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Characterization of Conjugative Plasmids Carrying Antibiotic Resistance Genes Encoding 16S rRNA Methylase, Extended-Spectrum Beta-Lactamase, and/or Plasmid-Mediated AmpC Beta-Lactamase

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In this study, we identified extended-spectrum ß-lactamase (ESBL) and plasmid-mediated AmpC ß-lactamase which were associated with 16S rRNA methylase gene on the conjugative plasmid. Among 82 clinical isolates of *Enterobacteriaceae* that carry 16S rRNA methylase gene (64 strains, *armA*, and 18 strains, *rmtB*), bla_{SHV-12} was detected either alone or combined with bla_{DHA-1}, $bla_{CTX-M-3}$, and $bla_{CTX-M-14}$ in 30 strains carrying *armA* and 6 strains carrying *rmtB*. The $bla_{CTX-M-3}$ was detected in 13 of 64 strains carrying *armA* but no strains carrying *rmtB*. Whereas $bla_{CTX-M-14}$ was detected in 15 of 18 strains carrying *rmtB* but only 2 of 64 strains carrying *armA*. Overall, bla_{SHV-12} and $bla_{CTX-M-14}$ was the most common ESBL gene which was associated with *armA* and *rmtB*, respectively. In addition, we found that $bla_{CTX-M-3}$ localized with *armA* on the same IncL/M plasmid and $bla_{CTX-M-14}$ localized with *rmtB* on the same IncA/C plasmid. Restriction fragment length polymorphism of conjugative plasmids and pulsed-field gel electrophoresis of genomic DNAs revealed that intercellular horizontal transfer of conjugative plasmid and clonal transmission have been occurred at the same time.

***Keywords*:** 16S rRNA methylase, extended-spectrum beta-lactamase, IncL/M, IncA/C, Enterobacteriaceae

Since the first plasmid-encoded 16S rRNA methylase, RmtA, was discovered from a *Pseudomonas aeruginosa* isolated in Japan (Yokoyama *et al.*, 2003), a series of plasmid-encoded 16S rRNA methylases have emerged in Gram-negative bacilli (Galimand *et al.*, 2003; Doi *et al.*, 2004; Wachino *et al.*, 2006). They confer an extremely high-level of resistance to almost all clinically important aminoglycosides by altering the microbial ribosome. So far six types of plasmid-encoded 16S rRNA methylases, RmtA, RmtB, RmtC, RmtD, NpmA, and ArmA, have been reported in several nosocomial pathogens, including *P. aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii*, and *Proteus mirabilis* (Galimand *et al.*, 2003; Yokoyama *et al.*, 2003; Doi *et al.*, 2004; Galimand *et al.*, 2005; Gonzalez-Zorn *et al.*, 2005b; Lee *et al.*, 2006; Wachino *et al.*, 2006; Wachino *et al.*, 2007; Yamane *et al*., 2008). In Korea, three recent reports demonstrated the dissemination of 16S rRNA methylases among Gram-negative bacilli (Lee *et al.*, 2006; Park *et al.*, 2006; Kang *et al.*, 2008).

It was reported that the dissemination of genes encoding 16S rRNA methylases was mediated by mobile genetic elements carried by large transferable plasmids and linked often to other resistance determinants such as bla_{TEM-1}, $bla_{CTX-M-3}$, $bla_{CTX-M-14}$, *sul1*, and *dfrXII* (Galimand *et al.*, 2003; Galimand *et al.*, 2005; Gonzalez-Zorn *et al.*, 2005a; Yamane *et al.*, 2005; Golebiewski *et al.*, 2007). In a Taiwanese study, most, but not all, of the *armA*- or *rmtB*-positive *E. coli* and *K. pneumoniae* isolates had extended-spectrum ß-lactamases (ESBLs) such as $bla_{CTX-M-3}$, $bla_{SHV-5-like}$, or $bla_{CTX-M-14}$ (Yan *et al.*, 2004). In a very recent study done in Belgium, all 16S rRNA methylase-bearing strains produced ESBLs, predominantly CTX-M-3, as well as various types of ß-lactamases (Bogaerts *et al.*, 2007). In our very recent study on the distribution of 16S rRNA methylases genes among the *Enterobacteriaceae* isolates in a Korean hospital, the association between 16S rRNA methylase gene and other antimicrobial resistance genes conferring resistance to extended-spectrum ß-lactams and/or quinolones on the conjugative plasmids was also suggested (Kang *et al.*, 2008). Here, we identified ESBL and plasmid-mediated AmpC ß-lactamase (pACBL) which were associated with 16S rRNA methylase gene on the conjugative plasmid. We also investigated phenotypic and genotypic characteristics of the conjugative plasmids carrying both 16S rRNA methylase gene and bla_{CTX-M}.

