always interrupted by IS4321. The Tn6317-like module was composed of a 702-bp sequence that included a gene encoding a DNA invertase and the IR (IR_{inv}) of the transposon, while the Tn21 module consisted of *tniBΔ1*, *tniA*, the *mer* operon, and the IR (IR_{mer}) of the transposon. Of note is that both the IR_{inv} of Tn6317-like and the IR_{mer} of Tn21 were found 1,711 bp upstream of an intact copy of the *rhs1* gene (type 1 version of the R1 region). This finding indicated that a 5,145-bp segment (nt 115126 to 120270 in pKp55) of the plasmid backbone was duplicated due to homologous recombination events.

In the internal structure of the transposon, each of the plasmids harbored unique features. These features are described below.

Plasmids pEc9, pEc19, pEc78, and pKp55 included a 28,063-bp segment (nt 130007 to 158069 in pEc19) harboring coding sequences for a sulfate transporter, an alcohol dehydrogenase, and ABC transporters. Parts of this segment have been previously found in the IncHI2 plasmid pIMP4-SEM1 (encoding IMP-4 MβL) from *Salmonella enterica* Typhimurium, isolated from a companion animal (19), and plasmid pKrDSM16656L from *Kosakonia radicincitans*, isolated from a plant (20), suggesting that this segment is involved in the survival of the microbe under environmental conditions.

Plasmid pEc78 carried an additional 53,169-bp sequence (nt 149967 to 203135) harboring coding sequences for proteins implicated in heat resistance, additional transporters, and proteins with unknown function. This sequence also contained copies of IS5708, IS26, IS4321, IS5075, and ISKpn26, as well as genes encoding putative transposases and resolvases. Fragments of this sequence have been previously characterized from isolates of diverse geographical and host origins, like *S. enterica* Senftenberg 775W (GenBank accession no. [CP016838](#)), *E. coli* S43 (GenBank accession no. [CP010237](#)), *C. freundii* B38 (GenBank accession no. [CP016763](#)), and *E. coli* G3/10 (GenBank accession no. [KM107842](#)).

In the transposon structure of plasmid pEc9, a 40,966-bp sequence (nt 134440 to 175405) punctuated by a variety of mobile elements (IS26 [$n = 8$], IS4321, IS4, IS629, and truncated copies of IS*Sen4* and IS10) was identified. Two of the copies of IS26 bracketed the latter sequence. Transposition of this sequence within the transposon structure was indicated by the presence of target site duplications of 8 bp (TTCTGTGC) at the boundaries of the two copies of IS26. An integron array similar to In37 (16) was found next to a Tn3-like segment that was disrupted by one of the outermost IS26 elements. Similar to In37 in pKp55, the integron array lacked the entire 5'CS due to insertion of another copy of IS26. A second integron that is similar to In-194-A from pNL194 (12), whose variable region comprised *dfra12*, *orfF*, and *aadA2* cassettes, was located upstream of In37. In-194-A lacked the entire 3'CS due to insertion of an *aphA1*-carrying Tn4352-like composite transposon into the coding sequence of *aadA2*. Upstream from the Tn4352-like composite transposon, a 9,854-bp fragment (nt 147909 to 157762) sharing extensive similarity with a contiguous region carried by pMRSN346647_113.1 (100% coverage; 99% identity) (GenBank accession no. [CP018207](#)) was found. This fragment was followed by an IncY-like sequence (nt 160789 to 169572), including the *repA* gene and *parAB* operon, being 99% similar to those found in the IncY plasmid pHYEC7-mcr1 (GenBank accession no. [KX518745](#)). An *aac(3)-Ild* resistance gene was identified downstream of the IncY-like sequence and next to the second outermost IS26.

In plasmids pCf52 and pCf53, a 6,961-bp sequence (nt 178207 to 184167 in pCf52) comprised of an ISCR1 element, the *qnrA1* resistance gene, *ampR*, and a copy of integron 3'CS bounded the Tn21 *mer* module (108 bp downstream of *tniBΔ1*). However, a similar sequence, differing by the presence of *bla*_{DHA-1} instead of *qnrA1*, was also found in plasmid pKp55 in exactly the same position. Upstream of ISCR1, plasmids pCf52 and pCf53 contained a 4,500-bp segment (nt 172498 to 176997 in pCf52) originating from the *K. pneumoniae* chromosome. This segment was bracketed by IS6100 and a second copy of the ISCR1 element.

