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IncN ST7 epidemic plasmid carrying bla_{IMP-4} in Enterobacteriaceae isolates with epidemiological links to multiple geographical areas in China

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Objectives: To characterize bla_{IMP-4}-carrying plasmids originating from inpatients in Hong Kong.

Methods: Sixteen bla_{IMP-4}-carrying plasmids identified among Enterobacteriaceae (nine *Escherichia coli*, four *Klebsiella pneumoniae*, two *Citrobacter freundii* and one *Enterobacter cloacae*) recovered from 15 patients were characterized. The isolates, collected during January 2010 to December 2013, were retrospectively investigated by plasmid sequencing, molecular and fitness studies.

Results: The bla_{IMP-4}-carrying plasmids belonged to the IncN ST7 lineage (~50 kb). Twelve of the 16 plasmids were epidemiologically linked to seven different regions in China. Alignment of the complete plasmid sequences showed identical plasmid backbones and two highly similar resistance regions, each carrying one of two resistance genes (bla_{IMP-4} and *qnrS1*). The bla_{IMP-4} was detected in a class 1 integron (containing bla_{IMP-4} and intron Kl.pn.13) that is part of an IS*6100*-IS*26* transposon-like structure. The nine *E. coli* carrying the epidemic plasmid belonged to multiple multilocus STs (six ST542, one ST131, one ST657 and one ST3177). Fitness assays performed on *E. coli* J53 recipients showed that the presence of the epidemic plasmid did not have a significant biological cost.

Conclusions: This study identified a bla_{IMP-4}-carrying IncN ST7 plasmid disseminated among multiple enterobacterial species originating from patients with epidemiological links to different regions in China.

Introduction

Acquired carbapenemases are a major public health threat and their dissemination among Enterobacteriaceae has followed complex pathways, involving both epidemic plasmids and bacterial clones with which these genes have become associated. Representative examples are the dissemination of bla_{KPC} mediated by $IncFII_K$ plasmids associated with the *Klebsiella pneumoniae* ST258 clone in many areas and the dissemination of bla_{NDM-1} mediated by the IncX3-type plasmid (pNDM-HN380) among Enterobacteriaceae in several countries.[1,2]

IMP-4 carbapenemases, first identified in Hong Kong and China in the 1990s and initially restricted to Asia and the Pacific, have since become the predominant carbapenemase type in Australia.[3,4] Certain plasmid types have been implicated in the endemic spread of IMP-4 in Australia. These include the IncM2 type (pEl1573) in Sydney, the IncA/C type in Melbourne and the IncHI2 type in Queensland.[3,5,6] In China, IMP-4 is also increasingly detected among Enterobacteriaceae from hospitals, but little is known about the bacterial clones and plasmid types involved in the dissemination of bla_{IMP-4}.[7–9] In Hong Kong, IMP-4 is also a common enzyme type among carbapenemase-producing Enterobacteriaceae (CPE) and has been found to be carried on IncA/C- and IncN-type plasmids.[9–11] We recently described an IncN ST7 plasmid encoding IMP-4 in an *K. pneumoniae* isolate.[11] In this work, we describe the epidemiological and molecular aspects of 16 IncN ST7 plasmids encoding IMP-4 among isolates originating from a territory-wide surveillance programme for carbapenemases in Hong Kong hospitals.

Materials and methods

Bacterial strains, susceptibility testing and plasmid replicon typing

In Hong Kong, public hospitals are divided into seven healthcare regions (A–G). A territory-wide surveillance programme for carbapenemases has been implemented since 2009.[12] During January 2010 to December 2013, the programme identified 29 bla_{IMP}-positive Enterobacteriaceae isolates from clinical specimens and rectal swabs of 28 patients. PCR and sequencing showed that 24 of the 29 isolates had bla_{IMP-4}. Plasmid replicon typing showed that 16 of the 24 bla_{IMP-4}-carrying plasmids were of IncN.[10,13,14] The 16 isolates included 9 *Escherichia coli*, 4 *K. pneumoniae*, 2 *Citrobacter freundii* and 1 *Enterobacter cloacae*. One of the IncN ST7 plasmids, i.e. pIMP-HZ1, originating from a *K. pneumoniae* isolate, was previously published.[11] The 16 isolates with IMP-4-encoding IncN plasmids (including the original isolate carrying pIMP-HZ1) were investigated in the present study. Susceptibility of the isolates was determined by the CLSI's disc diffusion method.[15]

