

Spread of *ISCR1* Elements Containing *bla*_{DHA-1} and Multiple Antimicrobial Resistance Genes Leading to Increase of Flomoxef Resistance in Extended-Spectrum- β -Lactamase-Producing *Klebsiella pneumoniae*[∇]

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Increasing resistance to quinolones, aminoglycosides, and/or cephamycins in extended-spectrum- β -lactamase (ESBL)-producing *Enterobacteriaceae* exacerbates the already limited antibiotic treatment options for infections due to these microbes. In this study, the presence of resistance determinants for these antimicrobial agents was examined by PCR among ESBL-producing *Klebsiella pneumoniae* (ESBL-KP) isolates that caused bacteremia. Pulsed-field gel electrophoresis was used to differentiate the clonal relationship among the isolates studied. Transferability and the location of the resistance genes were analyzed by conjugation experiments, followed by DNA-DNA hybridization. Among the 94 ESBL-KP isolates studied, 20 isolates of flomoxef-resistant ESBL-KP were identified. They all carried a DHA-1 gene and were genetically diverse. CTX-M genes were found in 18 of the isolates. Among these DHA-1/CTX-M-producing *K. pneumoniae* isolates, *ISCR1* was detected in 13 (72%) isolates, *qnr* genes (1 *qnrA* and 17 *qnrB* genes) were detected in 18 (100%), *aac(6′)-Ib-cr* was detected in 11 (61%), and 16S rRNA methylase (all *armA* genes) was detected in 14 (78%). Four transconjugants were available for further analysis, and *qnrB4*, *aac(6′)-Ib-cr*, *armA*, and *bla*_{DHA-1} were all identified on these self-transferable *bla*_{CTX-M}-carrying plasmids. The genetic environments of *ISCR1* associated with *armA*, *bla*_{DHA-1}, and *qnrB4* genes in the four transconjugants were identical. Replicon-type analysis revealed a FIIA plasmid among the four self-transferable plasmids, although the other three were nontypeable. The cotransfer of multiple resistance genes with the *ISCR1* element-carrying plasmids has a clinical impact and warrants close monitoring and further study.

Klebsiella pneumoniae is an opportunistic pathogen associated with a wide spectrum of infections. Increasing resistance to multiple antimicrobial agents in the bacterium has compromised the effectiveness of treatment. Production of extended-spectrum cephalosporinases, either extended-spectrum β -lactamases (ESBLs) or plasmid-mediated AmpC β -lactamases (PMABLs), confers resistance to broad-spectrum penicillins and cephalosporins (13, 27, 29). While the coexistence of ESBL and PMABL genes in *Enterobacteriaceae* is common (42), multidrug resistance to other antimicrobial agents was less frequently studied among *K. pneumoniae* isolates that simultaneously produced both ESBLs and PMABLs.

Plasmid-mediated quinolone resistance (PMQR) determinants, such as *qnr*, *aac(6′)-Ib-cr*, and *qepA* genes, might cause low-level resistance or reduced susceptibility to quinolones because they could facilitate the selection of mutants with higher-level resistances (31, 40). Aminoglycoside resistance

usually resulted from the presence of aminoglycoside-modifying enzymes (33). Recent reports highlighted the significance of emerging plasmid-mediated 16S rRNA methylase genes (*armA* and *rmtB*) in conferring high-level resistance to all aminoglycosides (3).

Integrations that contain resistance genes, such as *aac(6′)-Ib-cr*-like genes in a class 1 integron cassette, could disseminate resistance characteristics (25). Interestingly, *qnr* is often present in complex class 1 integrons, located between common region 1 (CR1) that comprised the *orf513* recombinase and a second copy of the 3′ conserved segment (3′-CS2), together with gene cassettes encoding resistance to other antimicrobial agents, such as aminoglycosides (31). Toleman et al. proposed that *orf513*, later termed insertion sequence common region 1 (*ISCR1*), may mobilize both a nearby sequence and a truncated 3′-CS from one integron to the 3′-CS of another integron using rolling circle (RC) transposition, thus facilitating the formation of complex class 1 integrons associated with *ISCR1* (36). In addition, *ISCR1* is associated with many antimicrobial resistance genes, including *qnr* (11) and other genes for aminoglycoside and β -lactam resistance (23, 36, 37). It was suggested that these antimicrobial resistance genes were added to the 3′-CS of the class 1 integron via comobilization with the nearby

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ISCR1 from other integrons, using RC transposition and homologous recombination (36).