Materials and Methods

Bacterial strains

Among the clinical isolates of *Enterobacteriaceae* collected between 1995 and 2005 at the Kyungpook National University Hospital in Korea, we selected 96 isolates which showed a high-level of resistance to amikacin (MIC>128 μg/ml) and were able to transfer amikacin resistance by conjugation experiment. MIC against amikacin was determined by the agar dilution method (CLSI, 2006). Conjugation assays were performed by using a broth mating method and *E. coli* RG

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488 Rif^r and J53 Azide^r were used as recipient bacteria. The transconjugants were selected on Muller-Hinton agar supplemented with amikacin (64 μg/ml) and rifampin (50 μg/ml) or sodium azide (200 μg/ml). Each isolate was from a single patient and bacterial species were identified by using GN cards on the VITEK®2 system (bioMérieux, France) or API 20E system (bioMérieux).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing and determination of MICs were performed by the agar dilution method (CLSI, 2006). The antimicrobial agents included were gentamicin and kanamycin (DUCHEFA, Netherland), amikacin and trimethoprim (ICN Biomedicals, USA), streptomycin (Sigma Chemical Co., USA), ampicillin (USB, USA), cefoxitin (Sigma), cefotaxime (Sigma), cefepime (Boryung Inc., Korea), aztreonam (Sigma), ceftazidime (Sigma), ciprofloxacin (Fluka, Switzerland), chloramphenicol (Sigma), tetracycline (Sigma), and sulfamethoxazole (Sigma). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Detection of antimicrobial resistance genes

The PCR method and primers described by Yan *et al*. (2004) were used to detect the genes encoding 16S rRNA methylases such as *rmtA*, *rmtB*, and *armA*. A multiplex PCR (Chia *et al.*, 2005) was performed to detect bla_{SHV}, $bla_{CTX\text{-}M\text{-}3\text{-}like}$, and $bla_{CTX\text{-}M\text{-}14\text{-}like}$ genes and a simplex PCR (Kim *et al.*, 2005b) was performed to detect bla_{TEM}, $bla_{DHA\text{-}1}$, and $bla_{CTX\text{-}M\text{-}1\text{-}like}$. The isolates carrying bla_{SHV}, $bla_{CTX\text{-}M\text{-}3\text{-}like}$ or $bla_{CTX\text{-}M\text{-}14\text{-}like}$ were re-subjected to a PCR using primers described by Kim *et al*. (2005a) and both strands of the PCR products were sequenced with the same primers by the dideoxy-chain termination method with an ABI 3700 autosequencer (Applied Biosystem, USA). Sequence analyses and comparison with known sequences were performed with the BLAST program at the National Center for Biotechnology Information.

PCR-based replicon typing

To identify the incompatibility group of plasmids, a PCR-based Inc/rep typing was performed as described by Carattoli *et al*. (2005). Plasmid DNAs were amplified by 5 multiplex- and 3 simplex-PCRs using eighteen pairs of primers, recognizing FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. As positive controls of the PCR reactions, R27 (HI1), R478 (HI2), R483 (I1), R446b (L/M), RN3 (N), Tp181 (FIme), RS-a (W), RP4(P), R40a (A/C), Rts1 (T), R124 (FIV), R387 (K), R16 (B/O), and R6K (X) were used.

Table 1. Detection of extended-spectrum ß-lactamase and plasmid-mediated AmpC ß-lactamase genes in the clinical isolates of *Enterobacteriaceae* and their transconjugants carrying a 16S rRNA methylase gene

Strains (No. of strains tested) and ESBL(s)[a]	No. of strains carrying 16S rRNA methylase gene	
	armA	*rmtB*
Clinical isolates (96)	**64**	**18**
***K. pneumoniae* (40)**	**28**	**9**
SHV-12	11	
SHV-12 and DHA-1	11	
SHV-12 and CTX-M-3	2	
CTX-M-3	1	
SHV-12 and CTX-M-14	1	6
CTX-M-14		3
***E. coli* (25)**	**10**	**9**
SHV-12	2	
CTX-M-3	3	
CTX-M-14	1	6
***E. cloacae* (18)**	**14**	
SHV-12	2	
***S. marcescens* (6)**	**6**	
SHV-12 and CTX-M-3	1	
CTX-M-3	5	
***C. freundii* (3)**	**3**	
CTX-M-3	1	
Others (4)	**3**	
SHV-12	0	
Transconjugants (96)	**64**	**18**
SHV-12	10	
SHV-12 and DHA-1	10	
DHA-1	1	
SHV-12 and CTX-M-3	1	
CTX-M-3	12	
CTX-M-14		15

[a] TEM-type β-lactamase is not described in this table.

