New complex class 1 integron carrying an *ISCR1* element in *Escherichia coli* clinical isolates harbouring the *bla*_{CMY-11} gene

Resistance to β -lactam antibiotics in Gram-negative bacteria is mainly mediated by the production of β -lactamases, which are divided into four major Ambler's molecular classes (A, B, C and D). In previous studies, we have demonstrated that CMY-11 from a virulent clinical strain is a plasmid-encoded extended-spectrum class C (AmpC) β -lactamase conferring resistance to cefoxitin and cefotetan, as well as to penicillins and oxyimino-cephalosporins (Lee *et al.*, 2002). We previously obtained 710 *Escherichia coli* clinical isolates from patients with various infections, 9 of which showed high levels of resistance to amoxicillin, amoxicillin-clavulanic acid, cefalotin, cefoxitin and cefotetan, as well as to cefotaxime, ceftazidime and aztreonam (Kim *et al.*, 2004). All nine *E. coli* clinical isolates produced CMY-11 (Kim *et al.*, 2004). A *bla*_{CMY-11} gene, responsible for this β -lactam resistance, was located on a ~130 kb conjugative plasmid harboured by the nine clinical isolates and their transconjugants (Kim *et al.*, 2004). Clonal and plasmid spread contributed to *bla*_{CMY-11} dissemination in Korean clinical isolates of *E. coli* (Kim *et al.*, 2004; Song *et al.*, 2009). This study was carried out to investigate the genetic environment surrounding the *bla*_{CMY-11} gene, which can play an essential role in the translocation of the gene from a chromosome (or plasmid) to the conjugative plasmid.

The BLASTN (basic local alignment search tool used against nucleotide sequence databases) program of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) was used for DNA sequence database searches of integron element genes and other genes. The primers for PCR amplification were designed by selecting consensus sequences based on multiple nucleotide alignments

The GenBank/EMBL/DDBJ accession number of the sequence of the complex class 1 integron determined in this work is FJ763641.

(CLUSTAL W program) of searched genes. PCR and DNA sequence analyses (using the primers shown in Table 1) of nine clinical isolates revealed that the *bla*_{CMY-11} gene was located in a new complex class 1 integron within the conjugative plasmid (Fig. 1a). To characterize this integron, recombinant plasmids with overlapping fragments were obtained as follows. Long

and accurate (LA) PCR amplification was performed with specific primers (Table 1). The LA PCR products were ligated with the pCR2.1-TOPO cloning vector (Invitrogen). The ligation mixture (recombinant plasmids) was introduced into competent *E. coli* DH5 α cells (Invitrogen) by transformation; transformant selection was by kanamycin