Flomoxef, a cephamycin unique in having a difluoromethylthioacetamido group at position 7, has been reported to be as clinically effective as a carbapenem in treating flomoxef-susceptible ESBL-producing *K. pneumoniae* (ESBL-KP) bacteremia (20). In 2004, a sudden increase from less than 10% to 20% in bloodstream infections caused by flomoxef-resistant ESBL-KP isolates at our hospital was noted. These isolates also presented concomitant resistance to ciprofloxacin and amikacin (18). The presence of PMQR determinants, PMABL, and plasmid-mediated 16S rRNA methylase genes among these isolates and the associated genetic structures was further studied.

MATERIALS AND METHODS

Setting, bacterial isolates, and clinical features. The study was conducted at Kaohsiung Chang Gung Memorial Hospital, a 2,300-bed medical center providing both primary care and tertiary referral service in southern Taiwan, between March 2004 and November 2005. Flomoxef-resistant, ESBL-producing *K. pneumoniae* clinical isolates recovered from blood cultures were collected for further laboratory investigation. Review of medical records among the patients from whom the isolates were recovered was performed.

Susceptibility testing. ESBL production was confirmed by the double-disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) (7). MICs of amikacin, cefotaxime, cefotaxime-clavulanate, ciprofloxacin, ertapenem, and tigecycline were examined with Etest (AB Biodisk, Solna, Sweden). The results were interpreted according to the CLSI's recommendations (8), except that breakpoints recommended by the U.S. Food and Drug Administration were used for tigecycline susceptibility (susceptible, ≤ 2 $\mu\text{g/ml}$; resistant, ≥ 8 $\mu\text{g/ml}$) (22). MICs of flomoxef were determined by a standard broth microdilution method and interpreted according to a previous report (12). Quality control for the MIC determination was performed using reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

PFGE. Genetic relationships of the flomoxef-resistant ESBL-KP isolates were characterized by pulsed-field gel electrophoresis (PFGE) as previously described (17). Genomic DNA of the bacteria precast in plugs was digested with XbaI and subjected to PFGE in a CHEF Mapper XA system (Bio-Rad Laboratories, Hercules, CA). An auto-algorithm mode was selected for running molecular masses ranging from 50 to 500 kb. Band patterns were analyzed using Fingerprinting II software, version 3.0 (Bio-Rad Laboratories), and interpreted according to previously defined criteria (35).

Analysis of the QRDRs among gyrase genes. PCR amplification of the quinolone resistance-determining regions (QRDRs) among *gyrA*, *gyrB*, *parC*, and *parE* genes was performed on ciprofloxacin-resistant isolates as previously described (9). DNA sequencing was performed in both directions among the purified PCR products. The results were compared with the published sequence of *K. pneumoniae* (GenBank accession no. AP006725).

Analysis of OMP profiles and PCR sequencing of specific porin and β -lactamase genes. Outer membrane protein (OMP) profiles of the isolates were examined with a method described previously (16). To examine the DNA sequences of the OmpK35 and OmpK36 genes, two pairs of previously described primers were used for PCR amplification and sequence analysis (17). For the PCR detection of specific ESBL genes (*bla*_{SHV}, *bla*_{TEM}, and *bla*_{CTX-M}), four sets of previously reported primers were used (19). Multiplex PCR was performed to detect the presence of plasmid-mediated AmpC β -lactamase genes using the method described previously (28). DNA sequencing was performed as described above with the primers used for the respective PCR amplification.