Plasmid analysis, Southern hybridization, and RFLP of plasmid DNA

Plasmids were isolated from transconjugants by the alkaline lysis method (Birnboim and Doly, 1979). Extracted plasmid DNAs were subjected to agarose gel electrophoresis and then transferred onto a positively charged nylon membrane (Boehringer Mannhein, Germany) using the capillary method. To prepare probes to be used for Southern hybridization analyses, the purified PCR products obtained from the PCR targeting *armA*, *rmtB*, $bla_{\text{CTX-M-3}}$, and $bla_{\text{CTX-M-14}}$ were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) according to the manufacturer's instructions. A digoxigenin DNA labeling and detection kit (Boehringer Mannheim) was used for hybridization analyses. The similarity of plasmid DNAs was evaluated by analyzing restriction fragment length polymorphism (RFLP) of plasmid DNAs using *Hin*dIII restriction enzyme (Boehringer Mannhein).

Pulsed-field gel electrophoresis (PFGE)

The genomic DNAs of the clinical isolates of *Enterobacteriaceae* were examined by the PFGE method, as described previously (Gautom, 1997). Briefly, after growth with shaking in a Luria broth (Invitrogen Ltd., United Kingdom) at 37°C overnight, genomic DNAs were digested with *Xba*I (Boehringer Mannheim) for 18 h and separated on a 1.0% agarose gel using a contour-clamped homogeneous-field apparatus (CHEF DRIII Systems, Bio-Rad Laboratories, USA) in a 0.5× TBE buffer (0.09 M of Tris, 2 mM of disodium EDTA; pH 8.5, 0.09 M of boric acid) at a constant temperature of 14°C at 6 V/cm for 20 h with an increasing pulse time between 5 and 40 sec.

Results

Among the 96 clinical isolates of *Enterobacteriaceae* showing a high-level resistance to amikacin, 64 isolates and 18 isolates carried *armA* and *rmtB*, respectively (Table 1). The 64 *armA*-carrying isolates included 28 *K. pneumoniae*, 14 *Enterobacter cloacae*, 10 *E. coli*, 6 *S. marcescens*, 3 *Citrobacter freundii*, 1 *Enterobacter sakazakii*, 1 *Klebsiella oxytoca*, and 1

Table 2. Phenotypic and genotypic characteristics of transconjugants and clinical isolates that carry both a 16S rRNA methylase gene and $bla_{\text{CTX-M}}$ gene

Transconjugants	Replicon type(s) of plasmid	Antimicrobial resistance pattern of transconjugants	Clinical isolates	Year of isolation
Transconjugants that received plasmid carrying *armA* and $bla_{\text{CTX-M-3}}$			*E. coli* AK168	2003
pK1681	L/M	AkKmGmTbApCtSu	*E. coli* AK10	2003
pK101	L/M	AkKmGmTbApCtCfAz	*E. coli* AK426	2004
pK4261	L/M	AkKmGmTbApCt	*S. marcescens* AK2	2003
pK21	L/M	AkKmGmTbApCt	*S. marcescens* AK4	2003
pK41	L/M	AkKmGmTbApCt	*S. marcescens* AK5	2003
pK51	L/M	AkKmGmTbApCt	*S. marcescens* AK6	2003
pK61	L/M	AkKmGmTbApCt	*S. marcescens* AK8	2003
pK81	L/M	AkKmGmTbApCt	*C. freundii* AK17	2003
pK171	L/M	AkKmGmTbApCt	*K. pneumoniae* AK423	2004
pK4231	L/M	AkKmGmTbApCtAz	*K. pneumoniae* AK18	2003
pK181	L/M, F	AkKmGmTbApCt	*S. marcescens* AK422	2004
pK4221	L/M, A/C	AkKmGmTbSmApCtSuTp		
Transconjugants that received plasmid carrying *rmtB* and $bla_{\text{CTX-M-14}}$				
pK5061	A/C	AkKmGmTbSmApCtCmTcSuTp	*K. pneumoniae* AK506	2005
pK5121	A/C	AkKmGmTbSmApCtCfCmTcSuTp	*K. pneumoniae* AK512	2005
pK5141	A/C	AkKmGmTbSmApCtCfCmTcSuTp	*K. pneumoniae* AK514	2005
pK1411	A/C	AkKmGmTbSmApCtCfCmTcSuTp	*K. pneumoniae* AK141	2001
pK1441	A/C	AkKmGmTbSmApCtCmTcSuTp	*K. pneumoniae* AK144	2001
pK1541	A/C	AkKmGmTbSmApCtCmTcSuTp	*K. pneumoniae* AK154	2001
pK1471	A/C	AkKmGmTbApCtCmTcSuTp	*K. pneumoniae* AK147	2001
pK1481	A/C	AkKmGmTbSmApCtCmTcSuTp	*K. pneumoniae* AK148	2001
pK1601	A/C	AkKmGmTbApCtCmTcSuTp	*K. pneumoniae* AK160	2001
pK1631	A/C	AkKmGmTbSmApCtCmTcSuTp	*E. coli* AK163	2002
pK2011	A/C	AkKmGmTbSmApCtCmTcSuTp	*E. coli* AK201	2002
pK2061	A/C	AkKmGmTbSmApCmTcSuTp	*E. coli* AK206	2002
pK2131	A/C, F	AkKmGmTbSmApCtCmTcSuTp	*E. coli* AK213	2002
pK4291	A/C	AkKmGmTbSmApCtCmTcSuTp	*E. coli* AK429	2004
pK5171	A/C, F, FIA	AkKmGmTbSmApCtCmTcSuTp	*E. coli* AK517	2005

