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Horizontal Transfer of Carbapenemase-Encoding Plasmids and Comparison with Hospital Epidemiology Data

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Carbapenemase-producing organisms have spread worldwide, and infections with these bacteria cause significant morbidity. Horizontal transfer of plasmids carrying genes that encode carbapenemases plays an important role in the spread of multidrug-resistant Gram-negative bacteria. Here we investigate parameters regulating conjugation using an *Escherichia coli* laboratory strain that lacks plasmids or restriction enzyme modification systems as a recipient and also using patient isolates as donors and recipients. Because conjugation is tightly regulated, we performed a systematic analysis of the transfer of *Klebsiella pneumoniae* carbapenemase (bla_{KPC})-encoding plasmids into multiple strains under different environmental conditions to investigate critical variables. We used four bla_{KPC}-carrying plasmids isolated from patient strains obtained from two hospitals: pKpQIL and pKPC-47e from the National Institutes of Health, and pKPC_UVA01 and pKPC_UVA02 from the University of Virginia. Plasmid transfer frequency differed substantially between different donor and recipient pairs, and the frequency was influenced by plasmid content, temperature, and substrate, in addition to donor and recipient strain. pKPC-47e was attenuated in conjugation efficiency across all conditions tested. Despite its presence in multiple clinical species, pKPC_UVA01 had lower conjugation efficiencies than pKpQIL into recipient strains. The conjugation frequency of these plasmids into *K. pneumoniae* and *E. coli* patient isolates ranged widely without a clear correlation with clinical epidemiological data. Our results highlight the importance of each variable examined in these controlled experiments. The *in vitro* models did not reliably predict plasmid mobilization observed in a patient population, indicating that further studies are needed to understand the most important variables affecting horizontal transfer *in vivo*.

Carbapenemase-producing organisms (CPO) have spread worldwide, and the Centers for Disease Control and Prevention have categorized these organisms as an "urgent" threat (1) with high mortality rates ranging from 30% to more than 70% among infected patients (2–4). These carbapenemases include *Klebsiella pneumoniae* carbapenemase (KPC, encoded by bla_{KPC}), NDM-1, IMP, VIM, and OXA family enzymes (5). Bacteria producing these enzymes are frequently resistant to nearly all antibiotics, because they harbor additional resistance genes carried on plasmids. bla_{KPC} is becoming prevalent in parts of the United States, Europe, Asia, and South America (6–8).

The global spread of bla_{KPC} has been due in part to vertical transmission through a relatively small set of successful bacterial host lineages (9, 10) such as *K. pneumoniae* sequence type 258 (ST258), which has disseminated globally (11, 12). Although the determinants behind the dramatic success of ST258 are unknown (13–15), genes transmitted in parallel with plasmids carrying bla_{KPC} genes may contribute to advantages either in pathogenicity or fitness (14–17) possibly through unique capsule antigens or type IV secretion systems (11, 14, 15, 18).

In addition to vertical transfer, horizontal gene transfer (HGT) of DNA can contribute to the spread of antibiotic resistance. Conjugative plasmids carry genes that enable gene transfer to a heterologous recipient cell (19). Also, the resistance gene bla_{KPC} is often located on the mobile transposon Tn*4401* found on diverse plasmids (20). The bla_{KPC}-containing plasmid backbone pKpQIL (IncFII replicon) examined here is endemic in Israel (8) and has been identified in outbreaks in the United States. IncN replicon plasmids carrying carbapenemase genes, also examined here, have been identified worldwide with increasing frequency (6, 21). Recently, it was suggested in a study analyzing clinical isolates that bla_{KPC}-IncN/ST-15 plasmids may have more efficient intra- and interspecies conjugation than the pKpQIL/FII_{K2} plasmids; however, this has not been proven, and the microbiology factors influencing this propensity to conjugate require further exploration (22).

The bla_{KPC} gene has been identified in at least 15 species in the *Enterobacteriaceae* family (21, 23–25) as well as several species not in the *Enterobacteriaceae* family (23, 24). One might speculate that the gastrointestinal (GI) environment within a patient, given its high bacterial densities, could potentially provide an ideal environment for gene transfer (26). However, despite this high potential for spread, there are relatively few reports of bla_{KPC} interspecies transfer within patients (22, 27–29). Plasmid transfer between bacteria within the hospital or community environment adds an additional layer of complexity (21). To better understand the clinical cases and outbreaks for which HGT appears to be frequent, compared to those for which HGT appears to be rare, we analyzed

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several conditions *in vitro* to approximate different settings, with a goal to fill in the knowledge gap surrounding the potential influences on conjugation.

In this work, we performed *in vitro* experiments to investigate factors that influence conjugation of four plasmids. The epidemic 114-kb pKpQIL plasmid was isolated during an outbreak at the National Institutes of Health Clinical Center (NIH) (4, 21). pKPC-47e, a 50-kb plasmid with an IncN replicon, was isolated from patients and the environment at NIH (21). At the University of Virginia Health System (UVA), an outbreak of carbapenemase-producing *Enterobacteriaceae* was partially attributed to an unprecedentedly high rate of proposed HGT of pKPC_UVA01, a nontypeable 43-kb plasmid, with transfer to at least 10 different species from seven genera (2, 30, 31). pKPC_UVA02, a 113-kb nontypeable plasmid, was also isolated during the outbreak and from subsequent surveillance cultures, providing evidence of possible HGT; however, it was identified in fewer species than pKPC_UVA01 (2, 30, 31). We analyzed these plasmids using *in vitro* and *in silico* methods along with hospital epidemiologic data to investigate factors that regulate their transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. Bacteria were grown at 37°C in tryptic soy broth (TSB) (Thermo Fisher Scientific, Waltham, MA), on blood agar (BA) (Remel, Lenexa, KS), or on tryptic soy agar (TSA) (Thermo Fisher Scientific) plates. The plates were supplemented with tetracycline (30 μg/ml), kanamycin (50 μg/ml), gentamicin (50 μg/ml), meropenem (0.5 μg/ml for *E. coli* XL-1 and 4 μg/ml meropenem for all other *Enterobacteriaceae*), where indicated.