Table 1. Primers used in this study

Target gene	Primer*	Sequence
<i>intI1</i>	intI1-F	5'-CTA CCT CTC ACT AGT GAG GGG CG-3'
	intI1-R	5'-ATG AAA ACC GCC ACT GCG C-3'
	intI1-F	5'-CCT CCC GCA CGA TGA TCG TGC-3'
	intI1-R	5'-TCC ACG CAT CGT CAG GCA TTG-3'
	C11-1F-1	5'-AAT CCA GAT CCT TGA CCC GCA G-3'
<i>dfrA12</i>	C11-1F-2	5'-CTT CTC CCA TGC TCA CGA AAC AG-3'
<i>orfF</i>	C11-1F-3	5'-GGC TTA CCT CGC CCG TTA GAC AT-3'
<i>aadA2a</i>	2-int-1-F	5'-AAG TTA GAC ATC ATG AGG GTA-3'
	2a-sul1-w2-F	5'-AGT GAT CTT CTT TTT GTC CCA-3'
	C11-1R-1	5'-TCT GAT CGG ACA GGG CGT CTA A-3'
	C11-1R-2	5'-GCT TAG TGC ATC TAA CGC CGG AG-3'
	<i>qacEA1</i>	qacEA1-F
<i>qacEA1</i>	qacEA1-R	5'-GTT ATC GCA ATA GTT GGC G-3'
		5'-AGC TTT TGC CCA TGA AGC AAC C-3'
<i>sul1</i>	sulf-F	5'-ATG GTG ACG GTG TTC GGC AT-3'
	sulf-R	5'-CTA GGC ATG ATC TAA CCC TCG GTC-3'
	sul1-F	5'-GAT TTT TCT TG AGC CCC GC-3'
	sul1-R	5'-TGG ACC CAG ATC CTT TAC AGG-3'
<i>ISCR1</i>	orf513-1F	5'-ATG TCG CTG GCA AGG AAC GC-3'
	orf513-2R	5'-TCA AAG AGA CGA CTC TGT GAT GGA TC-3'
	orf513-3F	5'-TGA CTC TTA TCC AAC GCT TTG GC-3'
	orf513-4R	5'-CTG GCC GAC TAA TGT AGC GAC AC-3'
	2-qac-w3-F	5'-TGC TCA GCT TTC CTT TCC AGC TAC G-3'
<i>bla</i> _{CMY-11}	C1-F	5'-GAG CAG ACC CTG TTC GAG AT-3'
	C2-R	5'-GAT TGG CCA GCA TGA CGA TG-3'
	2-CMY-11-1-R	5'-ATG AAT CCA CCT CCT CGG G-3'
	2-CMY-11-w2-R	5'-AAT TCC CTC ACT CGT TTA CCG CTC-3'
	2-CMY-11-w2-F	5'-CTG CGT GAT GAC ATG GGG TTC CTT A-3'
<i>yqgF</i>	C11-4F-1	5'-CTA TGC TGA TGG CGA CAA TGG C-3'
<i>yqgE</i>	2-CMY-11-w3-F	5'-GCT TGA GCT TGG TCA GCA TGG TGT C-3'
	C11-4F-2	5'-GCA TGG CGA GCA GGA AGT GA-3'
	2-sul1-w3-R	5'-ACC GAG TTC ATC TCA GCC ATC TAC A-3'
<i>gshB</i>	C11-4F-3	5'-GTT GGC GTC ATC CTC CTT CAT G-3'
	C11-4R-2	5'-CCG AGC ACA AGG ACG TGA TCC-3'
	2c-sul1-w2-R	5'-CGC CCT ACA GCA TCA ACA CCC T-3'
<i>orf97, orf105</i>	C11-4R-1	5'-GCC CGT ACC GTG CAG GAA G-3'

*Orientation of each primer: F, forward; R, reverse.

Fig. 1. (a) Schematic representation of the backbone structure of the complex class 1 integron harbouring novel genes on a 130 kb conjugative plasmid. White arrows, ORFs; white ovals, 59 base element (be); black oval, *attI1* recombination site. P_c (5'-TGGACATAAGCCTGTTGCGTTCGTAAGT-3') and P_{int} (5'-AGTCTATGCCTCGGCGATCCAAGCAGCAA-3') are promoters for cassette genes and integrase-encoding gene (*intI1*), respectively. The -35 and -10 motifs of the *bla*_{CMY-11} gene promoter are indicated by boxes. The RCS is underlined. *orfS* (the initiation site of *ISCR1* transposition) is indicated by a box and underlined. *qacEΔ1(140)* shows a 140 bp deletion at the 5' end of *qacEΔ1*, and the remainder of the two genes are identical. (b) Three subclones that were used for sequencing.

(50 $\mu\text{g ml}^{-1}$; Sigma-Aldrich). In Fig. 1(b), the recombinant plasmids are pTO21-33 D (4050 bp, a recombinant plasmid carrying the *sul1*-type integron), pTO21-33E (3755 bp, a recombinant plasmid carrying a region from within *sul1* to within *bla*_{CMY-11}) and pTO21-33F [5036 bp, a recombinant plasmid carrying a region from within *bla*_{CMY-11} to *qacEΔ1(140)/sul1*]. Consequently, the plasmids covered, together, a 11 839 bp region containing *bla*_{CMY-11}. To avoid repetition of information about the integron, only the first characterized sequence of this integron was submitted to the GenBank database.