Molecular analysis

The major carbapenemase genes (bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC} and bla_{OXA-48}) were detected by PCR and sequencing.[9,10] *E. coli* phylogroups and multilocus STs of *K. pneumoniae* and *E. coli* isolates were determined by using previously described methods.[10,14] PFGE and conjugation experiments and sizing of plasmids were carried out using published methods.[14]

Plasmid sequencing

Sixteen IncN plasmids from *E. coli* transconjugants or transformants were sequenced on Illumina MiSeq or 454 GS FLX platforms.[9,12] The reads were assembled by the SPAdes assembler (v. 3.5.0) and gaps in the plasmids were closed by PCR and Sanger sequencing (Table S1, available as Supplementary data at *JAC* Online). Additional bioinformatics analyses were performed as previously described.[1,9,16,17]

Plasmid stability and fitness cost on bacterial host

The test strains were *E. coli* J53 and a J53 transconjugant carrying pIMP-1495 (a representative of the bla_{IMP-4}-carrying IncN plasmids characterized in this study). Plasmid stability was determined as previously described.[18] Fitness cost was determined by pairwise competitive growth experiments using a lactose-positive J53 carrying pIMP-1495 and a lactose-negative reference strain (*E. coli* DH5α or a phylogroup B2 clinical *E. coli* strain 1-I8).[19] Inoculated LB broth was incubated at 37°C for 24 h with shaking (225 rpm) and bacterial counts were enumerated by subculture onto cysteine lactose electrolyte-deficient plates. Absence of plasmid conjugation to the reference strain was confirmed by replica plating of 100 colonies to plates containing 1 mg/L meropenem.

Results

Patient demographics, strain characteristics and conjugation

Sixteen isolates from 15 patients were investigated. Eleven of the 15 patients had recently been hospitalized in mainland China before the IMP-4-producing strains were detected in Hong Kong (Table 1). The interval between previous hospitalization in mainland China and local admission was ≤3 days in eight patients

Table 1. Patient demographics, molecular features and resistance patterns of 16 bacterial strains with bla_{IMP-4}-carrying plasmids

Strain[a]	Source[b]	Collection date	Sex/ age (years)	Place of medical care abroad (date)[c]	MLST (*E. coli* phylogroup)	Resistance pattern[d]	Plasmid name
Ec 1058	A	Sep 2012	male/0.2	Guangzhou (Sep 2012)	ST131 (B2)	TET	pIMP-GZ1058
Ec 1502	B1	Jan 2012	male/1[b]	Shenzhen (Jan 2012)	ST657 (F)	CHL, CIP, TET, SXT	pIMP-SZ1502
Ec 1504	B1	Jul 2012	male/82	none	ST542 (A)	CHL, TET	pIMP-HK1504
Ec 1505	B1	Jul 2012	male/54	Foshan (Jul 2012)	ST542 (A)	CHL, TET	pIMP-FS1505
Ec 1509	B1	Nov 2012	male/80	none	ST542 (A)	CHL, TET	pIMP-HK1509
Ec 1510	B1	Nov 2012	female/43	Shenzhen (Nov 2012)	ST542 (A)	CHL, TET	pIMP-SZ1510
Ec 1515	B1	Mar 2013	male/53	Shenzhen (Mar 2013)	ST542 (A)	CHL, TET	pIMP-SZ1515
Ec 1516	B1	Aug 2013	male/79	Danshui (Aug 2013)	ST542 (A)	CHL, TET	pIMP-DS1516
Ecl 1506	B2	Aug 2012	male/90	Shanghai (Aug 2012)	—	CIP, GEN, TET, SXT	pIMP-SH1506
Kp 1495	B1	Feb 2010	male/58	unknown	ST273	CIP	pIMP-1495
Kp 1496	B1	Jul 2010	male/75	China (Jun 2010)	ST644	AMK, CHL, CIP, GEN, TET, SXT	pIMP-1496
Kp 1501	B2	Jan 2012	male/1[b]	Shenzhen (Jan 2012)	ST35	CIP, GEN, TET, SXT	pIMP-SZ1501
Kp 1	C	Aug 2010	male/49	Huizhou (Apr 2010)	ST11	CHL, CIP, SXT	pIMP-HZ1
Cf 1500	D	Nov 2011	male/78	none	—	CIP, TET	pIMP-HK1500
Cf 1503	E	Jun 2012	female/64	Fujian (Jun 2012)	—	CHL, CIP, GEN, TET	pIMP-FJ1503
Ec 1517	F	Sep 2013	male/74	Guangzhou (Aug 2013)	ST3177 (F)	CIP, GEN	pIMP-GZ1517

