



Original Research Article

Don't overlook the little guy: An evaluation of the frequency of small plasmids co-conjugating with larger carbapenemase gene containing plasmids

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ABSTRACT

As the spread of antimicrobial resistance (AMR) genes becomes an increasing global threat, improved understanding of mobile genetic elements which contribute to the spread of antimicrobial resistance genes, becomes more critical. We created transconjugants from the mating of three chromosomally isogenic *Klebsiella pneumoniae* carbapenemase (*bla*_{KPC}) positive *Citrobacter freundii* isolates with a laboratory strain of *Escherichia coli* and evaluated the movement of small cryptic plasmids (SCPs), p3223 and p1916, when larger *bla*_{KPC}-plasmids were transferred. In all of the 143 transconjugants, multiple plasmids, both large and small, transferred with each mating. When two *bla*_{KPC}-plasmids were present in the host, frequently (87%; 98/113) both would be transferred during mating. p3223 is found in a wide range of bacterial hosts that harbor AMR genes; p1916 has been identified in only a limited number of publicly available sequences to date. From our evaluation, there is still much to learn about SCPs, and the high rate of co-transfer of multiple plasmids from real-world carbapenemase-producing Enterobacteriales.

1. Introduction

Carbapenemase-producing Enterobacteriales (CPE) are considered an urgent threat to modern medicine because of increased mortality in infected patients due to the lack of therapeutic options and their rapid emergence globally over the last decade (Center of Disease Control, 2013). Carbapenemase genes in Enterobacteriales most often reside on mobile genetic elements (MGE), such as plasmids, which can be easily shared across many species (Sheppard et al., 2016a). *Klebsiella pneumoniae* carbapenemase (KPC, encoded by *bla*_{KPC}) is an Ambler Class A serine carbapenemase, first isolated in the United States in 1996 (Yigit et al., 2001), and now accounts for the largest proportion of global CPE infections (Barria-Loaiza et al., 2016; Partridge et al., 2015; Paul et al., 2015; Pulcrano et al., 2016; van Duin and Doi, 2017). *bla*_{KPC} is located on transposon Tn4401, which is about 10 kb in size. Tn4401 is capable

of mobilization at a high frequency (Cuzon et al., 2011), and is almost always located on a plasmid which increases the likelihood of spread of *bla*_{KPC}.

After the introduction of KPC-Enterobacteriales (KPCE) to our institution in 2007, and in the context of on-going transmission, the screening of high-risk patients for asymptomatic carriage of KPCE was implemented in 2009 to try and reduce colonization and infection rates (Mathers et al., 2014). Unlike many institutions, which at the time described clonal transmission of KPC-producing *K. pneumoniae*, we witnessed sustained multispecies transmission of KPCE (Sheppard et al., 2016a). In 2013, there was recognition that wastewater premise plumbing was colonized with KPCE and we began environmental surveillance and mitigation strategies (Mathers et al., 2018). Hospital wastewater premise plumbing is increasingly recognized as a reservoir for CPE (Decraene et al., 2018; Kizny Gordon, 2014), and an ideal niche

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for the horizontal gene transfer of antimicrobial resistance (AMR) genes (Conlan et al., 2014).

For plasmids to be considered conjugative, they need to encode the necessary machinery. Conjugation apparatus includes a type IV secretion system (T4SS) to form the mating channel, and a mobility (MOB) module, which includes an origin of transfer (*oriT*), a relaxase, and a type IV coupling protein (Cabezón et al., 2015). Traditionally, for a plasmid to be characterized as mobile, it only needs an *oriT* and a relaxase, as it can hijack T4SS systems encoded by other MGEs within the same bacterial cell (Smillie et al., 2010). Until recently, if a plasmid has neither, it was considered non-transmissible. It is now understood that non-transmissible plasmids can undergo relaxase-*in trans*, where a relaxase from another plasmid in the same cell can be utilized to mobilize the non-transmissible plasmid (Moran and Hall, 2018; Ramsay and Firth, 2017). In most cases, the non-transmissible plasmid must have a highly similar *oriT*, called a mimic *oriT*, for this to work (O'Brien et al., 2015). This was shown to not be an absolute requirement if there was a relaxosome accessory factor (RAF) that could alleviate the need for a close *oriT* match for mobilization to occur (Moran and Hall, 2017). Non-transmissible plasmids have also been theorized to propagate *via* conjugation (Clark and Warren, 1979), or by transduction (Smillie et al., 2010).

Plasmids impose a cost on their host organism, so their long-term existence is deemed the plasmid paradox (Harrison and Brockhurst, 2012) as the beneficial genes should be incorporated into the chromosome or the costly plasmid should not propagate and persist (Baltrus, 2013). Plasmid persistence can occur by multiple mechanisms such as host cell adaptations and addiction systems (Bustamante and Iredell, 2017; Loftie-Eaton et al., 2017). Small antimicrobial resistance (AMR) plasmids that co-infect hosts with large AMR plasmids have increased stability when the larger plasmids are present (San Millán et al., 2014). Plasmids that do not carry any AMR genes, nor any other genetic information that is advantageous to the host, but are maintained due to a high copy number can be deemed small, abundant cryptic plasmids (SCPs) (Burian et al., 1997). The experimental data on SCPs remains limited, and generally, the incidence of co-transfer of SCPs is likely underreported as it may easily be overlooked in experimental evaluations if there are no identified genes of interest on the SCPs.