Plasmid elimination experiments. Plasmid elimination experiments were performed by serial passage at elevated temperature as described previously (32).

Construction of GFP-tagged plasmids and strains. Plasmid DNA was extracted and purified from *K. pneumoniae* KPNIH1760 with a QIAfilter Midi-Prep kit (Qiagen, Valencia, CA). To fluorescently tag pKpQIL, a GFP:kanamycin (GFP stands for green fluorescent protein) cassette was cloned into *aadA* of pKpQIL, encoding a truncated form of AadA (33). Primers were designed to add 40 nucleotides of *aadA* sequence (AadAGFP For [For stands for forward] [AGG CAG GCT TAT CTT GGA CAA GAA GAT CGC TTG GCC TCG CGC GCA ACG CAA TTA ATG TGA] and AadAGFP Rev [Rev stands for reverse] [TGA TTG GGA GAG TGG CGG AAA CGA ACA CCA ACA TAT GCA GCA GAA AGC CAC GTT GTG TCT CAA]) to a GFP:kanamycin construct under the control of a *lac* promoter. The resulting construct *aadA*:GFP*kan*:*aadA* was cloned into a TOPO TA cloning vector (Invitrogen), amplified by PCR, and purified by QIAquick PCR purification kit (Qiagen). An *E. coli* strain (NM1100) that expresses lambda proteins (kindly provided by Susan Gottesman) was transformed with pKpQIL and plated on CRE medium (RambaChrom KPC; Gibson Biosciences, Lexington, KY). Lambda red recombination procedure was performed with NM1100(pKpQIL) and transformed with *aadA*:GFPkan:*aadA*. Colonies were isolated on kanamycin selective media and confirmed to express GFP by fluorescence microscopy. Plasmid DNA (pKpQ-GFP) was sequenced for confirmation. The same procedure was repeated using fluorescently tagged pKPC-47e isolated from *Enterobacter cloacae* ECNIH3 to generate p47e-GFP by insertion of GFP:kanamycin into the truncated form of *aadA* on pKPC-47e (AadAGFPIncN For [TCG TAA ACT GTA ATG CAA GTA GCG TAT GCG CTC ACG CAA CGC GCA ACG CAA TTA ATG TGA] and AadAGFPIncN Rev [TTG TAG GTC AAT GCT TTA AAA AGT AGC TGC TCC CCT TTC GAA AGC CAC GTT GTG TCT CAA]). Strain KPNIH1760 was cured of the plasmid pKpQIL to generate Kpn1760CURE, followed by transformation with pKpQIL or pKpQ-GFP to generate Kpn1760CT_pKpQIL (CT stands for cured and transformed) or Kpn1760CT_pKpQGFP, respectively. PCR and susceptibility testing were performed on isolates to confirm transformation. PCR was used to confirm retention of the two other plasmids (pAAC154-a50 and pKPN-498) carried by strain KPNIH1760 (21) (data not shown). The two isogenic strains exhibited growth rates similar to KPNIH1760 (data not shown). There was no significant difference in the conjugation rates of KPNIH1760, Kpn1760CT_pKpQIL, and Kpn1760CT_pKpQGFP to two *Enterobacteriaceae* recipients (see Table S1 in the supplemental material). Similar results were seen with Kpn1760CT_pKPC47e and Kpn1760CT_p47eGFP (data not shown). All subsequent conjugation studies utilized the GFP-tagged isolate Kpn1760CT_pKpQGFP or Kpn1760CT_p47eGFP.

Plasmids pKPC_UVA01 and pKPC_UVA02 were originally derived from strains Kpn1016 and Kox1015 (2). These plasmids were electroporated into strain Kpn1760CURE to generate Kpn1760CT_pUVA01 and Kpn1760CT_pUVA02, which were confirmed by sequence analysis and susceptibility testing.

Strain CAV1193 harbors five plasmids: the pKPC_UVA01 slightly modified derivative pKPC_CAV1193 (34) that contains bla_{KPC}, two plasmids containing gentamicin resistance genes, and two additional plasmids. To generate a bla_{KPC} plasmid-free strain of CAV1193 (C1193_KPCCURE), gentamicin selection was maintained during plasmid elimination experiments. CAV1193 was cured of its plasmids that carry genes that encode gentamicin resistance to generate the gentamicin-sensitive strain C1193_GentCURE. Finally, C1193_KPCCURE was passaged at elevated temperature to generate the carbapenem- and gentamicin-sensitive strain C1193_CURE. Plasmid loss and/or retention were confirmed by PCR and susceptibility testing (see Table S2 in the supplemental material). pKpQ-GFP was transformed into the double cured strain, generating C1193CURE_pKpQGFP.