Furthermore, plasmid pCf52 contained a *bla*_{TEM-1} resistance gene next to IS*Cfr1*. Additionally, an integron that is similar to In127 from *Aeromonas* sp. strain MM.1.10 (21), in which *aadA2* was the sole gene cassette, was identified into the transposon structure of pCf52. The 3'CS of In127 was disrupted by one of the copies of ISCR1.

Prevalence of a hybrid IncA/C₂-type plasmid, encoding IMP-like MβLs, in Enterobacteriaceae isolates from gulls. In order to further examine which of the characterized plasmids was the most prevalent among the isolates of our collection, all IMP-encoding transconjugants were PCR screened for the presence of seven regions being characteristic of the IncA/C₂ plasmids sequenced during this study. PCR results showed that all transconjugants were positive for the presence of the *xerD* gene (also positive for *floR* and *sul2* genes present in the ARI-B region) (Table 1), indicating that pEc158 was not represented in isolates of our collection. Additionally, most of the transconjugants were negative for *traO* and *merP* genes (also negative for the IncY-like *repA* gene) but positive for the *atpA* region, suggesting that IMP-producing isolates of wildlife origin harbored IncA/C₂ plasmids that are close relatives of pEc9. The coding region for alcohol dehydrogenase (*ald2*) was found only in eight of the transconjugants. However, PCR profiles of nonsequenced isolates were not the same as the respective profiles from the isolates used for Illumina sequencing. These results indicate that IMP-encoding IncA/C₂ plasmids exhibited a high diversity, which could be explained by further evolution of the plasmids through rearrangements facilitated by ISs.

Concluding remarks. To our knowledge, this is the first report of complete nucleotide sequences of IncA/C₂ plasmids carrying In809-like integrons with carbapenemase genes recovered from wildlife. Of note, plasmid analysis did not justify the hypothesis that the same type of IncA/C₂ plasmids carrying In809-like elements circulate among isolates of wildlife and clinical origins. However, this conclusion is limited by the fact that isolates of wildlife origin were from 2012, while the isolate of human origin was recovered 10 years earlier. Despite these limitations, sequencing revealed that the

plasmid backbone of pEc158 of *E. coli* ST752, recovered from clinical material of a patient treated in the University Hospital of Melbourne (Australia), showed extensive similarity to type 2 IncA/C₂ plasmids (2), like pIP1202 (10). This finding is in discordance with the results of a previous study that characterized the complete nucleotide sequence of pIMP-PH114, a type 1 IncA/C₂ plasmid encoding IMP-4 from a *K. pneumoniae* isolate recovered from a patient who was hospitalized in the Philippines (7). Considering also the different insertion sites of the resistance island in pEc158 and of ARI-A in pMP-PH144, it is clear that the In809-carrying regions appeared in the two distinct IncA/C₂ lineages by two different acquisition events. On the other hand, the IMP-like-encoding plasmids of wildlife origin were very similar to each other and probably were fusion derivatives of type 1 and type 2 A/C₂ plasmids that contained class II transposon structures carrying In809-like integrons. The internal structure of the transposons was different in each plasmid, indicating that they have further evolved via *in situ* gain and/or loss of transposons, insertion sequences, and resistance genes. Of note, plasmids pEc9, pEc19, and pEc78 carried large segments with no resistance genes but encoding several transporters, suggesting an involvement of these regions in the environmental survival of the microbes.

These findings, in addition to previous reports from Australia (3, 4, 6), revealed that IncA/C₂ plasmids encoding IMP-like MβLs have played a major role in both human and animal societies, although with different dynamics. The broad-range characteristic of IncA/C plasmids, the rapid evolution of these molecules, and their role in the dissemination of multiple resistance genes, including *bla*_{CMY}, *bla*_{NDM}, *bla*_{VIM}, and *bla*_{IMP}, underscore the increasing clinical importance of this plasmid family, as well as the spreading potential of large multidrug resistance segments through reshuffling of enterobacterial plasmids by homologous recombination.

MATERIALS AND METHODS

Bacterial strains. Thirteen *E. coli*, two *K. pneumoniae*, and two *C. freundii* strains from cloacal samples collected from gull chicks on Five Islands, Australia (6), which were previously identified to harbor IncA/C plasmids carrying the integron In809, were studied. Furthermore, an *E. coli* isolate (Ec158) cultured in 2002 from a patient treated in a hospital in Melbourne (Australia) (8) was also included in this study for comparative purposes.