A *sul1*-type class 1 integron, containing *dfrA12*, *orfF* and *aadA2a* gene cassettes, was identified upstream of *bla*_{CMY-11} and ended with a 3' conserved segment (3'-CS) (*qacEΔ1-sul1*). The sequence past the end of the 3'-CS was almost identical to the 2.15 kb common regions for In6 of pSa (Stokes *et al.*, 1993) and for integrons of pAr-32 (Sorum *et al.*, 2003), pSAL-1 (Verdet *et al.*, 2000), pAJE0508 (Bae *et al.*, 2007), pCMXR1 (Doi *et al.*, 2002) and pKO56 (Jeong *et al.*, 2005). All common regions carried an *ISCR1* element. Recently it has been suggested that the *ISCR1* element is a member of an extended family of IS91-like elements that can transpose

adjacent DNA sequences by a mechanism termed rolling-circle transposition and are responsible for the mobilization of many classes of antibiotic-resistance genes, including *bla*_{CMY-9}, *bla*_{DHA-1}, *bla*_{CTX-M-14}, *catA2* and *qnr* genes, but never *bla*_{CMY-11} thus far (Toleman *et al.*, 2006). A recombination cross-over site (RCS) (33 bp DNA sequence containing *orfS*) at which insertion of resistance genes into the complex class 1 integron containing *ISCR1* takes place was observed downstream of *ISCR1* (Bennett, 2008). A 182 bp region that was identified between the RCS and the start codon of the *bla*_{CMY-11} gene had no identity to any known nucleotide sequences. This unique region was first identified in the integron harbouring the *bla*_{CMY-11} gene. Furthermore, the genetic environment surrounding the 3' end of RCS revealed that, compared to the upstream sequence of *bla*_{CMY-1} (GenBank accession no. X92508), the upstream sequence of *bla*_{CMY-11} had an additional 18 bases. On the other hand, the upstream sequences of *bla*_{CMY-9} (Doi *et al.*, 2002) and *bla*_{CMY-8} (Chen *et al.*, 2007) lacked 50 bases compared with the upstream sequence of *bla*_{CMY-1}. These nucleotide sequence analyses of *bla*_{CMY-1}, *bla*_{CMY-8}, *bla*_{CMY-9} and *bla*_{CMY-11} genes indicate that there might have been transposition events mediated by the *ISCR1* element upstream

of *bla*_{CMY-11} in the past because of the different genetic environments surrounding the 3' end of RCS in these genes. A published report suggested that this variation is indicative of past transposition events (Doi *et al.*, 2002).

A unique gene array (*yqgF-yqgE-gshB-orf97-orf105*) was first identified between *bla*_{CMY-11} and a duplicate [*qacEΔ1(140)-sul1*] of the 3'-CS (Fig. 1). The third ORF (*gshB*) encoded a 316 amino acid protein, which shared 95 % identity with the amino acid sequence of glutathione synthetase (GshB) of *Aeromonas hydrophila* (Seshadri *et al.*, 2006). The nucleotide sequence identity of the *bla*_{CMY-11} gene with the *bla*_{AmpC} gene (GenBank accession no. GQ152601) from *Aeromonas punctata* was 85 %, which suggests that this gene may have originated from the DNA of *A. punctata*. The functions of YqgF, YqgE, Orf97 and Orf105 are unknown.