The application of next generation sequencing techniques to KPCE from our institution revealed that the majority of isolates contained multiple plasmids in addition to *bla*_{KPC}-plasmids (Sheppard et al., 2016a). Although little directed effort has been focused on the mobility of non-resistance plasmids from “real-world” highly drug-resistant Gram-negative bacteria, previous work to understand plasmid populations has demonstrated that co-existence and interactions amongst plasmids in a single bacterial cell is common and that these interactions play an important role in plasmid stability and mobilization (Christiansen et al., 1973; Laufs and Kleimann, 1978; San Millán et al., 2014). Co-transfer of plasmids was proposed to be a non-independent event that is limited primarily by the plasmid with the lowest conjugation efficiency (Gama et al., 2017). Thus, once conjugation is initiated, multiple conjugative plasmids from the same cell can transfer jointly. Other work has shown that strains carrying multiple plasmids may be more fit than strains carrying single plasmids (Silva et al., 2011), which can help explain why multiple plasmids might persist in a host over generations. Although conjugative plasmids do not make up the majority of plasmid types (Smillie et al., 2010) many laboratory studies regarding the interaction or transmission of multiple plasmids have been focused solely on conjugative plasmids. Small plasmids are often non-conjugative and rely on a high copy number to avoid segregational loss (Summers, 1998); they frequently depend on other plasmids to facilitate their horizontal transfer (Ramsay et al., 2017).

To better understand the conjugation frequency of small and large plasmids from “real world” CPE isolates, we used *bla*_{KPC}-positive *Citrobacter freundii* collected from patients and the wastewater

environment at our institution. *C. freundii* is a recognized nosocomial pathogen (Samonis et al., 2009) and is the fourth most common species to carry *bla*_{KPC} at our institution (Sheppard et al., 2016a). We describe the frequency and patterns of transfer of two SCPs commonly seen in *C. freundii* with larger plasmids carrying *bla*_{KPC}. Improving our understanding of the dissemination of MGEs and AMR genes in patients and wastewater niches is of importance in developing future approaches to reducing their emergence and spread in the healthcare environment.

2. Materials and methods

2.1. DNA extraction, whole genome sequencing, assembly and annotation

DNA was extracted utilizing a commercial kit (QuickGene DNA Tissue Kit S, Fujifilm, Japan) as previously described (Stoesser et al., 2013) and sequenced by both long read (Pacific Biosciences, CA) and short read (HiSeq 2000, Illumina, San Diego, CA) sequencing technologies. A hybrid, complete assembly was generated for each isolate using methods previously described (Sheppard et al., 2016b). Assemblies were annotated using Serial Cloner 2.6.

2.2. Conjugation

Single colonies of J53^{rif} *Escherichia coli* and *bla*_{KPC}-positive *C. freundii* were grown at 37 °C overnight in Luria broth (LB) without and with meropenem (3.3 µg/mL) respectively. Overnight cultures were added to a sterile nitrocellulose membrane atop a sheep's blood agar plate (Thermo Scientific Remel, San Diego CA) at a 1:2 donor to recipient ratio and incubated at 37 °C for 8 h. The filter was aseptically removed from the agar plate with forceps and placed in 5 mL of Phosphate Buffer Solution (PBS) and then vortexed to dislodge the bacteria. 200 µL of the liquid suspension was plated on meropenem (1 µg/mL) and rifampicin (600 µg/mL or 250 µg/mL) LB plates. Single colonies were selected and a biochemical Indole test (Becton, Dickinson, and Co, Sparks, MD) that can differentiate between *C. freundii* and *E. coli* was done to ensure that all transconjugant colonies were *E. coli* and not *C. freundii*.

2.3. Real-time PCR to assess plasmid presence in transconjugants

Based on the hybrid assemblies, primers were designed (Eurofins Genomics, Louisville, KY) to target stable regions of each study plasmid that were unique when compared with other plasmids in the host cell (Table 1). The PCR master mix was made using PowerUp SYBR Green Master Mix (Applied Biosystems, Austin TX) according to the manufacturer's directions for a 20 µL reaction. Each bacterial colony underwent a crude boil prep extraction (95 °C for 10 min) (Mathers et al., 2011) and 2 µL of the supernatant was added as the template. The PCR assay was run on the BioRad CFX96 Real Time PCR System using the following parameters: 50 °C for 2 min, 95 °C for 2 min, and cycling 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s for 40 cycles with a melt curve step (65–95 °C, 0.5 increments for 5 s). Each transconjugant was then tested for the presence/absence of all potential plasmids individually by PCR.

2.4. Southern gel electrophoresis

Selected isolates were grown overnight in a shaking culture at 37 °C in 50 mL LB with a 1 µg/mL meropenem concentration and then processed using the Qiagen CompactPrep Plasmid Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was then quantified (Nanodrop, ThermoFisher Scientific).

All samples were loaded on a 0.7% 0.5× TBE gel and run at 70 V overnight at 4 °C. Purified *bla*_{KPC} PCR product, was added for the last 10 min at 100 V to use as a positive control. The gel was then processed, blotted, labelled, hybridized, and underwent detection using the

Table 1
PCR primers.