Detection of genes for the class 1 integron, plasmid-mediated quinolone resistance, and 16S rRNA methylases. PCR amplification was performed to detect the presence of the class 1 integron structure and genes *qnrA*, *qnrB*, *qnrS*, *qepA*, *aac(6')-Ib*, *armA*, and *rmtB*, with primers listed in Table 1 (1, 21, 32, 34, 39–41). PCR-restriction fragment length polymorphism (PCR-RFLP) was used to examine the presence of *qnrB* variants, the wild-type allele *aac(6')-Ib*, and the mutant allele *aac(6')-Ib-cr*, as previously described (1, 34). RFLP analysis of the 496-bp *qnrB* PCR products was performed by using MvaI (Fermentas Inc., Hanover, MD), BglII (New England BioLabs, Beverly, MA), HindIII (New England BioLabs), and FokI (New England BioLabs). The 514-bp *aac(6')-Ib* PCR products were digested by the NdeI and TaaI enzymes (Fermentas Inc.). All amplified

products were sequenced using an automated sequencer (ABI Prism 3730 XL), and the results were compared with sequences published in the GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast/).

Conjugation. Transferability of the antimicrobial resistance among the clinical isolates of *K. pneumoniae* was performed using an azide-resistant strain, *E. coli* J53Az^r, as the recipient. Donors and the recipient were inoculated separately into LB broth and incubated at 37°C for 5 h. The bacterial cultures were then mixed together in a ratio of 1:3 (by volume) and were incubated overnight at 37°C. A portion (0.1 ml) of the overnight broth culture was spread onto an LB agar plate containing cefotaxime (2 $\mu\text{g/ml}$) and sodium azide (100 $\mu\text{g/ml}$). Transconjugants were then selected from the agar plate.

Plasmid analysis, replicon typing, and DNA-DNA hybridization. Plasmids of the transconjugants were extracted by the method described by Kado and Liu (15), followed by electrophoresis on a 0.8% agarose gel (SeaKem LE agarose). A multiplex PCR replicon typing system was used to analyze the conjugative resistance plasmids (4). DNA-DNA hybridization was performed with a digoxigenin labeling and detection kit (Roche, Mannheim, Germany).

Analysis of the genetic environments of transferable resistant determinants. The genetic context of the mobile element (ISCR1) associated with *qnrB* and *armA* was investigated by PCR mapping and subsequent sequencing. Plasmid DNA was extracted from the transconjugants by alkaline lysis (2) and amplified by PCR using the primers listed in Table 1 (21, 38, 41).

RESULTS

Bacterial strains and clinical features of the patients. During the study period, 546 patients were reported to have *K. pneumoniae* bacteremia. ESBL-producing *K. pneumoniae* isolates were recovered from the blood culture of 94 (17.2%) patients. Among them, 20 (22.9%) isolates were resistant to flomoxef and were included for further study. The median age of these patients was 42 years (range, 30 to 91 years), and 13 (65%) were male patients. No temporal or geographical relationship between the subjects was found. The majority of the patients were seriously ill with underlying diseases, had a prolonged hospital and/or intensive care unit stay, and received multiple antimicrobial agents before the development of the *K. pneumoniae* bacteremia. Lungs were the most common infection source of bacteremia (35%). β -Lactam antibiotics were the most common agent for empirical therapy. Three of the five patients treated with carbapenems as empirical therapy had favorable outcomes. The raw mortality rate was 70%.

Susceptibility testing, genotyping, β -lactamases, and OMP analysis. The 20 flomoxef-resistant ESBL-KP strains were fully susceptible to tigecycline and showed high resistance to ciprofloxacin (18 isolates; 90%) and amikacin (16; 80%) and some resistance to ertapenem (6; 30%). PFGE revealed 20 genotypes, indicating the diversity of the isolates. Several ESBLs were identified: SHV-5 ($n = 1$), SHV-12 ($n = 4$), CTX-M-3 ($n = 4$), and CTX-M-14 ($n = 14$). DHA-1 was the only PMABL discovered among the 20 isolates. OmpK35 deficiency was found in six isolates that carried either *bla*_{CTX-M-14} alone ($n = 5$) or *bla*_{CTX-M-14} plus *bla*_{SHV-12} ($n = 1$); three of them were resistant to ertapenem at low MICs of 1 to 2 $\mu\text{g/ml}$ (8). Another isolate that harbored both *bla*_{CTX-M-3} and *bla*_{SHV-5} genes was found to be deficient in both OmpK35 and OmpK36 and was resistant to ertapenem (MIC, 4 $\mu\text{g/ml}$) (8). In three of these isolates, a single nucleotide deletion at different positions, leading to a frameshift in the amino acid sequence, was found to be the major mechanism for the OmpK35 deficiency. For the remaining four isolates, no apparent mutational changes were found in either the promoter or the coding regions of the OmpK genes.