Conjugation experiments. Conjugation experiments were performed with modifications from a previously described procedure (35). Briefly, *Enterobacteriaceae* donor and recipient cultures were grown overnight with agitation at 37°C in TSB. Saturated cultures were subcultured into fresh TSB and incubated with agitation for approximately 2 h, until cultures reached an absorbance (600 nm) of 0.4 to 0.6. For broth culture matings, donor and recipient cultures were mixed at a 1:10 ratio in fresh TSB and incubated at 37°C without agitation for 20 h. Experiments were conducted using time points from 1 h to 20 h for some combinations (see additional Materials and Methods in the supplemental material). The endpoint of 20 h was selected given the lack of conjugation for many combinations at shorter time points. The culture was diluted in phosphate-buffered saline (PBS) and used for enumeration of transconjugants on TSA plates containing either tetracycline or gentamicin and meropenem. Recipients were enumerated on TSA plates containing tetracycline or gentamicin only. For filter matings, donor and recipient cultures were mixed at a 1:10 ratio and then spotted onto 0.22-μm nitrocellulose filters placed on BA plates for 20 h. Transconjugants were confirmed by PCR and/or fluorescence microscopy. To confirm expression and function of the pKpQ-GFP-encoded resistance elements following conjugation, susceptibility profiles of recipients and transconjugants were compared. Recipient strains Kpn223 and Kpn731 were susceptible to doripenem, meropenem, imipenem, and ertapenem prior to conjugation (Table S3). Kpn223 and Kpn731 pKpQ-GFP transconjugants were resistant to doripenem, ertapenem, imipenem, and meropenem, as well as to several other antibiotics in accordance with known resistance mechanisms encoded by pKpQIL (33). Conjugation frequency is given as the number of CFU of transconjugants/number of CFU of recipients. When no transconjugants were detected, the limit of detection was set at 9 CFU/ml, and used to calculate the conjugation frequency, which was denoted using open circles. Mann-Whitney tests were performed using Graphpad Prism (San Diego, CA), where a P value of <0.05 was considered significant.

IncFII and IncN typing by multiplex PCR. Bacterial strains used as conjugation recipients were screened for the presence of the IncN bla_{KPC}

TABLE 1 Plasmids and bacterial strains used in this study

Plasmid or bacterial strain	Description or relevant characteristic[a]	Purpose in study	Source and/or reference
Plasmids			
pKpQIL	pKpQIL-6e6 from strain KPNIH14	Donor plasmid	NIH Clinical Center (4)
pAAC154-a50	From KPNIH14	Extra plasmid in donor	NIH Clinical Center (4)
pKPN-498	From KPNIH14	Extra plasmid in donor	NIH Clinical Center (4)
pKPC-47e	pKPC-47e from strain ECNIH3	Donor plasmid	NIH Clinical Center (21)
pKpQ-GFP	pKpQIL-6e6 tagged with GFP	Donor plasmid	This study
p47e-GFP	pKPC-47e tagged with GFP	Donor plasmid	This study
pKPC_UVA01	pKPC_UVA01 from Kpn1016	Donor plasmid	UVA Medical Center (2)
pKPC_UVA02	pKPC_UVA02 from strain Kox1015	Donor plasmid	UVA Medical Center (2)
pKPC_CAV1193	pKPC_CAV1193 from strain CAV1193	Donor plasmid	UVA Medical Center (34)
pCAV1193-3741	From CAV1193	Extra plasmid in donor	UVA Medical Center (34)
pCAV1193-78	From CAV1193	Extra plasmid in donor	UVA Medical Center (34)
pCAV1193-166	From CAV1193; confers Gent^r	Extra plasmid in donor	UVA Medical Center (34)
pCAV1193-258	From CAV1193	Extra plasmid in donor	UVA Medical Center (34)
pKPN-065	From Kpn223	Extra plasmid in recipient	This study
pKPN-7c3	From Kpn555	Extra plasmid in recipient	This study
pKPN-d90	From Kpn555	Extra plasmid in recipient	This study
Bacterial strains			
KPNIH1760	*K. pneumoniae* patient isolate	Donor	4; this study
ECNIH3	*E. cloacae* patient isolate	Intermediate	21
ECONIH1	*E. coli* patient isolate	Donor	21
ECONIH2	*E. coli* patient isolate	Donor	This study
KPNIH39	*K. pneumoniae* patient isolate	Donor	This study
Kpn1760CURE	KPNIH1760 cured of pKpQIL-6e6	Intermediate	32
Kpn1760CT_pKpQIL	KPNIH1760 cured and transformed with pKpQIL-6e6	Donor	32
Kpn1760CT_pKpQGFP	KPNIH1760 cured and transformed with pKpQ-GFP	Donor	This study
Kpn1760CT_pKPC47e	KPNIH1760 cured and transformed with pKPC-47e	Donor	This study
Kpn1760CT_p47eGFP	KPNIH1760 cured and transformed with p47e-GFP	Donor	This study
Kpn1760CT_pUVA01	KPNIH1760 and transformed with pKPC_UVA01	Donor	This study
Kpn1760CT_pUVA02	KPNIH1760 cured and transformed with pKPC_UVA02	Donor	This study
ECONIH2CURE	ECONIH2 cured of bla_{KPC} plasmid	Recipient	This study
XL-1	*E. coli* XL-1 Blue	Recipient	Stratagene
Kpn223	*K. pneumoniae* thigh wound isolate, Sept. 2013	Recipient	NIH Clinical Center
Kpn555	*K. pneumoniae* urine isolate, Feb. 2013	Recipient	NIH Clinical Center
Kpn808	*K. pneumoniae* urine isolate, March 2014	Recipient	NIH Clinical Center
Kpn731	*K. pneumoniae* sputum isolate, March 2013	Recipient	NIH Clinical Center
Kpn889	*K. pneumoniae* urine isolate, Feb. 2014	Recipient	NIH Clinical Center
Kpn164	*K. pneumoniae* skin lesion isolate, Aug. 2013	Recipient	NIH Clinical Center
Kpn110	*K. pneumoniae* urine isolate, Aug. 2013	Recipient	NIH Clinical Center
Kpn331	*K. pneumoniae* urine isolate, Sept. 2013	Recipient	NIH Clinical Center
Eco385	*E. coli* blood isolate, Aug. 2013	Recipient	NIH Clinical Center
Eco889	*E. coli* urine isolate, Feb. 2014	Recipient	NIH Clinical Center
Eco046	*E. coli* urine isolate, Oct. 2013	Recipient	NIH Clinical Center
Eco067	*E. coli* sputum isolate, Oct. 2013	Recipient	NIH Clinical Center
Eco207	*E. coli* tracheal aspirate isolate, Feb. 2013	Recipient	NIH Clinical Center
Eco452	*E. coli* urine isolate, Nov. 2013	Recipient	NIH Clinical Center
Eco556	*E. coli* urine isolate, Feb. 2013	Recipient	NIH Clinical Center
Eco603	*E. coli* blood isolate, Oct. 2013	Recipient	NIH Clinical Center
CAV1193	*K. pneumoniae* pKPC_CAV1193; Mero^r Gent^r	Donor	UVA Medical Center (34)
C1193_KPCCURE	Mero^s Gent^r; cured of pKPC_CAV1193	Recipient	This study
C1193_GentCURE	Mero^r Gent^s; cured of Gent^r plasmids	Intermediate	This study
C1193_CURE	Mero^s Gent^s; cured of pKPC_CAV1193 Gent^r plasmids	Intermediate	This study
C1193CT_pKpQGFP	Mero^r Gent^s; transformed with pKpQ-GFP	Donor	This study
CAV1205	*K. pneumoniae* pKPC_UVA02	Donor	UVA Medical Center (31)
CAV1237	*K. pneumoniae* pKPC_UVA01	Donor	UVA Medical Center (31)
Kpn1016	*K. pneumoniae* pKPC_UVA01	Donor	UVA Medical Center (2)
Kox1015	*K. oxytoca* pKPC_UVA02	Donor	UVA Medical Center (2)

