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2 Short communication

3 **Plasmid-mediated fosfomycin resistance in *Escherichia coli* isolated from pig**

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Abstract

Previous studies have reported plasmid-mediated *fosA3* among *Escherichia coli* originating from human and companion animals. In this study, the plasmid, designated pHK23a originating from a multidrug-resistant *E. coli* isolate recovered from a slaughter pig in December 2008 in Hong Kong, China was sequenced. In conjugation, the plasmid readily transferred to *E. coli* J53 at high frequencies. It belongs to the narrow host range IncFII incompatibility group and is 73607 bp in length. Sequence alignment showed that pHK23a has a 59.1 kb backbone which shares high homology with the prototype R100 plasmid and a 14.5 kb variable region. The variable region includes three genes mediating antimicrobial resistance (*fosA3*, Δbla_{TEM-1} , $bla_{CTX-M-3}$), ten mobile genetic elements (four copies of IS26, *insA*, $\Delta insB$, $\Delta Tn2$, *ISI*, $\Delta ISEcp1$, $\Delta int11$), the *tir* transfer inhibition protein, the *pemI/pemK* addiction system and eight ORFs of unknown functions (*orf1*, *orf2*, $\Delta orf3$, *orf20*, *orf23*, *orf24*, *ycdA* and *ycdB*). The three resistance genes were organized in a novel IS26-composite transposon-like structure. In conclusion, this is the first report of *fosA3* containing plasmid in an isolate of pig origin. Since IncFII plasmids spread efficiently in *Enterobacteriaceae*, the detection of *fosA3* with bla_{CTX-M} is worrisome and might become a public health concern.

Key words: pigs, plasmid, extended-spectrum beta-lactamases, fosfomycin resistance

35 **1. Introduction**

36 Plasmid-mediated extended-spectrum β -lactamases (ESBLs) are increasing worldwide. It is
37 of note that the CTX-M type ESBLs have spread explosively among isolates of both human
38 and animal origins and high prevalence of their presence have been reported in many
39 countries (Ho et al., 2012a; Naseer and Sundsfjord, 2011). In *Enterobacteriaceae*, the CTX-
40 M type ESBLs are often encoded on plasmids which also carried genes mediating resistance
41 to other drug classes such as aminoglycosides, tetracyclines, sulphonamides and trimethoprim
42 (Naseer and Sundsfjord, 2011). However, the great majority of CTX-M producing
43 *Escherichia coli* remain susceptible to the cell wall active agent, fosfomycin. Consequently,
44 there is rekindled interest in using fosfomycin for treatment of multidrug-resistant infections
45 in human and veterinary medicine. In pigs, fosfomycin is sometimes used for treatment of
46 infectious diseases or for preventing infections during the weaning period (Food and
47 Agriculture Organization et al., 2008). In China, there is preliminary evidence that
48 fosfomycin resistance is emerging among *E. coli* isolates originating from companion
49 animals in pet hospitals and was associated with dissemination of the fosfomycin resistance
50 gene *fosA3* carried on IncFII plasmids (Hou et al., 2012). In this study, we report the
51 complete sequence of an IncFII plasmid, designated pHK23a carrying *fosA3* and *bla*_{CTX-M}
52 originating from a slaughter pig in Hong Kong, China.

53

54 **2. Materials and Methods**

55 **2.1 Bacterial strains and susceptibility testing**

56 The *E. coli* isolate P0022_S_T in this study originated from a surveillance program for
57 antimicrobial resistance in food animals that was started in the last quarter of 2008 (Ho et al.,
58 2011a). In the first 3 months, a total of 39 *E. coli* isolates were recovered from rectal swabs
59 of 39 pigs. Susceptibility testing showed that four of the 39 isolates were fosfomycin-resistant.

60 PCR screening revealed that all four isolates were positive for *fosA3* and *bla*_{CTX-M} (Wachino
61 et al., 2010). Plasmid replicon typing and hybridization revealed that in one of the isolates,
62 *fosA3* was carried on an IncFII plasmid. The plasmid, designated pHK23a was further
63 analysed by sequencing.