TABLE 1. Sequences of primers used for specific gene detection

Target	Primer	Sequence (5'-3')	Annealing temp (°C)	Position	GenBank accession no.	Reference
<i>qnrA</i>	qnrA-1A	TTCAGCAAGAGGATTTCTCA	60	325–344	AY070325	39
	qnrA-1B	GGCAGCACTATTACTCCCAA				
<i>qnrB</i>	qnrB-F	GATCGTGAAAGCCAGAAAGG	53	175–194	DQ351241	32
	qnrB-R	ACGATGCCTGGTAGTTGTCC				
<i>qnrS</i>	qnrS-1A	CAATCATAACATATCGGCACC	60	9748–9767	AB187515	39
	qnrS-1B	TCAGGATAAAACAACAATACCC				
<i>qepA</i>	qepA-F	GCAGGTCCAGCAGCGGGTAG	60	4179–4198	EF150886	40
	qepA-R	CTTCCTGCCCGAGTATCGTG				
<i>aac(6')-Ib</i>	qac1	GGCATCACTGCGTGTTCGCTCG	65	1862–1841	AF034958	1
	qac2	GACTGAGCATGACCTTGCG				
<i>armA</i>	armA-F	CCGAAATGACAGTTTCTATC	55	1931–1950	AY220558	41
	armA-R	GAAATGAGTGCCTTGAGG				
<i>rmtB</i>	rmtB-F	ATGAACATCAACGATGCCCT	50	1410–1429	AB103506	41
	rmtB-R	CCTTCTGATTGGCTTATCCA				
Class 1 integron	5'CS-F	GGCATCCAAGCAGCAAG	55	1236–1252	M73819	21
	3'CS-R	AAGCAGACTTGACCTGA				
<i>bla_{DHA-1}</i>	DHA-MF	AACTTTCACAGGTGTGCTGGGT	65	1244–1265	Y16410	28
	DHA-MR	CCGTACGCATACTGGCTTTGC				
<i>ISCR1</i>	orf513-F	CTTTTGCCCTAGCTGCGGT	60	7329–7347	DQ831141	21
	orf513-R	CTCACGCCCTGGCAAGGTTT				
<i>ISCR1</i> and <i>armA</i> junction	orf513-F	CTTTTGCCCTAGCTGCGGT	53	8953–8971	FJ715937	21
	armA-R	GAAATGAGTGCCTTGAGG				
3'-CS2	sul1-F1	GTATTGCGCCGCTCTTAGAC	55	7618–7637	FJ715937	This study
	orf513-R1	CGGTAAATGCCAGACGTTTT				
	ampR-F	GATTACTGCCGGTGCTCAAT	55	6164–6183	FJ715937	This study
	sul1-R1	AGGGTTTCCGAGAAGGTGAT				
<i>pap</i> operon and <i>ampC</i> junction	pspD5'	CCGGCAATCAGGCTAAATAA	55	19982–20001	AJ971344	38
	ampC3'	TCCGGA AAAACAGGTGGCGA				
<i>pap</i> operon and <i>qnrB</i> junction	qnrB5'	ACAGTGCCTAACGTTTGTCTG	50	67–86	FJ715937	This study
	pspA3'	ACGAACATCACGGGAAGAAC				

Detection of genes for the class 1 integron, plasmid-mediated quinolone resistance, and 16S rRNA methylase. An aminoglycoside-modifying gene *aac(6')-Ib*-containing class 1 integron was detected in 13 (65%) of the 20 isolates. The presence of *qnrB4* was detected in 17 isolates, including one coexisting with a *qnrA1* gene. In another single isolate, *qnrB2* alone was detected. The *aac(6')-Ib-cr* gene was present in 11 (55%) isolates that also harbored a *qnr* gene. No *qepA* was found. DHA-1-producing ESBL-KP isolates carrying plasmid-mediated quinolone resistance genes all harbored *bla_{CTX-M}* genes, and three of them also possessed a *bla_{SHV-12}* gene. Among the 16 amikacin-resistant isolates, 2 carried the *aac(6')-Ib* gene, and the other 14 carried the plasmid-mediated 16S rRNA methylase gene *armA*. Various combinations of *bla_{CTX-M}*, *bla_{DHA-1}*, *qnr*, *aac(6')-Ib-cr*, and *armA* genes were found in 11 isolates.