[a] Gent^r and Gent^s, gentamicin resistance and sensitivity, respectively; Mero^r and Mero^s, meropenem resistance and sensitivity, respectively. The isolation date (month and year) of some isolates are given. The months were abbreviated as follows: Feb., February; Aug., August; Sept., September; Oct., October; Nov. November.

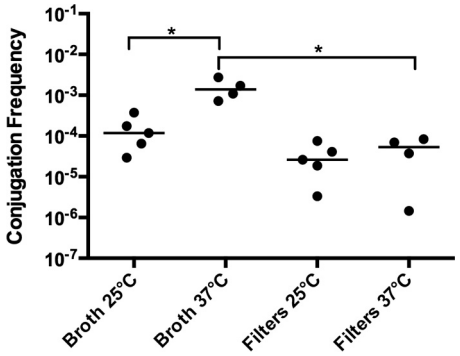

FIG 1 Effects of temperature and substrate on pKpQ-GFP conjugation frequency. The donor is Kpn1760CT_pKpQGFP, and the recipient is *E. coli* XL-1. Conjugation assays performed for 20 h in either broth culture or on filters placed on blood agar plates at 25°C or 37°C. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. Each symbol represents the value for an individual ($n \geq 4$). Each bar indicates the median value for a group. Values that are statistically significantly different ($P \leq 0.05$) are indicated by a bar and asterisk.

plasmid by multiplex PCR (36) and IncFII bla_{KPC} plasmid by PCR with FII_K FW/RV (forward/reverse) primers (37).

Genomic DNA sequencing and analysis. Strains KPNIH1760, ECNIH3, CAV1193, CAV1205, Kpn1016, and Kox1015 have been previously sequenced (4, 21, 31, 34). Kpn223, Kpn555, and Eco889 were sequenced as part of this study. Genomic DNA was prepared for each isolate using the Promega Maxwell 16 nucleic acid purification system with the tissue DNA purification kit. DNA was further purified using the Zymo Genomic DNA Clean and Concentrator-10 kit. Libraries were constructed using the SMRTbell template kit, version 1.0. The DNA was size selected for the range 7 to 50 kb using a BluePippin system with a 0.75% gel cassette. Sequencing was performed on the PacBio RSII instrument using P5 polymerase binding and C3 sequencing kits with magnetic bead loading and 180-min acquisition. Genome assemblies were performed using HGAP and Quiver as part of SMRTAnalysis version 2.3.

Nucleotide sequence accession numbers. The whole-genome sequencing data for strains Kpn223, Kpn555, and Eco889 can be retrieved at NCBI BioProject accession no. PRJNA279655, PRJNA279656, and PRJNA279654, respectively.

RESULTS

The temperature and substrate affected pKpQ-GFP conjugation. The conjugation frequency of pKpQ-GFP from strain Kpn1760CT_pKpQGFP to the *E. coli* cloning strain XL-1 Blue (XL-1) is 10^{-3} at 37°C in broth culture (Fig. 1). This rate is 10 times greater ($P < 0.05$) than the conjugation rate at 25°C. To determine whether mating substrate contributes to conjugation efficiency, we compared conjugation of pKpQ-GFP in broth to nitrocellulose filters to mimic a biofilm environment. The conjugation rate of pKpQ-GFP from Kpn1760CT_pKpQGFP to XL-1 was ~1 log unit higher in broth culture compared to filter matings at 37°C ($P < 0.05$ [Fig. 1]). These data indicate that conjugation of pKpQ-GFP varied significantly based on environmental factors.

The recipient strain influenced pKpQ-GFP conjugation efficiency. Many studies of conjugation use modified laboratory strains of *E. coli* as recipients; however, the applicability of the data are unclear (21). Therefore, we selected multiple clinical strains of *K. pneumoniae* as recipients (Table 1 and Fig. 2A). Transfer from strain Kpn1760CT_pKpQGFP was detected in six of eight *K. pneumoniae* patient isolate recipients, indicating that some *K. pneumoniae* strains are refractory to pKpQ-GFP conjugation under these experimental conditions (Fig. 2A). Of the recipients capable of receiving pKpQ-GFP, we observed a range of plasmid transfer frequencies from low (10^{-7}) to high (10^{-2}).