Typing. All isolates were typed by multilocus sequence typing (MLST) (22–24). The databases at <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>, <http://bigsdbs.web.pasteur.fr/klebsiella>, and <http://pubmlst.org/cfreundii> were used to assign STs.

Integron analysis. Variable regions of class 1 integrons with *bla*_{IMP}-like genes were amplified in two parts, from the 5' conserved segment (5'CS) to *bla*_{IMP} and from *bla*_{IMP} to the 3' conserved segment (3'CS) (25). Whole-gene arrays were sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). The Integrall integron database (<http://integrall.bio.ua.pt>) (26) was used to analyze and assign integron sequences.

Plasmid analysis. Conjugal transfer of *bla*_{IMP}-like genes from all wild-type strains into a plasmid-free rifampin- and sodium azide-resistant *E. coli* MT102 laboratory strain was carried out at exponential phase using a filter-mating method (27). Transconjugants were selected on Luria-Bertani agar plates supplemented with rifampin (25 μg/ml), sodium azide (100 μg/ml), and cefotaxime (2 μg/ml). The frequency of plasmid transfer was calculated as the proportion of transconjugants per donor cell.

To define the genetic units of the *bla*_{IMP}-like genes, the plasmid contents of donor strains and their transconjugants were analyzed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease (Promega, Madison, WI, USA) (28). Following PFGE, the DNA was transferred to a BrightStar-Plus positively charged nylon membrane (Applied Biosystems, Foster City, CA) and hybridized with digoxigenin-labeled *bla*_{IMP}-like probes.

Plasmid incompatibility (Inc) groups were determined by the PCR-based replicon typing (PBRT) method (29), using total DNA from transconjugants.

PMLST of IncA/C replicons was carried out on *repA*, *parA*, *parB*, and *053* genes as described previously (8). Sequence types (STs) were assigned at <https://pubmlst.org/>.

Detection of resistance determinants. All transconjugants were screened for the presence of various *bla* genes, including *bla*_{TEM-1}, *bla*_{OXA-1}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{CMY-2}, and *bla*_{DHA}, and of plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*, and *qepA*). Additionally, transconjugants were also examined for the presence of genes encoding resistance to aminoglycosides [*aac(3)-IId*, *aadA1*, *aadA2*, *aadA5*, and *strA-strB*], chloramphenicol (*catA1*, *cmIA*, and *floR*), sulfonamides (*sul1*, *sul2*, and *sul3*), tetracyclines [*tet(A)* and *tet(B)*], and trimethoprim (*dfrA1*, *dfrA12*, and *dfrA17*). Primers and conditions for PCR amplification were used as described previously (27).

Based on the results from Illumina sequencing (see below), six PCRs targeting characteristic regions of all IncA/C plasmids sequenced during this study were designed. The selected regions included the following: (i) an ORF of the plasmid backbone, encoding an ATPase (*atpA*; absent from pEc78), (ii) the

IncN-like *traO* gene, (iii) *orf39* from pCTX-M3, (iv) the coding sequence for alcohol dehydrogenase (*ald2*), (v) *merP* of the Tn21 *mer* module, and (vi) the *xerD* gene of the plasmid backbone that was found in all plasmids except pEc158. All transconjugants were screened for the presence of the regions described above by the use of specific primers (see Table S1 in the supplemental material).

Plasmid sequencing and bioinformatics. Seven plasmids were selected for complete sequencing. Among these, six were selected as representatives of all different plasmid sizes, replicons, and resistance determinants (observed as mentioned above). Additionally, plasmid pEc158, of human origin, was selected for complete sequencing.

Plasmid DNAs from *E. coli* transconjugants were extracted using a HiPure plasmid midiprep kit (Invitrogen, Carlsbad, CA). Plasmid DNA libraries were prepared by the NexteraXT kit and sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Initial paired-end reads were quality trimmed using the Trimmomatic tool, v0.32 (30). For assembly of the plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096), using the BWA-MEM v0.7.12 algorithm (31) to filter out the chromosomal DNA. All of the unmapped paired-end reads then were assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v3.6.0 (32). The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the ORF finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve (version 2.3.1) program (33).

Additionally, all sequenced plasmids were further analyzed by a cgPMLST scheme using the database hosted at the University of Queensland (8).

Accession number(s). The nucleotide sequences of the pEc9, pEc19, pCf52, pCf53, pKp55, pEc78, and pEc158 plasmids have been deposited in GenBank under accession numbers KY887590, KY887591, KY887592, KY887593, KY887594, KY887595, and KY887596, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01093-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We have no conflicts of interest to declare.