Conjugation and plasmid restriction enzyme digestion profile. Conjugation experiments were performed on all 20 isolates, and only four transconjugants were generated successfully (Table

2). The four donor strains expressed different PFGE patterns but all carried the *bla_{CTX-M-14}*, *bla_{DHA-1}*, *qnrB4*, *aac(6')-Ib-cr*, and *armA* genes simultaneously. After conjugation, all these resistance genes were transferred together into the respective transconjugants, as evidenced by PCR amplification using specific primers. A *bla_{SHV-12}* gene was also carried by two of the donors, KP134 and KP148, but was not found in their transconjugants. Accordingly, the MICs of cefotaxime, cefotaxime-clavulanate, flomoxef, and amikacin all increased considerably compared to those of the recipient *E. coli* J53. Two donor strains, KP148 and KP243, were found to have 2 to 3 mutations in their QRDRs of the *gyrA* and *parC* genes, hence contributing to the high MICs of ciprofloxacin observed.

Replicon typing of plasmid and DNA-DNA hybridization. Figure 1 shows plasmid analysis and DNA-DNA hybridization for the four transconjugants and their donors. Although multiple plasmids were found among the donor isolates, a single plasmid that concomitantly harbored the resistance determi-

TABLE 2. Antimicrobial resistance genes and susceptibility profiles of four DHA-1/ESBL-producing *K. pneumoniae* isolates and their transconjugants in *E. coli* J53

Bacterium	Resistance gene profile					MIC ($\mu\text{g/ml}$) ^a						Porin deficiency ^b	
	<i>bla</i>	<i>qnr</i>	<i>aac(6')-Ib</i>	16S rRNA methylase	QRDR ^c <i>gyrA</i> <i>parC</i>	CTX/CTX-CLA	FLO	CIP	AMK	TGC	ERT		
KP105	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	WT	>16/1	128	2	>256	0.5	0.5	None	
J53/pKP105	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	ND	>16/1	8	0.5	>256	0.25	0.05	ND	
KP134	<i>bla</i> _{SHV-12} <i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	WT	>16/1	128	2	>256	0.5	0.5	None	
J53/pKP134	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	ND	>16/1	64	0.25	>256	0.25	0.5	ND	
KP148	<i>bla</i> _{SHV-12} <i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	S83I	S80I	>16/1	>128	>32	>256	0.5	2	OmpK35
J53/pKP148	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	ND	>16/1	8	1	>256	0.25	0.012	ND	
KP243	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	S83F D87A	S80I	>16/1	64	>32	>256	1	1	OmpK35
J53/pKP243	<i>bla</i> _{CTX-M-14} <i>bla</i> _{DHA-1}	<i>qnrB4</i>	<i>aac(6')-Ib-cr</i>	<i>armA</i>	ND	>16/1	32	0.5	>256	0.5	1	ND	
<i>E. coli</i> J53	ND					0.25/0.032	0.125	0.008	2	0.25	0.012	ND	

^a CTX, cefotaxime; CLA, clavulanic acid; FLO, flomoxef; CIP, ciprofloxacin; AMK, amikacin; TGC, tigecycline; ERT, ertapenem.

^b ND, not done.

^c QRDR, quinolone resistance-determining region; WT, wild type; ND, not done; S, serine; I, isoleucine; F, phenylalanine; D, aspartic acid; A, alanine.

nants, *bla*_{CTX-M-14}, *aac(6')-Ib*, *qnrB*, *armA*, and *bla*_{DHA-1}, in the four transconjugants was found. In strain KP105, two bands were demonstrated by the DNA-DNA hybridization, using the amplicons of *qnrB4*, *armA*, and *bla*_{DHA-1} as the probes (Fig. 1B, lane 2). Enzyme digestion of the plasmid DNA by EcoRI, KpnI, and SalI (all obtained from New England BioLabs) was performed. The subsequent DNA-DNA hybridization using the same probes as mentioned above each produced only one single band with the use of each respective probe (data not shown), indicating that the two bands were very likely from the same plasmid. PCR-based replicon typing analysis indicated that the self-transferable plasmid from strain KP243 was a FIIA plasmid, while the remaining three transferable plasmids were nontypeable by the multiplex system.