Primer name	Sequence 5'–3'	Target	CAV1321	CAV1741	CAV1857
1916F	CATCCTGGGAGTAAACCATG	Hypothetical protein	p1916	p1916	p1916
1916R	ATCTATCCACCCGAGCATATAGG				
3223F	GCTGCCTTTTATCTGCTTTCCC	Multidrug transporter	p3223	p3223	p3223
3223R	GCTAACCAACCAACAGCTAG				
KPC_244F	GACGATTGGAGGACACTACTGA	Tellurium resistance protein	pKPC_CAV132-244	–	pCAV1857_208
KPC_244R	CATTACTGACTTCACGAGCCAG				
CcdA F	CGTCATCTGTAGCATTCAGC	Post segregational antitoxin CcdA	pKPC_CAV1321-45	pKPC_CAV1741	pKPC_CAV1857-43
CcdA R	GATAAGCCCGAGTATGGACATG				
KPC_105F	AAGTAATCGCAACATCCGCATT	Hypothetical protein	–		pKPC_CAV1857-85
KPC_105R	ATGGCTGTAATTATGACGACG				
KPC_F	GATTGGCTAAAGGAAACACGA	<i>bla_{KPC}</i>	<i>bla_{KPC}</i>	<i>bla_{KPC}</i>	<i>bla_{KPC}</i>
KPC_R	GCTGTGCTTGTCTCCTTGTTA				

Amersham ECL Direct Nucleic Acid Labelling and Detection Systems (GE Healthcare, United Kingdom) according to the manufacturer's protocol. The same primers that were described in Table 1 were used to run PCR on the parent strain, and the PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified PCR product was used to create probes for the blotting.

2.5. NCBI query

Using nucleotide BLASTn, both the sequences for p3223 and p1916 were queried in the NCBI database using > 99% identity and across > 80% coverage as thresholds for a match.

2.6. Alignment of plasmid sequences

Comparative analysis between plasmids carried by CAV1321, CAV1741 and CAV1857 was performed using progressive MAUVE (Darling et al., 2004) with a match seed weight of 15 and a minimum LCBscore of 30,000.

2.7. In silico tools used for characterizing isolates

All plasmid sequences were imported into the web based program, PlasmidFinder (Carattoli et al., 2014) 2.0, for *in silico* plasmid typing. The plasmids were typed using the Enterobacteriaceae database, with minimum thresholds of 80% identity and 60% coverage. As recommended, small plasmids (< 20 kb) were typed with greater stringency at > 80% identity and > 96% coverage (Carattoli et al., 2014).

The putative *oriT*s for the target plasmids were identified *in silico* using the web based tool *oriT*finder (Li et al., 2018) and named from the *oriT*DB. All putative *oriT* sequences identified were then compared using ClustalW (<https://www.genome.jp/tools-bin/clustalw>).

Typing of multiple loci for the *C. freundii* strains was performed using the web tool Multi-Locus Sequence Typing (MLST) v2.0 (<https://cge.cbs.dtu.dk/services/MLST-2.0/>) (Larsen et al., 2012).

2.8. SNV distance analysis to determine chromosomal relatedness

Illumina paired-end reads for CAV1321, CAV1741 and CAV1857 were mapped against the chromosome sequence of CAV1321 (accession: CP011612.1) using BWA MEM (Li and Durbin, 2009) and single nucleotide variants (SNVs) were called using BCFTools v1.7 mpileup (Li, 2011). A core SNV multifasta alignment was generated using snippy-core (<https://github.com/tseemann/snippy>). Phage regions on the CAV1321 chromosome were identified by PHASTER (<http://phaster.ca/>), and removed from the alignment. A Maximum likelihood tree was constructed using IQ-TREE (Nguyen et al., 2015), incorporating a reversible polymorphism-aware phylogenetic model (Schrempf et al., 2016), and joint ancestral state reconstruction

algorithm (Pupko et al., 2000). The in-house scripts can be accessed from GitHub repositories Bushwalk, Purge, Germie and PotPlant at <https://github.com/alexwailan>.

2.9. Estimating genomic resemblances between plasmids

The genomic resemblances between circularized plasmids from the 3 *C. freundii* isolates CAV1321, CAV1741 and CAV1321 was estimated using the MinHash algorithm implemented in MASH (Ondov et al., 2016).

2.10. Data availability

Complete genome assemblies have been deposited in the GenBank database under BioProject PRJNA246471.