REFERENCES

- Carattoli A, Miriagou V, Bertini A, Loli A, Colino C, Villa L, Whichard JM, Rossolini GM. 2006. Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerg Infect Dis* 12:1145–1148. <https://doi.org/10.3201/eid1207.051555>.
- Harmer CJ, Hall RM. 2015. The A to Z of A/C plasmids. *Plasmid* 80:63–82. <https://doi.org/10.1016/j.plasmid.2015.04.003>.
- Espedido BA, Partridge SR, Iredell JR. 2008. *bla*_{IMP-4} in different genetic contexts in *Enterobacteriaceae* isolates from Australia. *Antimicrob Agents Chemother* 52:2984–2987. <https://doi.org/10.1128/AAC.01634-07>.
- Peirano G, Lascols C, Hackel M, Hoban DJ, Pitout JD. 2014. Molecular epidemiology of *Enterobacteriaceae* that produce VIMs and IMPs from the SMART surveillance program. *Diagn Microbiol Infect Dis* 78:277–281. <https://doi.org/10.1016/j.diagmicrobio.2013.11.024>.
- Papagiannitsis CC, Dolejska M, Izdebski R, Giakkoupi P, Skálová A, Chudějová K, Dobiasova H, Vatopoulos AC, Derde LP, Bonten MJ, Gniadkowski M, Hrabák J. 2016. Characterisation of IncA/C₂ plasmids carrying an Inc416-like integron with the *bla*_{VIM-19} gene from *Klebsiella pneumoniae* ST383 of Greek origin. *Int J Antimicrob Agents* 47:158–162. <https://doi.org/10.1016/j.ijantimicag.2015.12.001>.
- Dolejska M, Masarikova M, Dobiasova H, Jamborova I, Karpiskova R, Havlicek M, Carlile N, Priddel D, Cizek A, Literak I. 2016. High prevalence of *Salmonella* and IMP-4-producing *Enterobacteriaceae* in the silver gull on Five Islands, Australia. *J Antimicrob Chemother* 71:63–70. <https://doi.org/10.1093/jac/dkv306>.
- Ho PL, Lo WU, Chan J, Cheung YY, Chow KH, Yam WC, Lin CH, Que TL. 2014. pIMP-PH114 carrying *bla*_{IMP-4} in a *Klebsiella pneumoniae* strain is closely related to other multidrug-resistant IncA/C₂ plasmids. *Curr Microbiol* 68:227–232. <https://doi.org/10.1007/s00284-013-0471-x>.
- Hancock SJ, Phan MD, Peters KM, Forde BM, Chong TM, Yin WF, Chan KG, Paterson DL, Walsh TR, Beatson SA, Schembri MA. 2017. Identification of IncA/C plasmid replication and maintenance genes and development of a plasmid multilocus sequence typing scheme. *Antimicrob Agents Chemother* 61:e01740–e01816.
- Fricke WF, Welch EJ, McDermott PF, Mammel MK, LeClerc JE, White DG, Cebula TA, Ravel J. 2009. Comparative genomics of the IncA/C multidrug resistance plasmid family. *J Bacteriol* 191:4750–4757. <https://doi.org/10.1128/JB.00189-09>.
- Welch TJ, Fricke WF, McDermott PF, White DG, Rosso ML, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, Leclerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J. 2007. Multiple