Prevalence of ISCR1 and the genetic environment flanking the ISCR1 element. The presence of ISCR1 was confirmed by PCR amplification among the four self-transferable resistance plasmids. Further investigation indicated that the insertion of the ISCR1 truncated the *su11*-type integrons' incomplete 3' conserved sequences (3'-CS). ISEc28 and the aminoglycoside resistance gene *armA* were found downstream of ISCR1, while the AmpC-type *bla*_{DHA-1} gene and its regulator, *ampR*, were located immediately after the incomplete 3'-CS. A phage shock protein operon and the *qnrB4* gene were located downstream of the *bla*_{DHA-1} gene (Fig. 2). The genetic environments flanking the ISCR1 element in the four studied transconjugants were identical.

ISCR1 was also detected in 9 of the remaining 16 clinical isolates tested. In 11 of the 13 ISCR1-containing plasmids,

various combinations of *bla*_{CTX-M}, *bla*_{DHA-1}, *qnr*, *aac(6')-Ib-cr*, and *armA* genes were also presented.

DISCUSSION

Currently, carbapenems are still regarded as the drug of choice for infections caused by ESBL-producing pathogens (27). However, the selective pressure from increasing use of carbapenems may lead to the development of carbapenem-resistant microbes or even the prevalence of other multidrug-resistant pathogens (6). An effective alternative to carbapenems, such as flomoxef, may relieve this selective pressure and offer an option to carbapenem-allergic patients, when necessary (20). Therefore, the increase of flomoxef resistance in ESBL-KP represents a clinical disturbance that requires further attention. Previously, we have found that patients with bacteremia caused by flomoxef-resistant ESBL-KP are more likely to receive inappropriate empirical therapy due to the multidrug resistance and are associated with a higher rate of mortality within 14 days (18). While no other clinical risk factors for the acquisition of flomoxef resistance among ESBL-KP isolates could be identified, laboratory investigation was conducted herein to reveal the related microbiological factors.

Most of the PMQR determinants or plasmid-mediated 16S rRNA methylase genes were shown to be associated with ESBL genes (31, 39, 43). To our knowledge, the present report is the first to show the simultaneous existence of *bla*_{DHA-1} with *bla*_{CTX-M}, *qnrB4*, *aac(6')-Ib-cr*, and *armA* genes carried by a

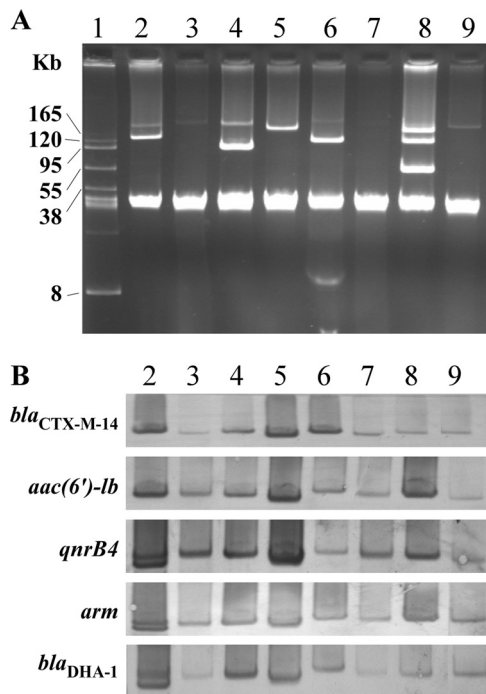


FIG. 1. Plasmid profiles (A) and DNA-DNA hybridization (B) of four DHA-1/ESBL-producing *K. pneumoniae* isolates and their transconjugants. The probes used are as indicated. Lane 1, molecular mass marker, BAC-Tracker-supercoiled DNA ladder (Epicentre, Madison, WI); lanes 2 to 9, *K. pneumoniae* KP105, *E. coli* J53/pKP105, KP134, J53/pKP134, KP148, J53/pKP148, KP243, and J53/pKP243, respectively.

single self-transferable plasmid. The diverse genotypes of the donor bacteria further indicated that the multidrug resistance among the organisms was associated with the resistance plasmids rather than the clonal expansion of a multiple resistant strain.