64 Antimicrobial susceptibility to fosfomycin, cefuroxime, ceftriaxone, ceftazidime,
65 imipenem, meropenem, ciprofloxacin, levofloxacin, chloramphenicol, cotrimoxazole,
66 gentamicin, amikacin, and tetracycline was determined by the disc diffusion method and
67 interpreted according to the CLSI M100-S21 document (Clinical and Laboratory Standards
68 Institute, 2011). Production of ESBL was determined by the double-disc synergy test using
69 cefotaxime, amoxicillin-clavulanic acid and ceftazidime at inter-disc distances (centre to
70 centre) of 20 and 25 mm (Ho et al., 1998). MIC for fosfomycin was determined by Etest.

71

72 **Molecular studies**

73 Conjugation was carried out in filters with *E. coli* J53Az^r as the recipient. Donor and
74 recipient cells were mixed at 1:2 ratio. Transconjugants were selected on trypticase soy agar
75 plates containing sodium azide (150 mg/L; Sigma Chemical Co., St Louis, MO, USA) for
76 counterselection and fosfomycin (4 mg/L; Sigma Chemical Co.) to select for plasmid-
77 encoded resistance. The plasmids were converted to the linear forms by incubation with
78 *Aspergillus oryzae* S1 nuclease (Sigma Chemical Co.) and were sized by pulsed-field gel
79 electrophoresis (PFGE) as previously described (Ho et al., 2011c). Plasmid-mediated *fosA3*
80 was investigated by PCR using specific primers (Wachino et al., 2010).

81

82 **Plasmid sequencing**

83 The Illumina Genome Analyzer IIx was used for sequencing of plasmids at >100-fold
84 coverage, as described previously (Ho et al., 2011c; Ho et al., 2011b). In brief, plasmids were

85 extracted from the transconjugants by using the Qiagen Large Construct kit (Qiagen, Hong
86 Kong, People's Republic of China). Purified plasmid DNA was fragmented by nebulization.
87 The fragments were amplified and a library was constructed as described previously (Ho et
88 al., 2011h). Based on the qPCR quantified concentration of the barcoded plasmid library, it
89 was diluted to generate approximately 500,000 clusters and seeded with other samples in the
90 same Solexa sample lane. Sequencing run of 76-base pair-end reads was performed according
91 to the manufacturer's recommendations. Additional rounds of PCR and Sanger sequencing
92 was used to complete the assembly (Table S1). The Illumina Off-Line Basecaller (version
93 v1.6), WebACT and Geneious Pro (Version 5.0.1, Biomatters Limited, Auckland, New
94 Zealand) softwares were used for bioinformatics analysis (Ho et al., 2011c).

95

96 **3. Results and discussion**

97 The *E. coli* isolate P0022_S_T originating from a slaughter pig in Dec 2008 in Hong
98 Kong was resistant to fosfomycin and multiple other antimicrobial agents including
99 cephalosporins (cefuroxime, ceftriaxone), fluoroquinolones (ciprofloxacin, levofloxacin),
100 chloramphenicol, cotrimoxazole, gentamicin and tetracycline and exhibited an ESBL
101 phenotype. The isolate was susceptible to amikacin, piperacillin-tazobactam and the
102 carbapenems (imipenem, meropenem). In conjugation experiment, the fosfomycin resistance
103 trait could be transferred to J53 recipient *E. coli* at frequency of 1.1×10^{-1} transconjugants per
104 donor cells. S1-PFGE showed that there was transfer of a single plasmid of ~70 kb size in the
105 transconjugants. Upon acquisition of the plasmid, the fosfomycin MIC of the recipient
106 increased from 0.75 mg/L to >1024 mg/L. In addition, there was co-transfer of cephalosporin
107 resistance and ESBL phenotype. The presence of the *fosA3* marker in the transconjugants was
108 confirmed by PCR and localized to the ~70 kb plasmid by hybridization using the
109 digoxigenin (DIG)-labelled *fosA3* PCR products as a probe.