3. Results

3.1. Strain and plasmid characteristics

271 *bla_{KPC}* positive *C. freundii* (90 from patients and 181 from the environment) were identified and sequenced (Illumina) between 2007 and 2017 and some were also selected for subsequent long-read sequencing (PacBio) to generate closed assemblies. Three *C. freundii* isolates with closed assemblies were selected for conjugation experiments because they had highly related chromosomes (< 100 SNVs) and harbored the two same SCPs, but varied with their remaining plasmid content and organization (Fig. 1 and Supplement Table S1). All 3 strains are sequence type (ST)22; they had no apparent direct epidemiologic link. The 3 *C. freundii* strains were as follows; CAV1321 (patient isolate November 2010), CAV1741 (patient isolate October 2012), and CAV1857 (environmental intensive care room sink isolate December 2013). Combined the three isolates carried 22 total circularized structures (Fig. 1). It should be noted that there appears to be a high amount of recombination between plasmids and thus traditional typing methods targeting plasmid backbone regions do not provide enough resolution to describe the variability here. All three strains harbor identical SCP structures, namely p3223 (3223 bp; accession no. NZ_CP011652.1) and p1916 (1916 bp; accession no. NZ_CP011651.1), which have not undergone recombination. p3223 is a mobilizable plasmid that encodes for a *MobC* gene, replication initiation protein, DNA polymerase, and a small multidrug resistance (SMR) transporter (Fig. 2). p1916 is annotated almost entirely of hypothetical proteins besides a TM2 domain-containing protein. Due to the lack of an identified relaxase, it could be considered traditionally non-transmissible (Smillie et al., 2010). Each of the SCPs has a putative *oriT* according to *in silico* analysis. p3223 has *oriT*_pH205 and p1916 has *oriT*_pNPO1. pCAV1741_KPC and pKPC_CAV1857-85 also have a recognizable putative *oriT* region (*oriT*_pCTXM360), while the other *bla_{KPC}* plasmids in this study do not have a known *oriT* that has been previously described.

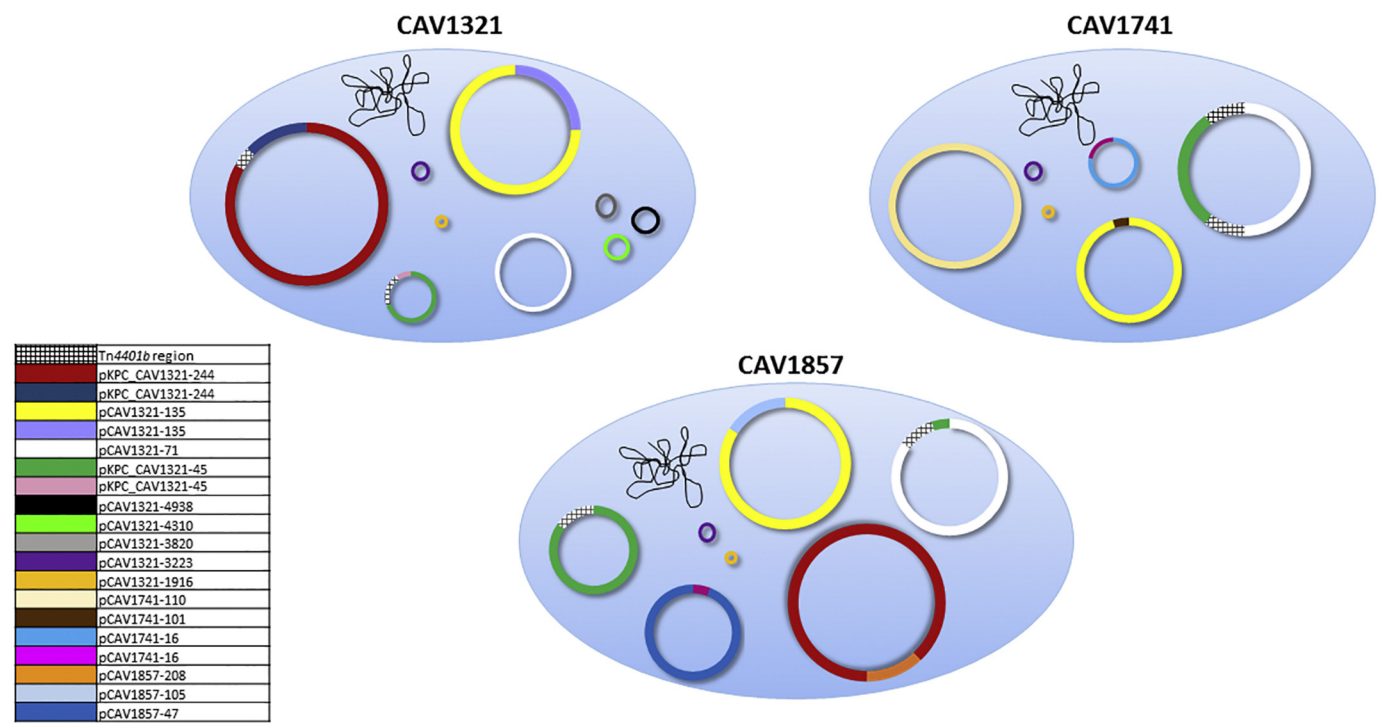


Fig. 1. Plasmid Fragment Map There is a wide amount of genetic variability amongst the plasmids within the 3 *Citrobacter freundii* isolates. Common colors illustrate areas of homology amongst plasmid fragments of each isolate. Fragments are initially colored using CAV1321 plasmid fragments as a reference, followed by CAV1741 then CAV1857.

Isolate CAV1321 harbors nine plasmid structures, two of which contain *bla*_{KPC}, namely pKPC_CAV1321-45 (accession no. [CP011608.1](#)) and pKPC_CAV1321-244 (accession no. [CP011611.1](#)) (Fig. 1). Isolate CAV1741 harbors six plasmid structures and only one of those plasmid carries *bla*_{KPC}, namely pKPC_CAV1741 (accession no. [CP011656.1](#)). Isolate CAV1857 harbors seven plasmid structures, with pKPC_CAV1857-85 (accession no. [CP037737.1](#)) and pKPC_CAV1857-43 (accession no. [CP037739.1](#)) both carrying *bla*_{KPC}. Both pKPC_CAV1321-45 and pKPC_CAV1857-43 are homologous to the plasmid pKPC_UVA01 (accession no. [NZ_CP017937.1](#)) which was our original index case plasmid from 2007 (Sheppard et al., 2016b). Amongst the three strains,

there are both homologous regions of the plasmids that are shared, and some plasmid regions that are unique to one parent strain (Fig. 3 and Supplementary Table S1).