[a]Cf, *C. freundii*; Ec, *E. coli*; Ecl, *E. cloacae*; Kp, *K. pneumoniae*.
[b]Healthcare regions A–F. B1 and B2 are two different hospitals within healthcare region B. Six strains were recovered from clinical specimens [respiratory (Kp 1495, Cf 1500 and Ecl 1506), urine (Kp 1496 and Ec 1517) and wound (Kp 1)]. The other 10 strains were identified in rectal swabs. Ec 1502 and Kp 1501 were recovered from the same patient.
[c]Eleven patients had previously been hospitalized in China. Four patients were transferred from hospitals in China to hospital B1 on the same day (Ec 1505, Ec 1515, Ec 1516 and Kp 1496). The intervals between previous hospitalization in China and local hospital admission were 2 days in two patients (Ec 1058 and Ec 1510), 3 days in two patients (Ec 1502/Kp 1501 and Cf 1503) and 1–4 months in three patients (Ecl 1506, Kp 1 and Ec 1517). The exact region of previous hospitalization in China was unknown in one patient (Kp 1496).
[d]For the following drugs: AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; and SXT, co-trimoxazole.

Figure 1. Comparison of 16 IncN plasmids in this study. (a) Circular map of the plasmid pIMP-1495. This plasmid was used to illustrate the backbone shared by all the analysed IncN plasmids and the location of the two variable regions. The outer circle shows the coding sequences, with arrows representing the direction of transcription. The middle circle indicates the functional sequence blocks. The G+C plot is indicated in the inner circle, ranging from high (grey) to low (black). (b) Comparison of the variable region containing bla_{IMP-4} in 16 plasmids. The target site duplications (DR) indicative of IS*6100* (5 bp), IS*Sen4* (4 bp) and IS*26* (8 bp) transposition are indicated. (c) Comparison of the variable region containing *qnrS1* in 16 plasmids. The target site duplications indicative of IS*Kpn19* (7 bp) and IS*26* (8 bp) transposition are indicated. Grey shading indicates shared sequence identities.

and 1–4 months in three patients. Ten patients had previously been hospitalized in Guangdong province (Danshui, Foshan, Guangzhou, Huizhou and Shenzhen), Fujian province and Shanghai, China. The nine *E. coli* strains belonged to four STs (one ST131, six ST542, one ST657 and one ST3177). The six ST542 strains originated from hospital B1 and they had the same banding patterns after XbaI digestion and PFGE (Figure S1). These strains included four imported cases from July 2012 to August 2013 and two local cases from July 2012 to November 2012. However, no epidemiological links could be identified for the two local cases (Ec 1504 and Ec 1509). The clones (ST11, ST35, ST273 and ST644) of the four *K. pneumoniae* were diverse (Table 1).

The bla_{IMP-4}-carrying plasmids could be transferred by conjugation to the J53 recipient at frequencies of 10^{-1}–10^{-5} per donor cell. Reduced susceptibility to ciprofloxacin (i.e. smaller inhibition zone diameters, by 4–9 mm) was the only resistance trait cotransferred with bla_{IMP-4}.

Sequence analysis of IncN plasmids carrying bla_{IMP-4}

Complete sequences of the 16 plasmids with sizes of ~50 kb were obtained (Table S2). They have a plasmid scaffold typical of IncN plasmids in general (Figure 1a). Query of our plasmids against the plasmid MLST database assigned all of them to plasmid ST7.

Two resistance genes, *qnrS1* and bla_{IMP-4}, were found in the variable regions 1 and 2, respectively (Figure 1a). The variable region containing bla_{IMP-4} was inserted between the *uvp1* gene and the *EcorII* gene (Figure 1b). The immediate bla_{IMP-4} genetic context was identical in 14 plasmids and minor variations were found in 2 plasmids (pIMP-FJ1503 and pIMP-HK1500). First, an array of genes associated with IS*6100* was inserted, leading to two flanking 5 bp direct repeats (AACAG). This includes the bla_{IMP-4}-associated integron (In823::Kl.pn.13). Second, an IS*26* element was inserted into and disrupted the integrase gene into two parts, *intI1*Δ1 and *intI1*Δ2 (Figure 1b).