Abbreviations: Ak, amikacin; Km, kanamycin; Gm, gentamicin; Tb, tobramycin; Sm, streptomycin; Ap, ampicillin; Ct, cefotaxime; Az, aztreonam; Cm, chloramphenicol; Tc, tetracycline; Su, sulfamethoxazole; Tp, trimethoprim

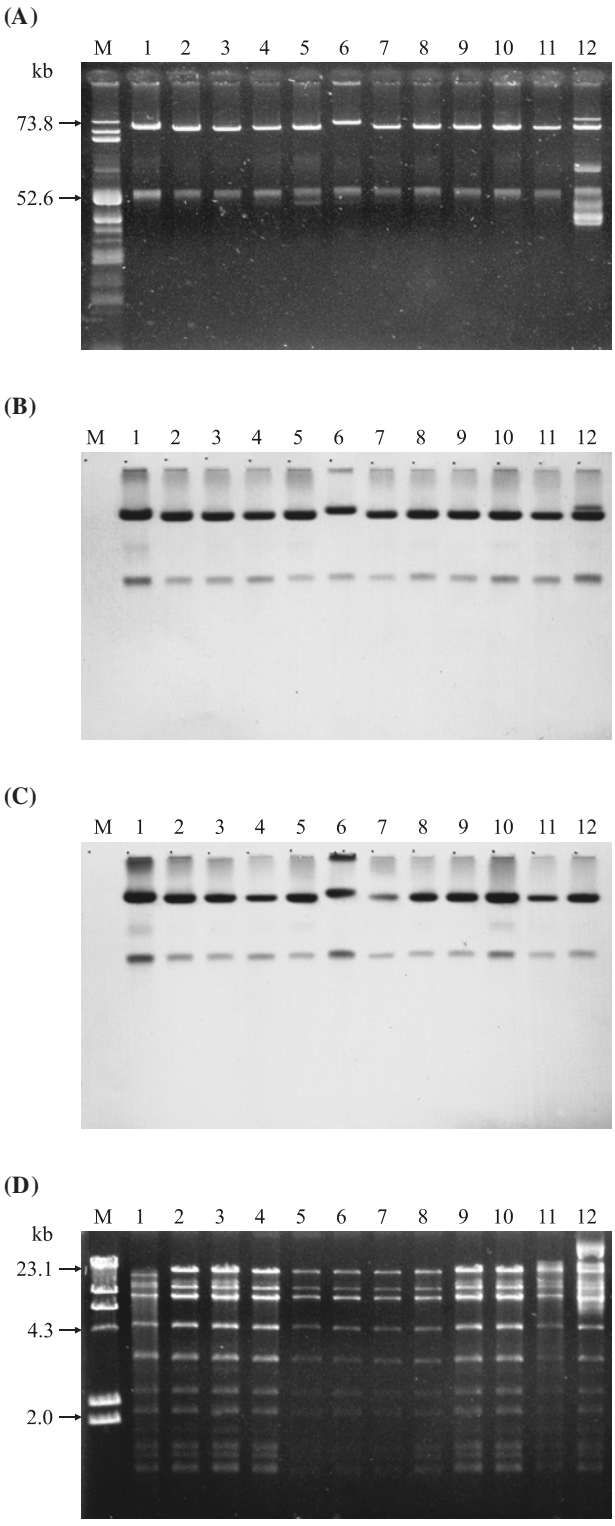

Fig. 1. Plasmid DNAs extracted from the conjugants carrying *armA*, *aacA4*, and $bla_{\text{CTX-M-3}}$. (A) Electrophoresis of the plasmid DNAs, (B and C) Hybridized plasmid DNAs with each probe specific for *armA* (B) and $bla_{\text{CTX-M-3}}$ (C), and (D) Digested fragments of the plasmid DNAs by *Hin*dIII. Lanes: M, KE327; 1, pK1681; 2, pK101; 3, pK4261; 4, pK21; 5, pK41; 6, pK51; 7, pK61; 8, pK81; 9, pK171; 10, pK4231; 11, pK181; and 12, pK4221.