- antimicrobial resistance in plague: an emerging public health risk. *PLoS One* 2:e309. <https://doi.org/10.1371/journal.pone.0000309>.
11. Harmer CJ, Hall RM. 2014. pRMH760, a precursor of A/C2 plasmids carrying *bla_{CMY}* and *bla_{NDM}* genes. *Microb Drug Resist* 20:416–423. <https://doi.org/10.1089/mdr.2014.0012>.
 12. Miriagou V, Papagiannitsis CC, Kotsakis SD, Loli A, Tzelepi E, Legakis NJ, Tzouvelekis LS. 2010. Sequence of pNL194, a 79.3-kilobase IncN plasmid carrying the *bla_{VIM-1}* metallo- β -lactamase gene in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 54:4497–4502. <https://doi.org/10.1128/AAC.00665-10>.
 13. Williams LE, Detter C, Barry K, Lapidus A, Summers AO. 2006. Facile recovery of individual high-molecular-weight, low-copy-number natural plasmids for genomic sequencing. *Appl Environ Microbiol* 72:4899–4906. <https://doi.org/10.1128/AEM.00354-06>.
 14. Golebiewski M, Kern-Zdanowicz I, Zienkiewicz M, Adamczyk M, Zylinska J, Baraniak A, Gniadkowski M, Bardowski J, Ceglowski P. 2007. Complete nucleotide sequence of the pCTX-M3 plasmid and its involvement in spread of the extended-spectrum beta-lactamase gene *bla_{CTX-M-3}*. *Antimicrob Agents Chemother* 51:3789–3795. <https://doi.org/10.1128/AAC.00457-07>.
 15. Partridge SR, Hall RM. 2003. The IS1111 family members IS4321 and IS5075 have subterminal inverted repeats of Tn21 family transposons. *J Bacteriol* 185:6371–6384. <https://doi.org/10.1128/JB.185.21.6371-6384.2003>.
 16. Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. *Antimicrob Agents Chemother* 47:2242–2248. <https://doi.org/10.1128/AAC.47.7.2242-2248.2003>.
 17. Sun F, Zhou D, Sun Q, Luo W, Tong Y, Zhang D, Wang Q, Feng W, Chen W, Fan Y, Xia P. 2016. Genetic characterization of two fully sequenced multi-drug resistant plasmids pP10164-2 and pP10164-3 from *Leclercia adecarboxylata*. *Sci Rep* 6:33982. <https://doi.org/10.1038/srep33982>.
 18. Liebert CA, Hall RM, Summers AO. 1999. Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* 63:507–522.
 19. Abraham S, O'Dea M, Trott DJ, Abraham RJ, Hughes D, Pang S, McKew G, Cheong EY, Merlino J, Saputra S, Malik R, Gottlieb T. 2016. Isolation and plasmid characterization of carbapenemase (IMP-4) producing *Salmonella enterica* Typhimurium from cats. *Sci Rep* 6:35527. <https://doi.org/10.1038/srep35527>.
 20. Witzel K, Gwinn-Giglio M, Nadendla S, Shefchek K, Ruppel S. 2012. Genome sequence of *Enterobacter radicincitans* DSM16656(T), a plant growth-promoting endophyte. *J Bacteriol* 194:5469. <https://doi.org/10.1128/JB.01193-12>.
 21. Moura A, Henriques I, Ribeiro R, Correia A. 2007. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *J Antimicrob Chemother* 60:1243–1250. <https://doi.org/10.1093/jac/dkm340>.
 22. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 60:1136–1151. <https://doi.org/10.1111/j.1365-2958.2006.05172.x>.
 23. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 43:4178–4182. <https://doi.org/10.1128/JCM.43.8.4178-4182.2005>.
 24. Bai L, Xia S, Lan R, Liu L, Ye C, Wang Y, Jin D, Cui Z, Jing H, Xiong Y, Bai X, Sun H, Zhang J, Wang L, Xu J. 2012. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One* 7:e33054. <https://doi.org/10.1371/journal.pone.0033054>.
 25. Papagiannitsis CC, Studentova V, Ruzicka F, Tejkalova R, Hrabak J. 2013. Molecular characterization of metallo- β -lactamase-producing *Pseudomonas aeruginosa* in a Czech hospital (2009–2011). *J Med Microbiol* 62:945–947. <https://doi.org/10.1099/jmm.0.056119-0>.
 26. Moura A, Soares M, Pereira C, Leitão N, Henriques I, Correia A. 2009. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* 25:1096–1098. <https://doi.org/10.1093/bioinformatics/btp105>.
 27. Dobiasova H, Dolejska M. 2016. Prevalence and diversity of IncX plasmids carrying fluoroquinolone and β -lactam resistance genes in *Escherichia coli* originating from diverse sources and geographical areas. *J Antimicrob Chemother* 71:2118–2124. <https://doi.org/10.1093/jac/dkw144>.
 28. Barton BM, Harding GP, Zuccarelli AJ. 1995. A general method for detecting and sizing large plasmids. *Anal Biochem* 226:235–240. <https://doi.org/10.1006/abio.1995.1220>.
 29. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 63:219–228. <https://doi.org/10.1016/j.mimet.2005.03.018>.
 30. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
 31. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:1303.3997 [q-bio.GN]*.
 32. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Pribelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comp Biol* 19:455–477. <https://doi.org/10.1089/cmb.2012.0021>.
 33. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. <https://doi.org/10.1371/journal.pone.0011147>.