The *qnrS1*-containing region was inserted within the *fipA* gene, splitting it into two fragments (*fipA*Δ1 and *fipA*Δ2, Figure 1c). This region was flanked by two 7 bp direct repeats (ATATAGG) and had likely resulted from an IS*Kpn19* insertion. The *qnrS1* gene was flanked by a *tnpR* resolvase downstream and a truncated IS*2 tnpR* resolvase upstream, followed by an IS*26* element. Sequence variations in this region included a second copy of IS*Kpn19* in seven plasmids, a second copy of IS*26* in two plasmds (pIMP-HK1500 and pIMP-SH1506), and partial or complete loss of the *fipA*Δ1-IS*Kpn19* direct repeat sequence in another two plasmids (pIMP-FJ1503 and pIMP-1496).

Plasmid stability and its effect on bacterial host fitness

Plasmid pIMP-1495 persisted *in vitro* for 300 generations in 100% of the daughter cells in the absence of antibiotic pressure. The MIC of meropenem for J53/pIMP-1495 after passage for 300 generations was 32 mg/L, which was identical to that at generation 0. The conjugation frequencies of pIMP-1495 from the donor strain J53 to the recipient JP995 at generation 0 and generation 300 were 1.2×10^{-5} and 3.5×10^{-5} per donor cell, respectively (Student's *t*-test, $P=0.35$). This indicates that bla_{IMP-4} expression and the plasmid transfer rate were not affected by the serial passage in the absence of antibiotic pressure.

In competitive growth experiments, J53/pIMP-1495 behaved like its plasmid-free counterpart (Figure S2). The relative fitness of J53/pIMP-1495 in mixed growth with *E. coli* DH5α and the clinical *E. coli* strain 1-I8 were similar, 0.92 ± 0.1 and 0.90 ± 0.2, respectively ($P=0.6$ for both).

Discussion

The presence of highly similar bla_{IMP-4}-carrying IncN ST7 plasmids in multiple Enterobacteriaceae strains was confirmed in 11 patients with a history of medical treatment in different parts of mainland China. Given the short intervals between previous hospitalization in mainland China and detection upon local admission, these plasmids likely represent importations. The importation of the same ST542 clone from multiple hospitals in China might reflect this to be a frequent lineage linked to IMP-4 dissemination in the country. *E. coli* ST131 and *K. pneumoniae* ST11 are widespread clones, but *E. coli* ST542 has not previously been reported from China.[20] The plasmid was also detected in isolates from several patients with no travel history. Therefore, it is possible that there is simultaneous nosocomial transmission of this plasmid in Hong Kong hospitals.

Our IncN plasmids belonged to plasmid ST7. Among 119 IncN plasmids deposited in the plasmid MLST database (http://pubmlst.org/plasmid/, 18 July 2016), only 2 plasmids from *K. pneumoniae* belonged to ST7 including pKOX105 carrying VIM-1 from Italy and p53500 not carrying any β-lactamase from India. In the plasmids, the bla_{IMP-4}-associated integron (In823::Kl.pn.13) was identified inside a novel composite transposon-like structure flanked by IS*6100* and IS*26* (Figure 1b). Several *E. coli* and *K. pneumoniae* isolates from Shanghai and Tianjin have been reported to have integron-associated bla_{IMP-4} in similar gene arrays.[7,8] IMP-4 has also been reported in another integron array (bla_{IMP-4}-*qacG-aacA4-catB3*) carrying IncM2 and IncA/C plasmids from Enterobacteriaceae in Sydney, Melbourne and Hong Kong.[3,9] Furthermore, the wider genetic contexts of sequences flanking the integron array are different from those we described here.[3,9]

We investigated pIMP-1495 as a representative and revealed that it has only a a minimal effect on fitness. In addition, neither the integron nor the bla_{IMP-4}-carrying plasmid was lost after prolonged passage in antibiotic-free medium. Nonetheless, this study does not offer sufficient data to determine the prevalence of this bla_{IMP-4}-carrying epidemic plasmid in China.

In conclusion, this study identified a bla_{IMP-4}-carrying IncN ST7 plasmid disseminated among multiple enterobacterial species originating from patients with epidemiological links to widely separated areas in China.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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