3.2. Conjugation frequency of p3223 and p1916

A total of 143 *bla*_{KPC}-PCR positive transconjugants (59 J53/CAV1321, 30 J53/CAV1741, and 54 J53/CAV1857) were generated from three separate mating experiments which were done multiple times to gather an adequate number of transconjugants for analysis and to minimize pseudoreplication effects. The transconjugants underwent

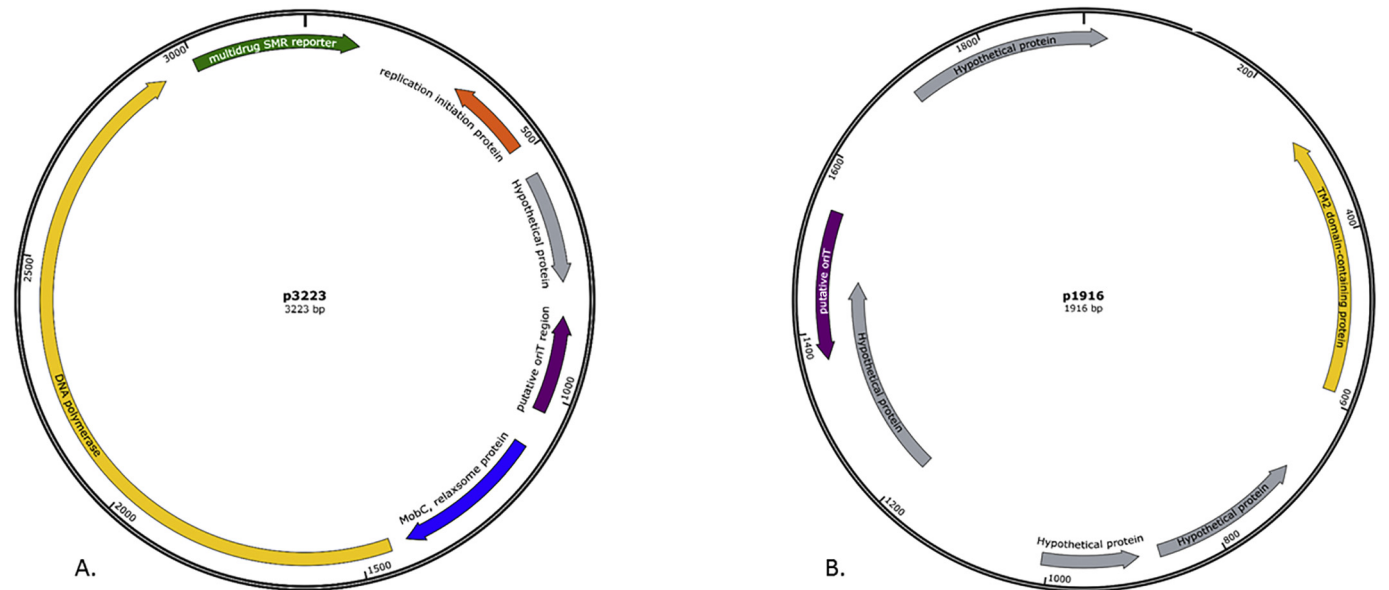


Fig. 2. Plasmid Annotation of p3223 and p1916. (A) Plasmid annotation for p3223 (B) Plasmid annotation for p1916.