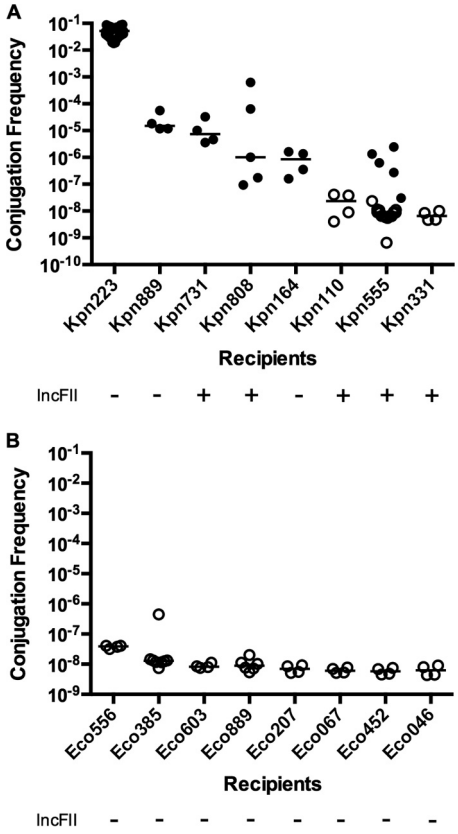

FIG 2 Effects of recipient strain and species on pKpQ-GFP conjugation frequency in broth. The donor is strain Kpn1760CT_pKpQGFP, and the recipients are *K. pneumoniae* (A) or *E. coli* (B) isolates from patients. Broth culture conjugations were performed for 20 h. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. Replicon typing was performed as described in Materials and Methods. Conjugation frequencies are indicated by solid circles. Open circles indicate that no transconjugants were observed at the limit of detection. Each symbol represents the value for an individual ($n \geq 4$). Each bar indicates the median value for a group.

Plasmid incompatibility groups alone do not explain differences in conjugation efficiency. This variation in transfer efficiencies could be due in part to plasmid incompatibility, namely, the presence of native plasmids that prevent the entry or maintenance of plasmids sharing the same replicon type (38). We analyzed by PCR whether *K. pneumoniae* recipients contained IncFII replicon plasmids. The most efficient recipient, Kpn223, lacked an IncFII plasmid, while the most refractory recipients (Kp110 and Kp331) contained at least one IncFII plasmid. However, Kpn731 and Kpn808 recipients contained at least one IncFII plasmid but still supported pKpQ-GFP conjugation. Kpn164 lacked an IncFII plasmid but only rarely produced pKpQ-GFP transconjugants (Fig. 2A). Furthermore, Kpn110, Kpn331, and Kpn555 were cured of their IncFII plasmids with minimal impact on conjugation efficiency (see Fig. S1 in the supplemental material).

***E. coli* was not an efficient recipient of pKpQ-GFP compared to *K. pneumoniae*.** Given that pKpQIL has been found in *E. coli* strains (39), we next tested eight patient *E. coli* clinical isolates as

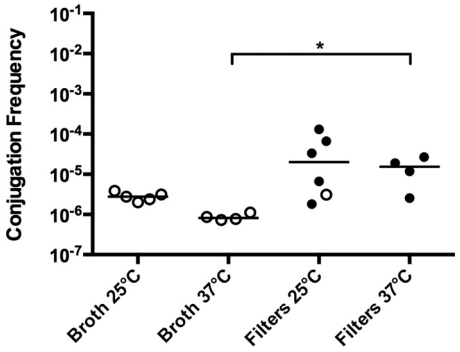

FIG 3 Effects of temperature and substratum on p47e-GFP conjugation frequency. The donor is Kpn1760CT_p47eGFP, and the recipient is *E. coli* XL-1. Conjugations were performed for 20 h in either broth culture or on filters placed on blood agar plates at 25°C or 37°C. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. Each symbol represents the value for an individual ($n \geq 4$). Each bar indicates the median value for a group. Symbols: solid circles, conjugation frequency; open circles, no transconjugants observed at the limit of detection; *, $P \leq 0.05$.

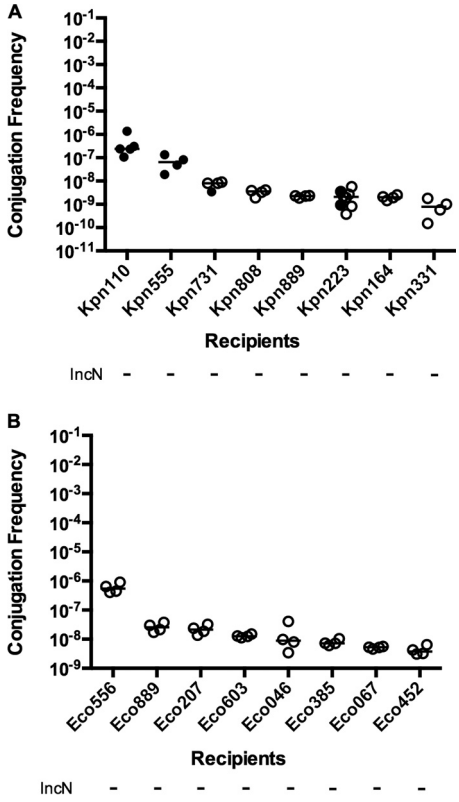

FIG 4 Effects of recipient strain and species on p47e-GFP conjugation frequency on filters. The donor is strain Kpn1760CT_p47eGFP, and recipients are *K. pneumoniae* (A) or *E. coli* (B) isolates from patients. Filter matings were performed for 20 h. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. Replicon typing was performed as described in Materials and Methods. Symbols: solid circles, conjugation frequency; open circles, no transconjugants observed at the limit of detection.

recipients to investigate species-specific factors in recipients. Strikingly, none of the eight *E. coli* patient isolates yielded a detectable number of transconjugants using strain Kpn1760CT_pKpQGFP as a donor (Fig. 2B). This indicated that with Kpn1760CT_pKpQGFP as a donor, clinical *E. coli* strains were more refractory recipients than *E. coli* XL-1 and multiple clinical *K. pneumoniae* strains. None of the *E. coli* recipients contained an IncFII plasmid (Fig. 2B). Given the inability of our *E. coli* recipients to accept plasmids from *K. pneumoniae*, we tested *E. coli* donors to determine whether intraspecies transfer would be more efficient. We used as donors the bacterial strain ECONIH1 containing the plasmid pKPC-47e (21), and the *K. pneumoniae* (KPNIH39) and *E. coli* (ECONIH2) strains containing pKpQIL plasmids. We used two of the previously tested *E. coli* strains and a plasmid-cured strain of ECONIH2 as recipients. We observed no difference in conjugation to *E. coli* recipients from either *E. coli* or *K. pneumoniae* donors (see Fig. S2 in the supplemental material).