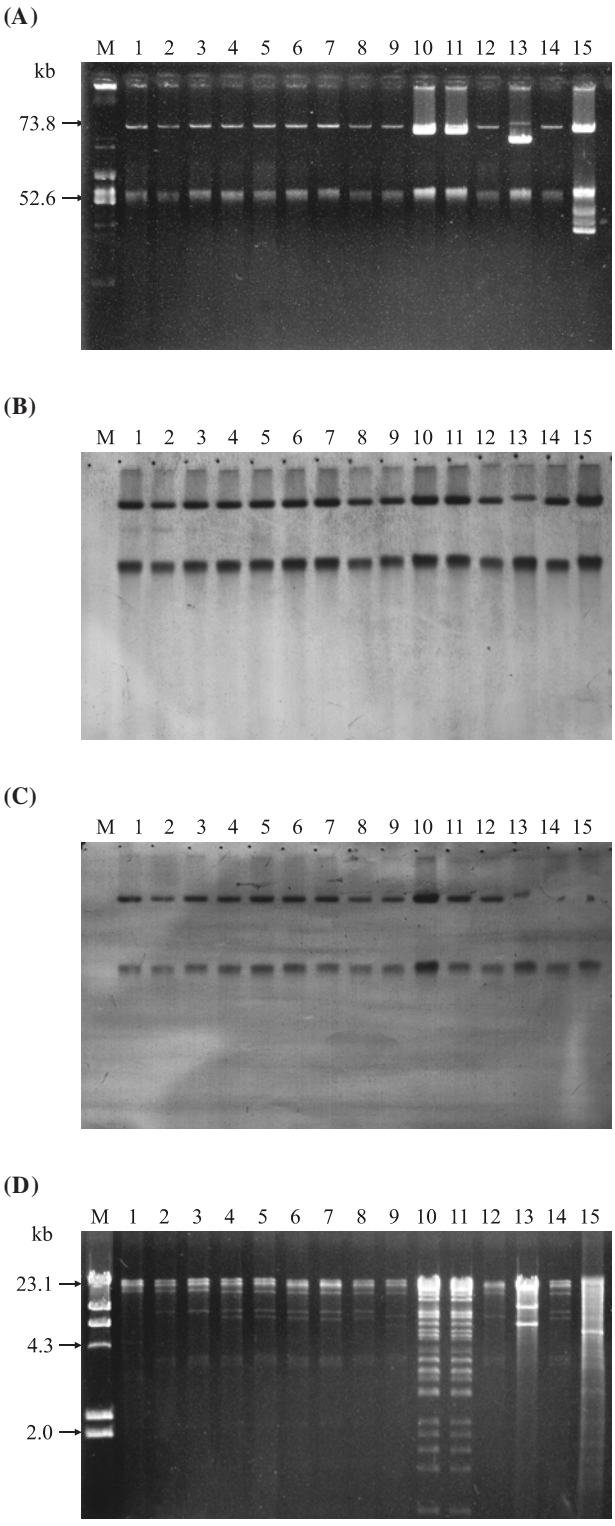

Fig 2. Plasmid DNAs extracted from the conjugants carrying both *rmtB* and $bla_{\text{CTX-M-14}}$. (A) Electrophoresis of the plasmid DNAs, (B and C) Hybridized plasmid DNAs with each probe specific for *rmtB* (B) and $bla_{\text{CTX-M-14}}$ (C), and (D) digested fragments of the plasmid DNAs by *Hin*dIII. Lanes: M, KE327; 1, pK5061; 2, pK5121; 3, pK5141; 4, pK1411; 5, pK1441; 6, pK1471; 7, pK1481; 8, pK1541; 9, pK1601; 10, pK1631; 11, pK2011; 12, pK2061; 13, pK2131; 14, pK4291; and 15, pK5171.

Morganella morganii. The 18 *rmtB*-carrying isolates included 9 *K. pneumoniae* and 9 *E. coli* isolates. All the isolates that carried *armA* or *rmtB* revealed a high level of resistance (MIC≥512 μg/ml) to aminoglycosides including amikacin, tobramycin, kanamycin, and gentamicin.

In PCR and sequencing experiments, $bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{CTX-M-14}}$ were identified in the *armA*-bearing isolates. $bla_{\text{CTX-M-14}}$ and $bla_{\text{SHV-12}}$ were identified in the *rmtB*-bearing isolates (Table 1). The $bla_{\text{SHV-12}}$ gene was detected either alone or combined with $bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{CTX-M-14}}$ in 30 of the 64 *armA*-bearing isolates and 6 of the 18 *rmtB*-bearing isolates. The $bla_{\text{CTX-M-3}}$ gene was detected in 13 of the 64 *armA*-bearing isolates and none of the 18 *rmtB*-bearing isolates. The $bla_{\text{CTX-M-14}}$ gene was detected in 2 of the 64 *armA*-bearing isolates and 15 of the 18 *rmtB*-bearing isolates. Therefore, $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-14}}$ was the most common ESBL gene which was associated with *armA* and *rmtB*, respectively. From the 21 of 64 transconjugants received *armA*, $bla_{\text{SHV-12}}$ was also detected. $bla_{\text{DHA-1}}$ and $bla_{\text{CTX-M-3}}$ were detected either alone or combined with $bla_{\text{SHV-12}}$ in 11 and 13 transconjugants received *armA,* respectively. $bla_{\text{CTX-M-14}}$ was detected in 15 of the 18 transconjugants received *rmtB* (Table 1).