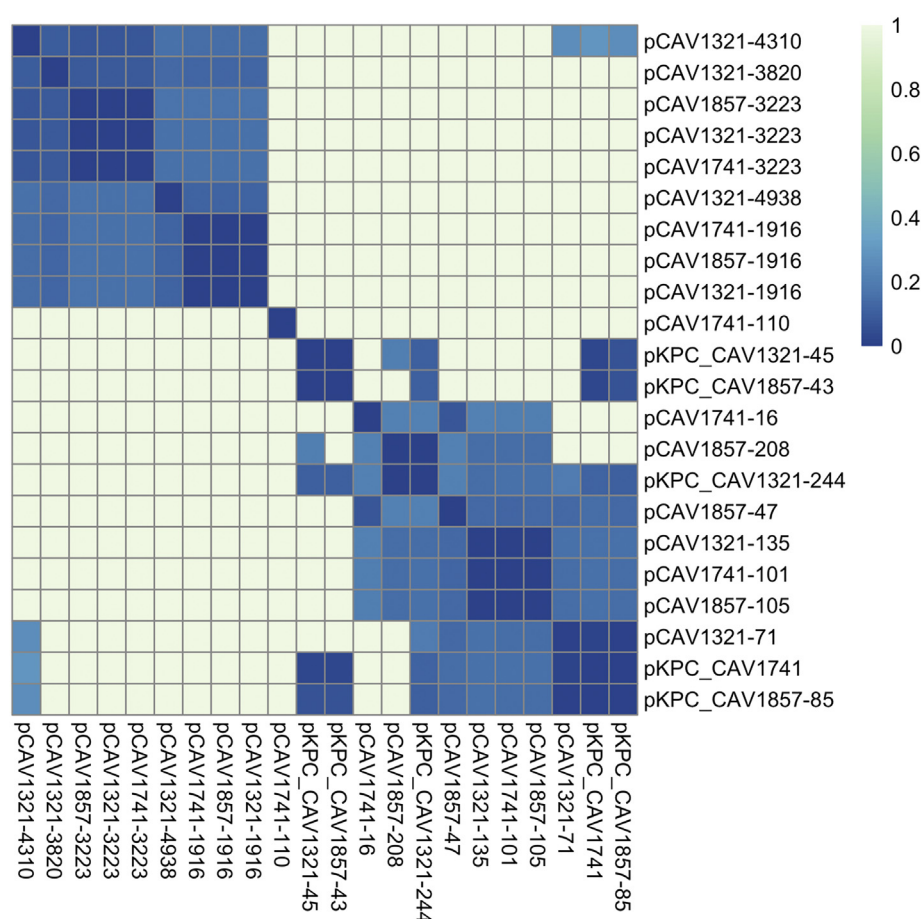


Fig. 3. Pairwise Differences Between the Plasmids MASH was used to identify the genomic distances between all plasmids in the three *C. freundii* strains. The distances range from 0 (identical) to 1 (highly unrelated).

Table 2
Target plasmids.

Plasmid name	NCBI accession No.	Size (bp)	Parent strain
pCAV1321_3223 (p3223)	NZ_CP011652.1	3223	CAV1321, CAV1741, & CAV1857
pCAV1321_1916 (p1916)	NZ_CP011651.1	1916	CAV1321, CAV1741, & CAV1857
pKPC_CAV1321-244	NZ_CP011611.1	243,709	CAV1321
pKPC_CAV1321-45	NZ_CP011608.1	44,846	CAV1321
pKPC_CAV1741	NZ_CP011656.1	129,196	CAV1741
pKPC_CAV1857-43	CP037739	43,621	CAV1857
pKPC_CAV1857-85	CP037737	85,575	CAV1857

PCR for the presence of regions of p3223, p1916, and parent plasmid backbones containing *bla*_{KPC} (Table 2). Eighty-three percent (118/143) of the transconjugants were PCR-positive for all targeted areas from all targeted plasmids indicating that both small plasmids, p3223 and p1916, transferred to the recipient cell along with plasmids carrying *bla*_{KPC}. Based on PCR targeting the backbone, the co-conjugation transfer of both p3223 and plasmids carrying *bla*_{KPC} was 92% (132/143) and 89% (127/143) for *bla*_{KPC} and p1916.

3.3. Plasmid movement when parent strain has multiple *bla*_{KPC}-plasmids

Isolates CAV1321 and CAV1857 each have two separate *bla*_{KPC}-plasmids. In the transconjugants created from those parent strains, both SCPs and both *bla*_{KPC}-plasmids had co-mobilized in 84% (95/113) cases. The other 18 transconjugants can be broken down into the follow categories: only one *bla*_{KPC}-plasmid transferred ($n = 7$), neither *bla*_{KPC}-

plasmid backbone transferred, but *bla*_{KPC} was detected ($n = 8$), or both *bla*_{KPC}-plasmids co-mobilized but p1916 did not ($n = 3$).

3.4. Transposition

An interesting phenomenon occurred in 14% (8/59) of the J53/CAV1321 transconjugants, where *bla*_{KPC} was detected in the transconjugants but the PCR primer target locations on the plasmid backbones observed to originally carry *bla*_{KPC} in the donor were not found. To help us understand this phenomenon, a second area of each plasmid was also targeted. However, all results for PCR of another region of the original *bla*_{KPC}-plasmids were also negative. Southern blotting was performed to investigate if the Tn4401 transposon mobilized onto another plasmid in the donor cell prior to conjugation. A southern blot of all eight transconjugants was probed for *bla*_{KPC} as well as the aforementioned plasmid area targets for pCAV1321-71 and all five small plasmids in CAV1321. The results showed various plasmids sizes from the transconjugants consistent with a transposition event occurring *in vitro*. We were able to get increased resolution on the transposon movement in 88% (7/8) of the transconjugants in question. The plasmids where the *bla*_{KPC} probe was co-localizing with on the southern blot were pCAV1321-71, pCAV1321-4938, and pCAV1321-3820.

3.5. NCBI query

In order to provide a global context for the prevalence of p3223 and p1916, the sequences were queried against the NCBI database using BLASTn. As of October 2018, there were 20 strains of eight species; *K. pneumoniae* (9), *C. freundii* (2), *Serratia marcescens* (2), *Enterobacter*