Substrate, but not temperature, affected p47e-GFP conjugation. pKPC-47e differs from pKpQIL in replicon type, size, and accessory genes (21). Conjugation was tested using strain Kpn1760CT_p47eGFP in broth culture and filter mating conditions using XL-1 as a recipient (Fig. 3). In this set of experiments, conjugation of p47e-GFP was observed only in the filter condition, in contrast to the result from pKpQ-GFP, which showed efficient conjugation in both broth and filters (Fig. 1).

Both recipient strain and species influenced p47e-GFP conjugation. IncN family plasmids typically have a broad host range (40), in contrast to IncFII plasmids such as pKpQIL. To test the species specificity of p47e-GFP, we analyzed conjugation of p47e-GFP to eight patient *K. pneumoniae* recipients and eight *E. coli* recipients (Fig. 4). Filter mating conjugation with strain Kpn1760CT_p47eGFP as the donor reproducibly produced transconjugants in only two of eight *K. pneumoniae* recipients, and the transfer rate was low (10^{-7} to 10^{-8}) (Fig. 4A). Transconjugants were not detected for any of the eight *E. coli* patient strain recipients (Fig. 4B). Broth mating gave similar results (see Fig. S3 in the supplemental material).

Donor and recipient strains contributed to the efficiency of UVA outbreak plasmid transfer *in vitro*. We next investigated whether pKPC_UVA01 would demonstrate a high conjugation efficiency *in vitro*, as this "promiscuous" plasmid was observed in at least 10 species at UVA (2, 30). We performed conjugation assays with strains Kpn1760CT_pUVA01 and Kpn1760CT_pUVA02 to the Kpn223 recipient in broth culture at 37°C (Fig. 5). We detected a lower conjugation efficiency of pKPC_UVA01 conjugation from strain Kpn1760CT_pUVA01 to strain Kpn223 compared to pKpQ-GFP. Conversely, the efficiency of pKPC_UVA02 transfer in the same donor background was high into Kpn223.

We next tested whether the donor background strain influenced conjugation by comparison of Kpn1760CT_pUVA01 or Kpn1760CT_pUVA02 to several unmodified patient donor strains. Original parent strains Kpn1016 and Kox1015 carrying pKPC_UVA01 and pKPC_UVA02, respectively, did not yield transconjugants, in contrast to Kpn1760CT_pUVA01 and Kpn1760CT_pUVA02 (Fig. 5), indicating donor influences on the process. Distinct differences in conjugation efficiency were observed between XL-1, Kpn555, and Eco385 as recipients (see Fig. S4 in the supplemental material), indicating additional recipient-level regulatory factors. We next asked whether the number of plasmids acquired by the original isolate CAV1193 was a consequence of enhanced recipient ability, as such hypothesized recipients would possibly contribute to the spread of resistant plasmids in the hospital. We generated a panel of CAV1193 isogenic strains

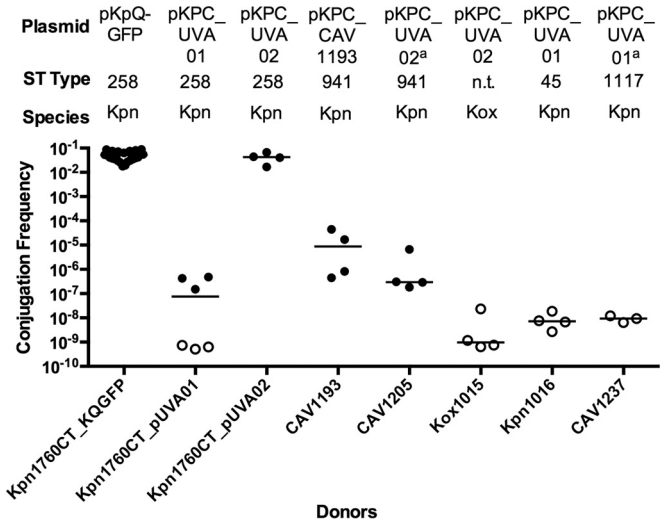

FIG 5 Conjugation ability of *Enterobacteriaceae* isolates containing pKPC_UVA01, pKPC_CAV1193, or pKPC_UVA02 to Kpn223. Donors are indicated on the *x* axis, and the recipient is Kpn223. Broth culture conjugations were performed for 20 h. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. The plasmid, sequence type, and species are given above the graph. The species are *K. pneumoniae* (Kpn) and *K. oxytoca* (Kox). Each symbol represents the value for an individual ($n \geq 3$). Each bar indicates the median value for a group. Symbols: solid circles, conjugation frequency; open circles, no transconjugants observed at the limit of detection. The superscript a for two plasmids indicates that the plasmid was determined by whole-genome sequencing on the Illumina HiSeq platform and long-range PCR (31). n.t., not tested.