110 The plasmid (designated as pHK23a) from the transconjugant P0022ST-T1 was
111 completely sequenced. The sequencing reads were assembled into six contigs and the gaps
112 were closed by PCR and Sanger sequencing. The sequences flanking the *fosA3* were
113 confirmed by PCR mapping and sequencing of the PCR products. The nucleotide sequence
114 was deposited in GenBank under accession number JQ432559. It is a 73607 bp circular
115 plasmid with an average GC content of 51.8% and 105 putative open reading frames (ORFs),
116 which are DNA sequences predicted to encode proteins (Aziz et al., 2008) (Figure S1). The
117 backbone sequence of pHK23a including the replication, partition, transfer leading and
118 transfer regions is almost identical to those for the classic resistance plasmid R100 (GenBank
119 accession AP000342) and the recent *bla*_{CTX-M} gene encoding variant, pHK01 (GenBank
120 accession HM355591). As in pHK01, the variable region in pHK23a (14.5 kb) was inserted
121 between the replication operon and the partition region. It has one addiction system
122 (*pemI/pemK*) and three resistance genes encoding resistance to fosfomycin (*fosA3*) and β -
123 lactams (Δ *bla*_{TEM-1}, *bla*_{CTX-M-3}).

124 In addition, the sequence of pHK23a was highly similar to the *fosA3*-containing
125 plasmid, pHN7A8 (Figure S2, GenBank Accession JN232517, originated from Guangdong
126 province, China, 2008) over the replication (90% coverage, 95% identity), partition (100%
127 coverage, 100% identity), transfer leading (99% coverage, 100% identity) and transfer (86%
128 coverage, 97% identity) regions. The variable region of pHK23a contained 24 ORFs of which
129 19 were found in pHN7A8 (Figure 1). The 19 ORFs common to the variable regions of both
130 plasmids include two resistance genes (*fosA3*, Δ *bla*_{TEM-1}), seven mobile genetic elements
131 (three copies of IS26, *insA*, Δ *insB*, Δ Tn2, IS1 and Δ ISEcp1), the *tir* transfer inhibition protein,
132 the *pemI/pemK* addiction system and six ORFs of unknown functions (*orf1*, *orf2*, *orf23*, *orf24*,
133 *ycdA* and *ycdB*).

The 4.1 kb region containing the IS26-*fosA3-orf1-orf2-Δorf3*-IS26 gene array was 100% identical to that reported in the plasmid p06607 (accession AB522970) originating from *E. coli* 08-642 in Japan (Figure 1). Truncated versions of the gene array were found in three *fosA3* harbouring IncFII plasmids (p1E1C, pN0863T and p3B9C) in *E. coli* isolates from the Guangdong province of China. The 1.8 kb region containing *orf1-orf2-Δorf3* had 78.6% and 78.0% homology with the chromosomes of *K. variicola* strain At-22 (accession CP001891) and *K. pneumoniae* strain 342 (CP000964), respectively. In both strain At-22 and strain 342, a *fosA* gene was separated from this homologous region by two ORFs (encoding LysR and NADPH). The *fosA* in strain At-22 and strain 342 had 75.0% and 74.3% nucleotide sequence identity to *fosA3* in pHK23a, respectively. Interestingly, the 4.1 kb *fosA3* gene-containing array was connected to the two β-lactamase genes (Δbla_{TEM-1} , $bla_{CTX-M-3}$), which were also flanked by two IS26 elements, forming a composite transposon-like structure with multiple IS26 elements.