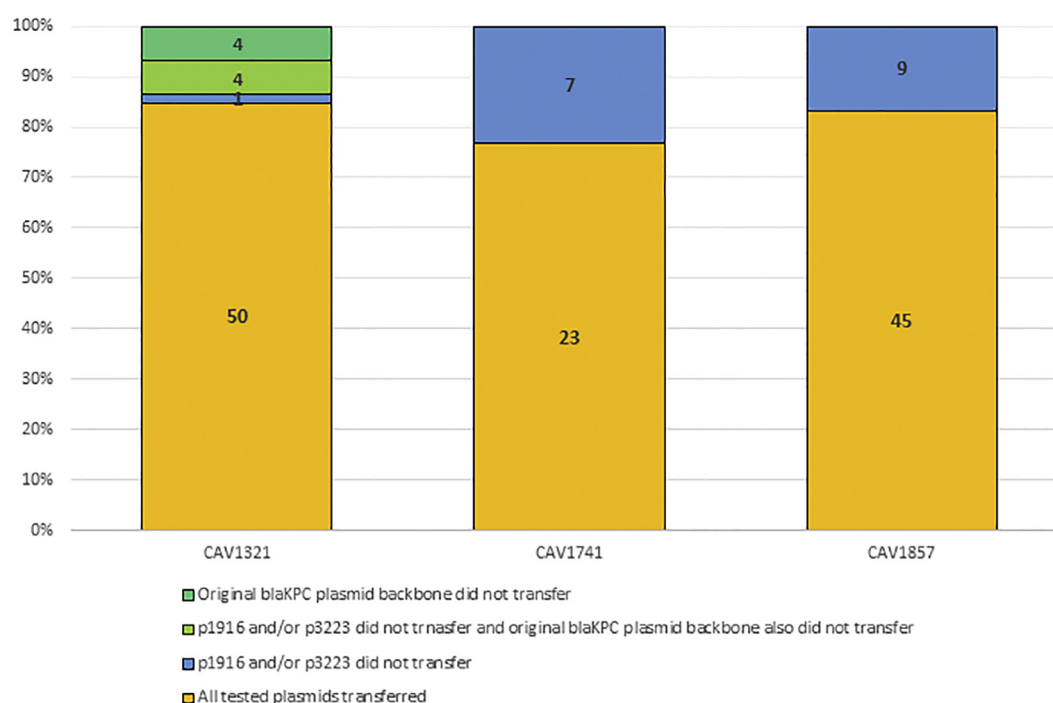


Fig. 4. Plasmid Movement in the Transconjugants This illustrates the distribution of plasmid transfer as determined by PCR in each set of transconjugants. In the majority of the transconjugants, all targeted plasmids (both SCPs and the *bla*_{KPC}-plasmids) transfer.

cloacae (2), *E. coli* (2), *Salmonella enterica* Serovar Typhimurium (1), *Raoultella ornithinolytica* (1) and *Enterobacter hormaechei* (1) that had a match to p3223. All of the matches were in isolates harboring other AMR genes. Specifically, 95% (19/20) of the hits were associated with isolates also carrying plasmids encoding beta-lactamases including *bla*_{CMY-2}, *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA}, *bla*_{CTX-M-15}, and *bla*_{TEM}. Thirty-five percent (7/20) were alignments with published isolates from our own collection.

The BLASTn query for the p1916 sequence had four hits - two of which were isolates from our institution and represented in this study, namely CAV1321 and CAV1741. The other two matches were to an *E. cloacae* (accession no. [CP030349.1](#)) and an *E. coli* (accession no. [KF992024.1](#)) sequence. All of these isolates also carried beta-lactamase genes: *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA}, *bla*_{VIM}, and *bla*_{TEM} on other plasmids.

3.6. In silico plasmid typing

Both p1916 and p3223 were typed as Col440I (96% identity, 100% coverage and 83% identity, 99% coverage respectively). Both pKPC_CAV1321-45 and pKPC_CAV1857-43 typed as a repA_CP013325 (previously non-typable). pKPC_CAV1741 also typed as repA_CP013325 as well as IncM at 100% and 99.4% identity and 100% coverage respectively. pKPC_CAV1857-105 also typed as IncM. The final *bla*_{KPC} plasmid, pKPC_CAV1321-244, typed as RepA_CP011611.

4. Discussion

Plasmids are diverse MGEs that can be readily shuttled between host strains through horizontal gene transfer; however, much of the focus has been on plasmids which carry identifiable genes which alter host function (i.e. antimicrobial resistance or virulence). The co-transfer and persistence of SCPs in Enterobacteriales has yet to be fully explained and remains largely overlooked. It was recently recognized that SCPs may not require their own unique relaxase and can transfer by utilizing another relaxase if the *oriT* demonstrates enough similarity or has a RAF that can help accommodate the differences in *oriT* (Moran and Hall, 2018). The *oriT*s of the SCPs (*oriT*_{pNPO1} and *oriT*_{pH205}) and

the *bla*_{KPC} plasmids pCAV1741_KPC and pKPC_CAV1857-85 (*oriT*_{pCTXM360}) are not homologous, therefore the traditional relaxase *in trans* method does not explain the mobilization of p1916. As previously mentioned, pKPC_CAV1321-45 and pKPC_CAV1857-43 are homologous to pKPC_UVA01, all which have no recognizable putative *oriT* or relaxase. pKPC_UVA01 has been shown to be transmissible but has lower conjugation efficiency *in vitro* than that observed epidemiologically (Hardiman et al., 2016). In our study, we have demonstrated very high rates of co-transfer of almost all targeted plasmids in “real-world” isolates of *C. freundii* with a diverse plasmid content including unique *bla*_{KPC}-plasmids, albeit under laboratory conditions.