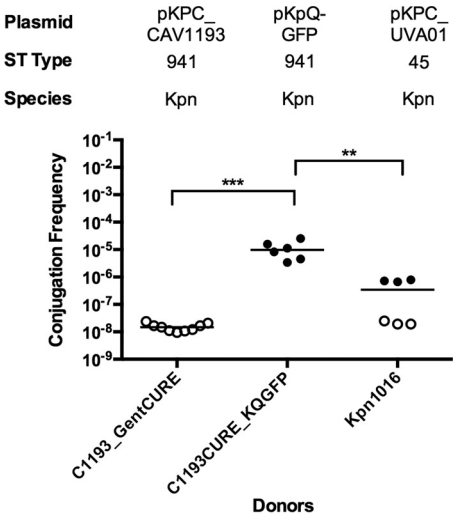

FIG 6 Effect of isogenic donor-recipient strains on conjugation. The recipient is strain C1193_KPCCURE, and donors are indicated on the *x* axis. Broth culture conjugations were performed for 20 h. The plasmid, sequence type, and species (*K. pneumoniae* [Kpn]) are given above the graph. The conjugation frequency is given by the number of CFU of transconjugants/number of CFU of recipients. Each symbol represents the value for an individual ($n \geq 6$). Each bar indicates the median value for a group. Symbols: solid circles, conjugation frequency; open circles, no transconjugants observed at the limit of detection. Values that are statistically significantly different are indicated by bars and asterisks as follows: **, $P < 0.005$; ***, $P < 0.0005$.

(see Table S2 and Fig. S5 in the supplemental material), which contain the pKPC_UVA01-like plasmid pKPC_CAV1193 (34) to obtain a set of donors that are carbapenem resistant due to a bla_{KPC} plasmid and gentamicin susceptible and a recipient that is carbapenem susceptible and gentamicin resistant. "Self-transfer" of pKPC_CAV1193 from the gentamicin-sensitive C1193_GentCURE into the plasmid-cured recipient C1193_KPCCURE was not detected (Fig. 6). The pKpQ-GFP plasmid transferred more easily into the cured CAV1193 strain than the original bla_{KPC}-carrying plasmid of the clinical isolate. The conjugation efficiency of pKPC_UVA01 from strain Kpn1016 was also low. These findings suggest that for our experimental conditions, CAV1193 may not be an inherently efficient recipient, although the effect of plasmids lost during curing on conjugation efficiency is unknown.

Sequence analysis of plasmids highlights complexity of genetics that regulate conjugation. Genomics is a powerful tool that has been utilized to track the origin of outbreaks (4) and infer plasmid evolution and host range (40). Thus, we sought to determine whether there were "signatures" that could predict conjugation efficiency. We analyzed plasmids from donor and recipient strains using Illumina MiSeq and single-molecule, real-time (SMRT) sequencing. We also included a few recently described plasmids from other studies that examined plasmids carrying carbapenemase genes, their conjugation ability, and HGT (29, 41). Using a candidate gene approach, we selected a set of 77 genes that are potentially associated with plasmid transfer, replication, or maintenance based on current literature, realizing that this is not an exhaustive list. We examined the plasmids for the presence of sequences matching our selected gene set. The presence/absence of predicted proteins was plotted as a heatmap, along with percent protein identity to show the variation in protein sequence and the limitations of annotation methods when separated from direct studies of protein function (see Fig. S6 in the supplemental material).

Plasmids from Kpn223 and Kpn555 recipients were analyzed due to strikingly different conjugation frequencies. Genes that mediate surface exclusion (*traS* and *traT*) were present in Kpn555 plasmids, but not predicted to be present in the Kpn223 plasmid. There was also a difference in the number of plasmids detected in the two recipients. Kpn555 contained two unique plasmids with *tra* genes, while Kpn223 carried one plasmid that appears to lack conjugation genes. Uncovering all of the significance differences between the strains would require an additional evaluation of the potential chromosomal regulatory factors and further *in vitro* experiments.

We next compared the sequences of donor carbapenemase gene-containing plasmids. Interestingly, the "promiscuous" plasmid pKPC_UVA01 carried many fewer transfer genes from our list based on protein comparison than most of the plasmids, including plasmids recently described in other studies (see Fig. S6 in the supplemental material). Chen et al. (41) describe the conjugative resistance plasmid pBK30683, and also pBK30661, which had lost many of the *tra* genes, providing evidence for the spread of resistance through both clonal spread and horizontal transfer. Dortet et al. (29) also describe a conjugative resistance plasmid, pOXA-48, and a related plasmid with a deletion in the *tra* operon. The set of proteins in pOXA-48, a plasmid reported to show very efficient conjugation in both intraspecies and interspecies experiments (22), has a set of predicted transfer genes that differ from those of pKPC_UVA01 and from plasmids shown in the heatmap. Our findings highlight the striking differences in pre-

sumed conjugation genes between this small set of plasmids and illustrate that it would be a challenge to use sequence analysis of the genes to fully explain the *in vitro* differences in plasmid transfer efficiency.

DISCUSSION

Nosocomial outbreaks of multidrug-resistant bacteria are of great concern due to their increasing incidence and associated mortality rate. Both vertical and horizontal transmissions contribute to these outbreaks (22, 30). HGT via conjugation is influenced by environmental and bacterial factors, but the precise role of each remains unknown. We used *in vitro* and *in silico* approaches to characterize variables affecting conjugal transfer of clinically isolated bla_{KPC}-containing plasmids. Commonly, bla_{KPC} plasmid transfer studies conflate the effect of donor strain and plasmid. We uncoupled the donor bacterial strain from the plasmid by generating a unique set of reagents through plasmid elimination and electroporation. We also compared our conjugation data with the observed plasmid molecular epidemiology.

IncN family plasmids accounted for four of the eight environmental plasmids isolated in a recent study (21), and our results may help explain the predominance of this plasmid in the environment, as p47e-GFP transfer was more efficient on filters than in broth. The filter method is a simplified simulation of an environmental biofilm, and a true biofilm may demonstrate even greater differences in conjugation efficiencies. Our data are consistent with previous work demonstrating that IncN plasmids had greater conjugation efficiencies on a solid substrate than in a liquid substrate (42). It is interesting to note the proposed HGT of the IncN/ST-15 plasmids in patients examined in the Adler et al. study (22). The HGT described in that study was proposed to be within the patient, not the environment; therefore, more-detailed conjugation studies of these plasmids would be valuable. It would also be of interest to directly examine IncN/ST-15 and IncN p47e-GFP under the same experimental conditions to compare conjugation efficiencies.