We reported here the complete sequence of pHK23a carrying *fosA3*. To our knowledge, this is the first time this resistance mechanism was identified in an isolate of pig origin while previous studies demonstrate the presence of *fosA3* in isolates in human, companion animals and poultry (Hou et al., 2012; Sun et al., 2012; Wachino et al., 2010). In pHK23a, multiple insertion sequences and mobile element relics including IS26, *insA*, *insB*, Tn2 and IS1 were detected in the variable region, indicating frequent insertion and deletion events. In agreement with all the plasmid-mediated *fosA3* described so far (Hou et al., 2012; Sun et al., 2012; Wachino et al., 2010), the *fosA3* gene in pHK23a was flanked by IS26. Similar genetic environment of *fosA3* in *E. coli* isolates of human and animal origins might indicate a similar mechanism of target site recombination events mediating incorporation into plasmids. In pHK23a, the *fosA3* gene was carried on the IncFII plasmid R100 backbone. In *Enterobacteriaceae*, IncFII plasmids occur widely among isolates of human, animal and

environmental origin (Carattoli, 2011) and have been association with the diffusion of genes mediating cephalosporin, carbapenem, aminoglycoside and quinolone resistance (Carattoli, 2011). IncFII plasmids likely contributed to the fitness of the bacterial host by providing virulence and antimicrobial resistance determinants (Carattoli, 2011; Ho et al., 2012b). Their maintenance and stability in the host cells are facilitated by presence of addiction systems. While some IncFII plasmids carry *bla*_{CTX-M} as the only resistance determinant (Ho et al., 2011b), this and recent studies showed that they could carry multiple resistance determinants (*fosA3*, *rmtB*, *aacC2*, *bla*_{NDM-1}).

4. Conclusion

This study described the complete sequence of an IncFII plasmid carrying *fosA3* and *bla*_{CTX-M} originating from commensal *E. coli* of a slaughter pig. Since IncFII plasmids are efficient vehicles for diffusion of antimicrobial resistance determinants in *Enterobacteriaceae*, the simultaneous presence of *fosA3* and *bla*_{CTX-M} genes in these plasmids is worrisome. The finding deserves public health attention because antimicrobial-resistant bacteria might be transmitted to human through direct animal contact and indirectly through contamination of meat products (Sheikh et al., 2012).

Acknowledgements

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Transparency Declaration

184 Conflicts of interest: Nothing to declare.

185

186 **Appendix A**

187 Supplementary data

188 **Figure 1.** Schematic representation of the variable region with *fosA3* gene in the plasmid pHK23a. Upper panel showed comparison of the
189 variable regions in pHN7A8 and pHK23a from the *tir* gene to the *ycdB* gene. The same color patterns were used to indicate the same genes or
190 homologous open reading frames (ORFs). The hypothetical relationship (insertion, deletion and exchange recombination) over three segments is
191 indicated by shading. Lower panel showed a comparison of the genetic organization of the *fosA3* region flanked by IS26 elements in pHK23a
192 with the one reported from Japan (100% identical) and three variants (with variable length of deletions downstream of *fosA3*) from Guangdong,
193 China. The name of the plasmid, the geographic source, collection year, source of the isolate and GenBank accession numbers are indicated on
194 the left.
195