PCR-based replicon typing of the plasmid incompatibility groups showed that the plasmids carrying *armA* and $bla_{\text{CTX-M-3}}$ belonged to the IncL/M group and the plasmids carrying *rmtB* and $bla_{\text{CTX-M-14}}$ belonged to the IncA/C group (Table 2). However, none of the replicon types of plasmid incompatibility group was detected from the transconjugants carrying *armA*, $bla_{\text{SHV-12}}$ and/or $bla_{\text{DHA-1}}$. Whereas transconjugants carrying the IncL/M plasmid revealed resistance to aminoglycosides and cefotaxime, transconjugants carrying the IncA/C plasmid revealed multiple drug resistances to chloramphenicol, tetracycline, sulfamethoxazole, and trimethoprim as well as aminoglycosides and cefotaxime (Table 2).

In all of the transconjugants that carried *armA* and $bla_{\text{CTX-M-3}}$, approximately 70 kb-sized plasmid was detected and hybridized to both *armA* and $bla_{\text{CTX-M-3}}$ probes (Fig. 1A to C). In all of the transconjugants that carried *rmtB* and $bla_{\text{CTX-M-14}}$, approximately 70 kb sized plasmid was hybri-

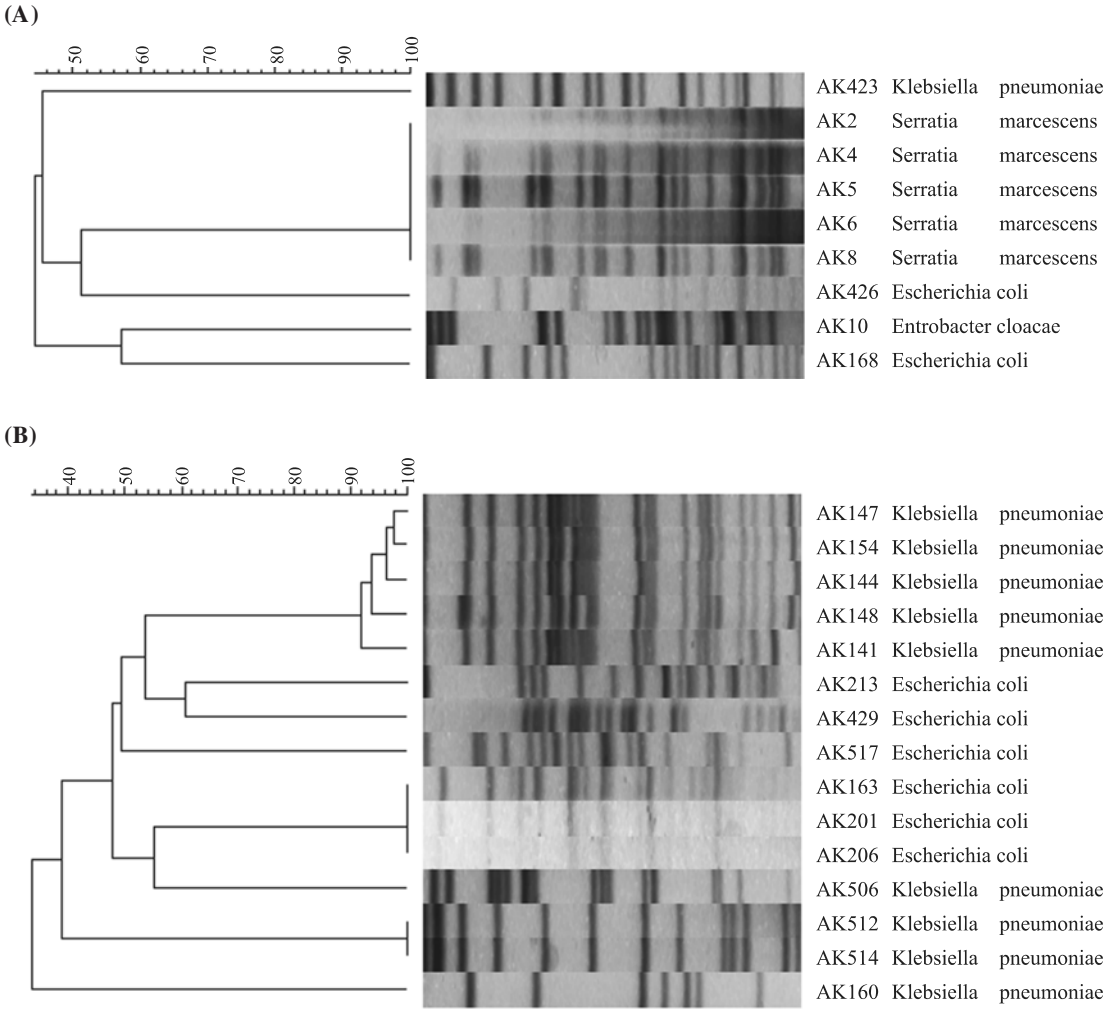

Fig 3. Dendrogram generated by Gel Compar II software showing the genomic relatedness of clinical isolates carrying *armA* and $bla_{\text{CTX-M-3}}$ (A) and clinical isolates carrying *rmtB* and $bla_{\text{CTX-M-14}}$ (B) determined by PFGE.