To our knowledge, this is the first analysis to investigate the role of the recipient strain on bla_{KPC}-containing plasmid transfer by testing several clinical isolate recipients. A similar study examining bla_{NDM-1} plasmids conjugated into multiple patient isolates yielded species-specific variations in conjugation frequencies (43). Species-level differences may be coordinated by shufflon genes (44), while strain-level differences may be due to outer membrane proteins (19, 45) or exclusion factors (46). The first report of plasmid-borne colistin resistance is a recent example illustrating the importance of testing a range of recipients for plasmid transfer studies (47). Liu et al. (47) showed conjugation of the colistin-resistant plasmid to the *E. coli* K-12 strain EC600, but no transconjugants were obtained from clinical isolate recipients, including *K. pneumoniae*, *E. coli*, and *Pseudomonas aeruginosa*. Our data highlight the manipulation required for *E. coli* cloning strains to become permissive plasmid recipients, as *E. coli* XL-1 received pKpQ-GFP with much higher efficiency than the tested clinical *E. coli* isolates. Less efficient conjugation into *E. coli* and subsequent instability of the plasmids has been noted in the past (48). Given the recovery of bla_{KPC}-containing plasmids in clinical *E. coli* strains in recent studies (22, 49), this issue deserves further examination to understand the critical factors regulating HGT in *E. coli*.

Our experiments targeted the possible origins of interspecies spread at UVA (30). As just one possible reason to explain the spread of resistance in the UVA Medical Center, we hypothesized that strain CAV1193 could be an inherently efficient recipient given that it had accepted and retained multiple plasmids. However, we did not detect transconjugants after mating a modified CAV1193 donor to a plasmid-cured CAV1193 recipient, and other donors to this recipient resulted in low to modest conjugation efficiency. We do not know the actual donor for the presumed HGT during the outbreak, and we cannot know how closely our contrived plasmid-cured CAV1193 matched the original recipient; furthermore, CAV1193 may have originally acquired pKPC_CAV1193 through a different mechanism of HGT. Other modes of HGT may be involved, as the role of transposons (20) and integrative and conjugative elements in bla_{KPC} transfer were not addressed by our experiments. Direct DNA import may also play an underappreciated role in bla_{KPC} transfer (50). Another limitation to note is that although our calculation of efficiency accounts for differences in recipient CFU, the analysis is limited by lack of growth rate data of donors, transconjugants, and recipients that may have some effect on the conjugation ratio. Our experimental protocol is commonly used for conjugation analyses, and this limitation is true for similar studies.

The bacterial strains Kpn223 and Kpn555 were strikingly different as recipients, prompting a comparison of the strains. The number of plasmids and type of replicon differed between strains Kpn223 and Kpn555, but the role of the number of recipient plasmids on conjugation efficiency is inconclusive (51–53). Kpn223 seems to carry only a plasmid with limited presumptive transfer genes and unclear conjugal ability, which may explain, in part, its high-efficiency recipient ability. Plasmid content data on other recipient strains were not determined. Finally, plasmid incompatibility is an important factor in plasmid inheritance based on previously published studies, but it is known not to be absolute (38, 54). pKpQ-GFP could transfer into certain IncFII-positive strains, and our experiments with strains cured of the IncFII plasmid clearly reveal that incompatibility groups are not the dominant determinant of conjugation efficiency in these donor/recipient combinations. Future refinement of incompatibility designations may be important.

Investigators in the past have tried to predict plasmid promiscuity based on genomic signatures, such as tracking specific trinucleotide frequencies (40). We wondered if there might be a pattern of genes that correlated with promiscuity, allowing us to discern important differences between pKPC_UVA01 and the other plasmids. As might be expected given the vast literature on conjugation, our sequence analysis of these plasmids did not reveal a definitive gene set that could easily predict conjugation efficiency. The analysis does highlight the significant differences seen in plasmid conjugation content; however, the sample size was quite small, so future studies are needed to investigate specific genes and gene redundancy further. Our findings are consistent with a study that did not identify recipient genes required specifically for conjugation (55). When comparing the plasmids in strain Kpn223 versus those in Kpn555, we see the surface exclusion genes, *traT* and *traS* (56), on Kpn555 plasmids. Sequence analysis revealed a wide range of transfer and maintenance genes among different donor plasmids. Our limited data suggest that it would be a challenge to predict the potential of plasmid spread *in vivo* based on the transfer-related genes carried on bla_{KPC} plasmids. Recent studies examine the spread of resistance as HGT or via a clonal transmission and describe modifications to the *tra* operon in plas-

mids, which highlights the increasing role of sequence and conjugation analyses in investigation of these clinically significant plasmids (29, 41).

Decades ago, investigators were studying factors relevant to plasmid transfer in the human gut (51), and we are still asking similar questions. When considering horizontal plasmid transfer in a patient, we know that the microbiome of the patient does not match the characteristics of a laboratory cloning strain. What parameters of donor, recipient, plasmid, and surrounding milieu are most critical for promoting or blocking conjugation and how important is regulation of conjugation compared to subsequent selective pressure?

To extend our understanding, animal models will be useful to investigate the role the microbiome plays in conjugation. In our study, only a limited number of donor/recipient combinations were tested, and a dense community of GI organisms, including uncultured bacterial donors, would allow a much higher number of transfer combinations. The recent clinical observation that the specific parent strain as well as the plasmid type may influence conjugation rate suggests that there is much to learn about HGT regulation (22). Elucidating these fundamental mechanisms of transfer may unlock new strategies for treatment and facilitate a broader understanding of the interchangeable components of bacterial genomes. The findings in the UVA Medical Center prompted a hypothesis about the ease of HGT of specific plasmids (2, 30), consistent with other publications in the field; however, our investigation of these clinically relevant plasmids clearly demonstrates the complexity of HGT regulation, as those plasmids did not easily conjugate in the experiments performed, and highlights the limits of our collective knowledge.

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