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252

253

Table S1. Primers use in the study

Primer name	Direction	Nucleotide sequence (5' to 3')	Position in Accession no. JQ432559	Source
rep_B	B	CCT GCT CCA TGA CAA ATC TGG CT	c2685-2663	This study
tir_458R	B	GCC ACA TCG TCC AGC AGT A	c3049-3031	This study
pem_F	F	GAG GGT TGG CGG CTC AGT TAT	3347-3367	This study
pem_B	B	GGA CAG GCG GCC AAG AAC TT	c3903-3884	This study
tnpIS26-R	F	AAC TCT GCT TAC CAG GCG	5241-5258	(Ho et al., 2012)
tnpIS26-R375	F	ACG ACG GGA GGA GAG ATA A	5604-5622	This study
tnpIS26-R182	F	CGT GGA GTG ATC GAC ATT CA	5769-5815	This study
tnpIS26-F	B	TGA CAT CAT TCT GTG GGC	c5901-5884	This study
fosA3-seq-FW	F	CAT AGA GCG GGG ATT AGT GTG	6167-6187	This study
fosA3-F	F	CCT GGC ATT TTA TCA GCA GT	6372-6391	This study
fosA3-R	B	CGG TTA TCT TTC CAT ACC TCA G	c6605-6584	This study
fosA3-seq-RV	B	GAG ATG TCC GAC AGC CAC GA	c6868-6849	This study
fosA3-2R	B	GAG AAC ATG GAC AAA GAG AAC G	c7120-7099	This study
pHK23a_node5_FW	F	CGC TTC TTC CAT GAT TGC CA	8405-8424	This study
int-F	F	GCC ACT GCG CCG TTA CCA CC	9531-9550	(Kern et al., 2002)
int-285B	B	GCA CAG CAC CTT GCC GTA GAA	c9806-9786	(Ho et al., 2012)
tem-149F	F	ACA GCG GTA AGA TCC TTG AG	11087-11106	This study
tem-607B	B	GCC GGG AAG CTA GAG TAA GTA G	c11545-11524	This study
CTX-M-F2	F	GAA TTT TGA CGA TCG GGG	12270-12287	(Leflon-Guibout et al., 2004)
CTX-M-F1	B	ATA AAA CCG GCA GCG GTG	c12750-12733	(Leflon-Guibout et al., 2004)
CTX-M-1B	F	AGC TTA TTC ATC GCC ACG TT	13035-13054	(Woodford et al., 2006)
CTX-M-1F	B	AAA AAT CAC TGC GCC AGT TC	c13449-13430	(Woodford et al., 2006)
ISEcplU1	B	AAA AAT GAT TGA AAG GTG GT	c13695-13676	(Saladin et al., 2002)
pHK23a_node2_RV	B	GTC TTA ACT CTG TCT GCC GTA G	c14892-14871	This study

Figure S1. An overview of the *fosA3* encoding plasmid, pHK23a. Starting from the outside, the first circle indicates the coordinate of the complete plasmid circle and the 14.5 kb variable region is shown in red. The open reading frames (ORFs) were annotated in the second circle with arrows representing the direction of transcription. The variable resistant region is coded by the same color scheme as in figure 1. The third circle indicates the functional sequence block. The G+C plot is indicated in the inner circle (mean 51.8%), ranging from high (grey) to low (black)