The *armA* gene is a part of the functional composite transposon Tn1548 in plasmid pIP1204 (10). Study of the flanking region of pIP1204 revealed that two insertion sequences, IS6100 and ISCR1, are located upstream of the *armA* gene (10). In the present study, ISEc28 and *armA* genes were found downstream of ISCR1 (Fig. 2), suggesting that the presence of ISCR1 may lead to the comobilization of the plasmid-mediated genes. Recently, researchers in China characterized the complete nucleotide sequence of *K. pneumoniae* plasmid pKP048 (GenBank accession no. FJ628167), which carries genes *bla*_{KPC-2}, *bla*_{DHA-1}, *qnrB4*, and *armA* (14). In the present study, ISCR1 was flanked by *armA*, *bla*_{DHA-1}, and *qnrB* genes in the four self-transferable *bla*_{DHA-1}-carrying plasmids (Fig. 2), similar to the 25-kbp region consisting of *armA*, ISCR1, *bla*_{DHA-1}, and *qnrB4* genes in the plasmid pKP048 detected in 2006 (14). Presumably, there is a relationship between ISCR1 and antimicrobial resistance genes. In our study, although self-transferable plasmids were successfully identified in four isolates, replicon typing revealed that the plasmids may not be derived from the same sources. On the other hand, 13 of the 20 isolates tested harbored ISCR1, and various combinations of *bla*_{CTX-M}, *bla*_{DHA-1}, *qnr*, *aac(6)-Ib-cr*, and *armA* genes were found in 11 of these isolates. It appears that ISCR1 plays an important role

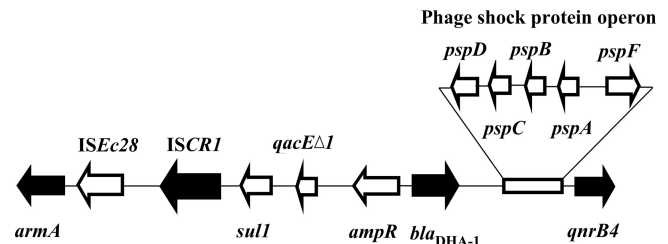


FIG. 2. Genetic environment of ISCR1, associated with *armA*, *bla*_{DHA-1}, and *qnrB4* genes (black arrows).

in the transfer of *bla*_{DHA-1}, together with an array of other resistance genes among our ESBL-KP isolates.

The transconjugants showed only low-level quinolone resistance, suggesting that high-level quinolone resistance in the original isolates may result from the coordinating action of several resistance mechanisms, such as QRDR point mutations (Table 2). The *qnr* genes alone may not confer resistance to quinolone (24), but they provide the first step toward further *in vivo* selection of high-level resistance against quinolones (30). Park et al. also observed the presence of *qnrB* in isolates with a wide range of quinolone MIC values, including full susceptibility (26). The wide range of MICs observed among *qnr*-positive bacteria suggests that the phenotypic detection of *qnr* genes in clinical isolates might be difficult. The presence of *aac(6)-Ib-cr* also does not always relate to high MICs of quinolones. Nine of the eleven isolates that possessed both *qnr* and *aac(6)-Ib-cr* genes demonstrated high quinolone MICs of over 32 μ g/ml. The phenomenon suggests that these two genetic factors may be synergistic in increasing quinolone resistance.

Our study revealed the coexistence of resistance genes encoding DHA-1, SHV, and CTX-M enzymes in *K. pneumoniae* and that the *bla*_{DHA-1} gene was associated with *aac(6)-Ib-cr*, *armA*, and *qnrB4* genes. The PCR-based replicon typing system provides a novel method to describe the dissemination and to follow the evolution of plasmid-mediated resistance (5). Through this method, one of the self-transferable plasmids identified in the present study was determined to be a rare FIIA type, while the remaining three remain to be defined. Because literature reports on plasmid replicon typings are still rare, further research is required to determine the influence of acquired resistance on plasmid trafficking. Horizontal gene transfer, especially that associated with ISCR1, appears to play an important role in the dissemination of the multidrug-resistant *K. pneumoniae* studied herein. Further investigations of the ISCR1 elements that may have contributed to the rapid dissemination of antibiotic resistance genes are warranted.

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