dized to the *rmtB* probe as well as the $bla_{\text{CTX-M-14}}$ probe (Fig. 2B and C). However, extraction of plasmid DNA from the transconjugants carrying *armA*, $bla_{\text{SHV-12}}$, and/or $bla_{\text{DHA-1}}$ was failed on the repeated attempts. For the reason, co-localization of *armA* with $bla_{\text{SHV-12}}$ and/or $bla_{\text{DHA-1}}$ could not be defined in this study.

The RFLP patterns of the IncL/M plasmids carrying *armA* and $bla_{\text{CTX-M-3}}$ were very similar to each other or the same although the host bacterial species were various including *K. pneumoniae*, *E. coli*, *S. marcescens*, and *C. freundii* (Fig. 1D and Table 2), suggesting intercellular horizontal transfer of the plasmid among the family *Enterobacteriaceae*. The RFLP patterns of the IncA/C plasmids carrying *rmtB* and $bla_{\text{CTX-M-14}}$ were the same or very similar to each other in the case of *K. pneumoniae* but various in the case of *E. coli* (Fig. 2D and Table 2). The RFLP pattern of plasmid (pK4291) carried by one *E. coli* strain (AK429) was same to the plasmids carried by five *K. pneumoniae* strains (AK141, AK144, AK147, AK154, and AK148).

In PFGE analysis of genomic DNAs, 5 strains of *S. marcescens* carrying *armA* and $bla_{\text{CTX-M-3}}$ revealed the same pattern, suggesting the same clone, but the patterns of 3 strains of *E. coli* carrying *armA* and $bla_{\text{CTX-M-3}}$ were different from each other (Fig. 3A and Table 2). In the case of *K. pneumonia* strains carrying *rmtB* and $bla_{\text{CTX-M-14}}$, 5 of 6 strains isolated in 2001 showed very similar PFGE pattern and 2 of 3 strains isolated in 2005 showed the same PFGE pattern. In the case of *E. coli* carrying *rmtB* and $bla_{\text{CTX-M-14}}$, 3 of 4 strains isolated in 2002 showed the same PFGE pattern (Fig. 3B and Table 2).

Discussion

In this study, ESBLs and pACBL which were associated with 16S rRNA methylase on the conjugative plasmid were identified; $bla_{\text{SHV-12}}$, $bla_{\text{DHA-1}}$, and $bla_{\text{CTX-M-3}}$ with *armA* and $bla_{\text{CTX-M-14}}$ with *rmtB*. This study also revealed the co-localization of $bla_{\text{CTX-M}}$ and 16S rRNA methylase gene on the conjugative plasmid of a broad-host-range; $bla_{\text{CTX-M-3}}$ and *armA* on the IncL/M plasmid and $bla_{\text{CTX-M-14}}$ and *rmtB* on the IncA/C plasmid.