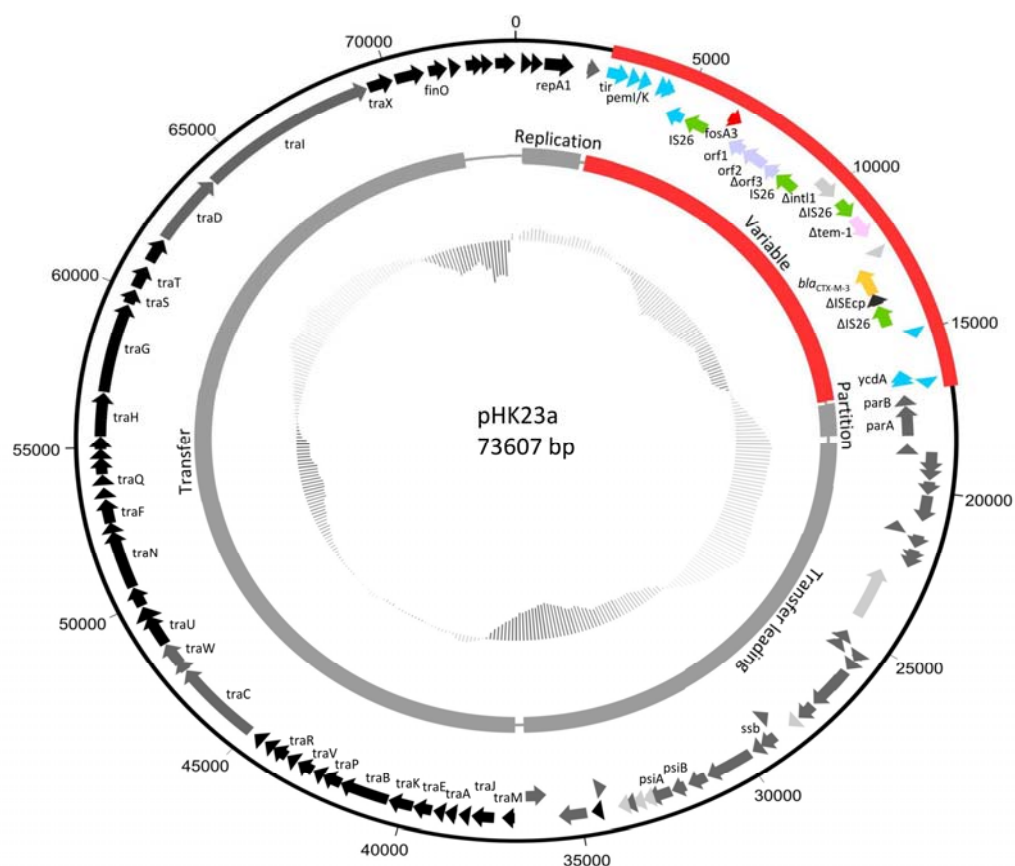


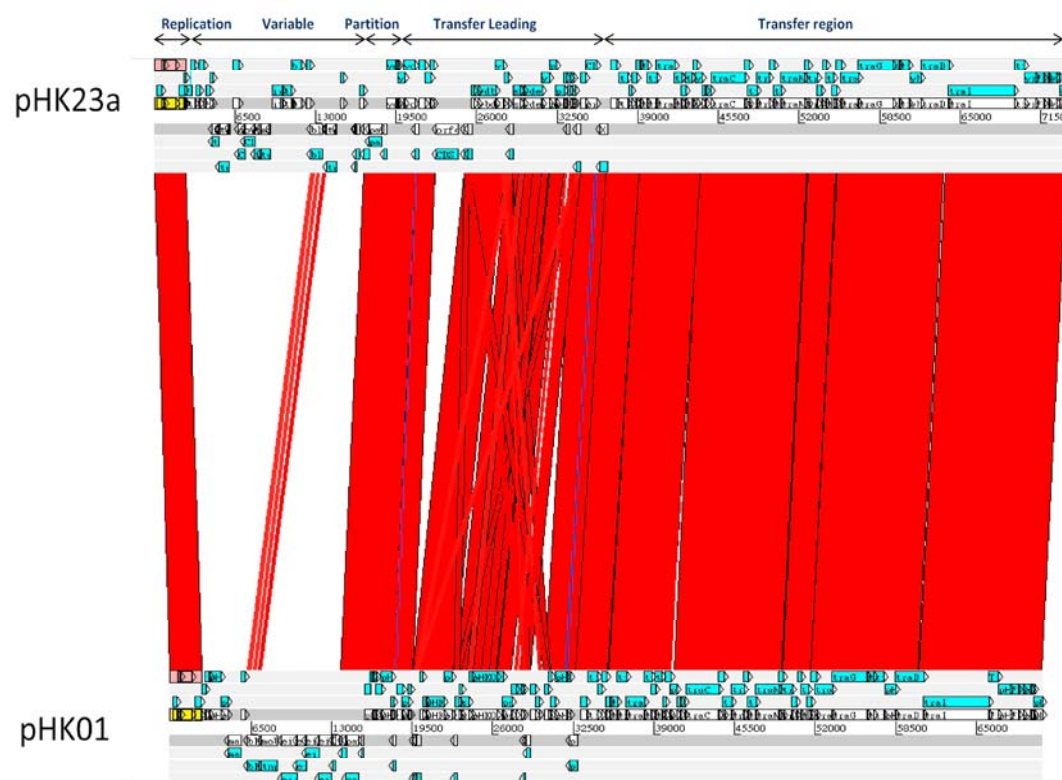
Figure S2. Sequence alignment of pHK23a (GenBank accession number JQ432559) to (A) R100 backbone CTX-M-14 harbouring plasmid pHK01 (IncFII) and (B) *fosA3* harbouring plasmid pHN7A8 (IncFII) using the Artemis Comparison Tool (ACT).

Red bars indicate sequence similarity, blue bars marks inversions, and white bars indicates no similarity.