Small plasmids can impose a fitness cost to the host that is similar to the high cost of a large plasmid (San Millan et al., 2009). If a plasmid imposes a high cost to the host, does not provide any benefit, and is without an addiction system, it should not persist. Since neither p3223 nor p1916 carry any AMR genes, identifiable addiction systems or any other obvious genes of benefit to the host bacterium and theoretically cannot mobilize on their own, their continued existence across numerous species and strains is hard to understand. They were not shown to be essential for *bla*_{KPC} plasmid conjugation in our experiments as they were not found in every transconjugant, but they did co-transfer with high frequency. We speculate that these SCPs frequently transfer with conjugative plasmids carrying AMR genes because they potentially could have a helper role, but this would require further testing outside the scope of this initial evaluation.

During *in vitro* conjugation of *bla*_{KPC}-plasmids from 3 *C. freundii* isolates with a J53-*E. coli* recipient, the larger *bla*_{KPC}-plasmids never transferred alone. Even when multiple large plasmids carried the same resistance gene, both large plasmids would transfer to the recipient the majority of the time (87%). Although this phenomenon has been previously recognized (Ramsay and Firth, 2017) it is not thought to occur so frequently largely limited by the idea that each plasmid contains a unique *oriT* and relaxase (Zechner et al., 2017). It has been hypothesized that coexisting AMR plasmids in a cell can produce positive epistatic effects on their host, thus minimizing the costs of carrying multiple plasmids (San Millan et al., 2014). This is concerning as it increases the ability of AMR plasmids to persist in the absence of

selective pressure (San Millan et al., 2014). Consistent with this, we demonstrate that multiple plasmids (of varying size and diversity) transferring during conjugation were the norm rather than the exception. Ultimately, we saw a high rate of transfer of SCPs as well as multiple large plasmids with redundant resistance (Fig. 4).

The amount of genetic diversity within the mobile elements ($n = 22$ plasmids) across three chromosomally similar strains from a single institution all with the same AMR gene is striking. There has not been widespread transmission of *bla*_{KPC}-*C. freundii* (Jimenez et al., 2017) but local outbreaks have been identified. We hypothesize that all of these isolates were locally acquired as this strain has been identified longitudinally in both patients and premise plumbing. Postulating about the evolution of this strain at our institution, three possibilities are: (1) the *C. freundii* strain acquired a single *bla*_{KPC} plasmid ancestor with subsequent genetic rearrangement, (2) there were multiple occasions where the *bla*_{KPC} was acquired, (3) the strain acquired multiple plasmids in a single transfer. Prior to our *in vitro* work described here, the first and second explanation may have seemed more likely, but based on our findings, the third explanation is also plausible. It is most likely a combination of genetic rearrangement events and uptake of multiple plasmids that explain the evolution of this strain. Such variability in plasmid content within the same strain over time has not been well characterized previously but we propose it can occur with high frequency. Additional work characterizing the mechanisms of co-transfer and persistence would be important to understanding horizontal gene transfer in a non-laboratory setting.

Another important finding was the relative high frequency with which *in vitro* transposition of a characterized transposon with a clinically relevant carbapenemase gene potentially occurred (Cuzon et al., 2011). It seemed to occur in 5% (8/143) of the transconjugants, but only when CAV1321 was the parent strain. CAV1321 is less susceptible to rifampicin than the other *C. freundii* strains used and thus the conjugation had to be done under a greater concentration of rifampicin (600 µg/mL vs 250 µg/mL). Increased antibiotic pressure could be the cause of why visible transposition events occurred in only the J53/CAV1321 transconjugants. Overall the frequency of transposition was likely underestimated because our experimental assay would not have picked up additional transposition events within the *bla*_{KPC} plasmids.

There are several shortcomings to the manuscript. First, we have only evaluated conjugation from a single strain and species. This was done intentionally in part to control the contribution of the host bacterium and focus solely on the plasmid DNA of clinical and environmental “real-world” strains. In addition, the SCPs were shown to encode a significant number of hypothetical proteins which could perform beneficial functions, but were not evaluated in our study. Also, though the BLASTn query in NCBI only returned a finite amount of hits, this number may actually be underrepresenting the abundance of p3223, and other potential important small plasmids, in the environment. As more sequence data are uploaded to public repositories we may see greater evidence of the presence of SCPs. Of note, with Pacific Biosciences SMRT sequencing, the library preparation frequently size selects for larger DNA fragments to optimize sequencing, thus potentially excluding small plasmid structures from the sequencing process which can only be identified with a hybrid assembly (George et al., 2017).

We have observed p1916 and p3223 being shared across many species of Gammaproteobacteria in both our isolate collection and NCBI's global collection. p3223 has found its way into multiple species carrying *bla*_{KPC} demonstrating that the finding of multiple plasmid transfer of SCP with large AMR plasmids can occur *in vivo* as well. We propose further evaluation needs to be done on the potential beneficial/synergistic relationship between large KPC plasmids and the SCPs during horizontal gene transfer as this could be crucial in the dissemination of antimicrobial resistance genes.

5. Conclusion

As more sequencing work is done globally, it is becoming more evident that bacterial isolates can harbor numerous plasmids and that experimental models need to account for interplay between plasmids in a host. Further work is needed to understand the relevance and function of SCPs, and their role in the dissemination of AMR plasmids.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plasmid.2019.03.005>.

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