Until the late 1990s, the major ESBLs found in Korea were TEM and SHV variants, especially TEM-52 and SHV-12 (Kim *et al.*, 1998; Pai *et al.*, 1999), but the predominance of CTX-M enzymes in the isolates of *E. coli* was observed in a survey of 2003 (Kim *et al.*, 2005a) after the first report of CTX-M-14 in 2001 in Korea (Pai *et al.*, 2001). As well in Korea, an extremely rapid worldwide dissemination of CTX-M enzymes has been reported in various geographic regions (Bonnet, 2004; Livermore *et al.*, 2007) and now the CTX-M enzymes comprise more than 60 variants (http://www.lahey.org/studies/webt.asp) belonging to 5 different clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25). Among the CTX-M-enzymes, CTX-M-1, -3, -9, -14, and -15 have been reported repeatedly and the dissemination of genes coding such enzymes is mediated by plasmid and/or mobile elements including IS*Ecp1*-like insertion sequences (Cao *et al.*, 2002; Bonnet, 2004; Lartigue *et al.*, 2004; Livermore *et al.*, 2007). The dissemination of CTX-M-3, -14, -9, and -15 in clinical populations of the family *Enterobacteriaceae* in Korea has also been demonstrated and a high level of resistance to amikacin (MIC≥512 μg/ml) was demonstrated in all of the CTX-M-3-producing strains but not in the strains producing other types of CTX-M enzymes (Kim *et al.*, 2005a). In the previous study reported by Galimand *et al.* (2005), *armA*-carrying strains were always associated with the production of CTX-M-3. In other studies, however, CTX-M-3 was not detected in all of the strains carrying *armA*; detection of CTX-M-3 in 21 of the 28 strains carrying *armA* in a Taiwanese study (Yan *et al.*, 2004), 17 of the 22 strains carrying *armA* in a Belgium study (Bogaerts *et al.*, 2007), 25 of the 36 strains carrying *armA* in a Korean study (Park *et al.*, 2006), none of the 40 strains carrying *armA* in another Korean study (Lee *et al.*, 2006), and 13 of the 64 strains carrying *armA* in the current study. $bla_{\text{CTX-M-3}}$, however, was transferred always with *armA* in the case of the strains carrying *armA* and $bla_{\text{CTX-M-3}}$ together, suggesting a co-localization of *armA* and $bla_{\text{CTX-M-3}}$ on the same conjugative plasmid. Hybridization analyses and replicon typing of the conjugative plasmids provided the evidence for the co-localization of *armA* and $bla_{\text{CTX-M-3}}$ on the large IncL/M plasmid. The physical link between *armA* and $bla_{\text{CTX-M-3}}$ on the conjugative IncL/M plasmid, very recently, has been demonstrated by Golebiewski *et al.* (2007). They reported complete nucleotide sequence of the pCTX-M3 plasmid, which carries 103 putative genes including $bla_{\text{CTX-M-3}}$, *armA*, $bla_{\text{TEM-1}}$, *aacC2*, *aadA2*, *dfrA12*, *sul1*, the IncL/M replicon, and the conjugal transfer genes. The current study also showed that the RFLP patterns of IncL/M plasmids carrying *armA* and $bla_{\text{CTX-M-3}}$ were the same or very similar to each other, suggesting intercellular horizontal transfer of the plasmid has been occurred in clinical populations of the family *Enterobacteriaceae*.

Besides CTX-M-3, other kinds of ESBLs, such as SHV-12, SHV-5-like, TEM-52, CTX-M-14, CTX-M-9, and CTX-M-15, and pACBLs, such as DHA-1 and CMY-2, have been found in the *armA*-carrying strains (Yan *et al.*, 2004; Park *et al.*, 2006; Bogaerts *et al.*, 2007). SHV-12 and DHA-1 were detected with a high proportion in *armA*-carrying strains in this study. CMY-2 and DHA-1 were detected in 8 and 5 strains carrying *armA*, respectively, in a Korean study (Lee *et al.*, 2006). SHV-5-like and CMY-2 were detected in 11 and 2 strains carrying *armA*, respectively, in a Taiwanese study (Yan *et al.*, 2004). Currently, however, no reports have provided a direct evidence for the co-localization of *armA* with genes coding such ESBL enzymes. In this study, genes coding SHV-12 or DHA-1 were transferred with *armA* in most strains carrying those genes but direct evidence to prove the co-localization of *armA* with genes coding SHV-12 or DHA-1 on the same conjugative plasmid could not be provided due to the repeated failures to extract plasmid from the transconjugants.

Although a few previous studies reported on the association of *rmtB* with CTX-M-14 (Yan *et al.*, 2004; Park *et al.*, 2006; Bogaerts *et al.*, 2007), this study revealed, for the first time, the co-localization of $bla_{\text{CTX-M-14}}$ and *rmtB* on a broad-host-range IncA/C plasmid. In addition, most of the transconjugants that received the IncA/C plasmid carrying *rmtB* and $bla_{\text{CTX-M-14}}$ revealed a resistance to chloramphenicol, tetracycline, streptomycin, sulfamethoxazole, and trimetho-

prim as well as aminoglycosides and ß-lactams. This finding suggested the co-existence of additional resistance genes on the IncA/C plasmid as well as *rmtB* and $bla_{\text{CTX-M-14}}$. These observations give rise to concern about a rapid increase of multi-drug resistant isolates by a spreading such plasmids among gram-negative bacilli. RFLP analysis of the IncA/C plasmids carrying *rmtB* and $bla_{\text{CTX-M-14}}$ and PFGE analysis of the clinical isolates suggested that the intercellular horizontal transfer of the IncA/C plasmids and the clonal transmission have been occurred at the same time.

In summary, it is suggested that the dissemination of 16S rRNA methylases and ESBLs among Gram-negative bacilli in Korea has been mediated in large part by a dissemination of conjugative plasmids that carry both genes. Especially, the IncL/M plasmids carrying *armA* and $bla_{\text{CTX-M-3}}$ and the IncA/C plasmids carrying *rmtB* and $bla_{\text{CTX-M-14}}$ might play an important role in a recent rapid dissemination of CTX-M enzymes and 16S rRNA methylases among clinical populations of the family *Enterobacteriaceae* in Korea.

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