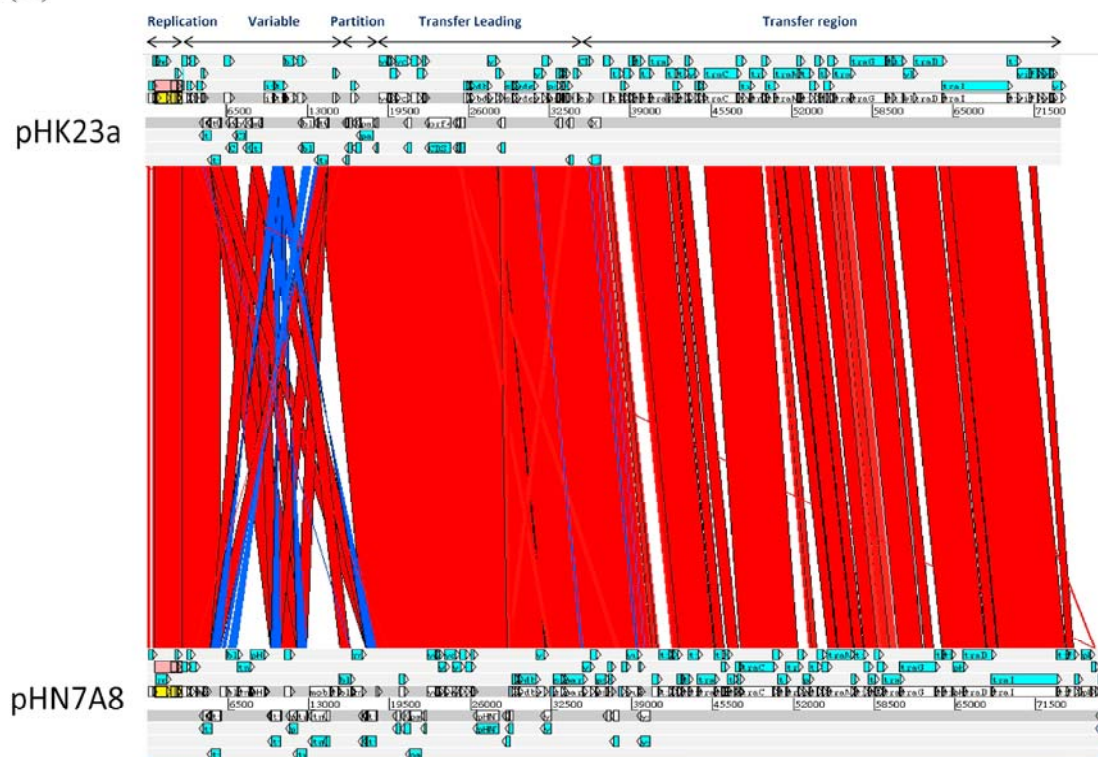
The plasmid backbone (replication, partition, transfer leading and transfer region) of pHK23a match about 83% of the 70262 bp plasmid sequence in pHK01 (GenBank accession number HM355591) with 100% sequence identity. The variable region in pHK23a and pHK01 had sizes of 14.5 kb and 11 kb, respectively. There was little similarity between the variable regions of the two plasmids.

pHK23a and pHN7A8 (JN232517) shared high similarities over the replication (90% coverage, 95% identity), partition (100% coverage, 100% identity) and transfer leading (99% coverage, 100% identity) regions. The similarity over the transfer regions were much lower (86% coverage, 97% identity). The variable region of pHK23a contained 24 open reading frames (ORFs) of which 19 were found in pHN7A8. The 19 ORFs common to the variable regions of both plasmids include two resistance genes (*fosA3*, Δbla_{TEM-1}), seven mobile genetic elements (three copies of IS26, *insA*, $\Delta insB$, $\Delta Tn2$, IS1 and $\Delta ISEcp1$), the *tir* transfer inhibition protein, the *pemI/pemK* addiction system and six ORFs of unknown functions (*orf1*, *orf2*, *orf23*, *orf24*, *ycdA* and *ycdB*).

(A)



(B)



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