When compared with nucleotide sequences of other complex class 1 integrons, the region (*intI1-aadA2a-qacEΔ1-sul1-ISCR1-RCS*) in the new integron harbouring the *bla*_{CMY-11} gene is significantly similar to those of In6 and of the integrons of pAr-32, pSAL-1 and pAJE0508 detected in Australia, Norway, France and South Korea, respectively. The region (*bla*_{CMY-11-yqgF-yqgE) between the}

RSC and *gshB* in the integron is very similar to those of integrons of pCMXR1 and pK29 (Chen *et al.*, 2007) in Japan and Taiwan. The region [*orf105-qacEΔ1(140)-sulI*] in the new integron containing the *bla*_{CMY-11} gene is identical to that of the integron of pKO56 in South Korea. These results suggest that the integron containing the *bla*_{CMY-11} gene might be derived from these integrons identified predominantly in East Asia (especially South Korea and Japan). This work highlights the genetic environment of the *bla*_{CMY-11} gene, which has been associated with a new complex class 1 integron bearing an *ISCR1* element for what is believed to be the first time.

Acknowledgements

This work was supported by research grants from the Korea Research Foundation (KRF-2008-313-C00790) and the Driving Force Project for the Next Generation of Gyeonggi Provincial Government in Republic of Korea.

**Jae Seok Song,^{1†} Seon Ju Jang,^{1†}
Il Kwon Bae,^{2†} Hyun Ji Lee,¹
Byeong Chul Jeong¹
and Sang Hee Lee¹**

¹Drug Resistance Proteomics Laboratory, Department of Biological Sciences, Myongji University, San 38-2 Namdong, Yongin, Gyeonggi-do 449-728, Republic of Korea

²Research Institute for Antimicrobial Resistance, Kosin University College of Medicine, Busan, Republic of Korea

Correspondence: Sang Hee Lee
(sangheelee@mju.ac.kr)

†Jae Seok Song, Seon Ju Jang and Il Kwon Bae contributed equally to this work.

Bae, I. K., Lee, Y. N., Lee, W. G., Lee, S. H. & Jeong, S. H. (2007). Novel complex class 1 integron bearing an *ISCR1* element in an *Escherichia coli* isolate carrying the *bla*_{CTX-M-14} gene. *Antimicrob Agents Chemother* **51**, 3017–3019.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* **153**, S347–S357.

Chen, Y.-T., Lauderdale, T.-L., Liao, T.-L., Shiau, Y.-R., Shu, H.-Y., Wu, K.-M., Yan, J.-J., Su, I.-J. & Tsai, S.-F. (2007). Sequencing and comparative genomic analysis of pK29, a 269-kilobase conjugative plasmid encoding CMY-8 and CTX-M-3 β -lactamases in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **51**, 3004–3007.

Doi, Y., Shibata, N., Shibayama, K., Kamachi, K., Kurokawa, H., Yokoyama, K., Yagi, T. & Arakawa, Y. (2002). Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. *Antimicrob Agents Chemother* **46**, 2427–2434.

Jeong, J. Y., Yoon, H. J., Kim, E. S., Lee, Y., Choi, S. H., Kim, N. J., Woo, J. H. & Kim, Y. S. (2005). Detection of *qnr* in clinical isolates of *Escherichia coli* from Korea. *Antimicrob Agents Chemother* **49**, 2522–2524.

Kim, J. Y., Song, J. S., Bak, S. H., Cho, Y. E., Kim, D. W., Jeong, S. H., Park, Y.-M., Lee, K. J. & Lee, S. H. (2004). Dissemination of *Escherichia coli*

producing AmpC-type β -lactamase (CMY-11) in Korea. *Int J Antimicrob Agents* **24**, 320–326.

Lee, S. H., Kim, J. Y., Lee, G. S., Cheon, S. H., An, Y. J., Jeong, S. J. & Lee, K. J. (2002). Characterization of *bla*_{CMY-11}, an AmpC-type plasmid-mediated β -lactamase gene in a Korean clinical isolate of *Escherichia coli*. *J Antimicrob Chemother* **49**, 269–273.

Seshadri, R., Joseph, S. W., Chopra, A. K., Sha, J., Shaw, J., Graf, J., Haft, D., Wu, M., Ren, Q. & other authors (2006). Genome sequence of *Aeromonas hydrophila* ATCC 7966^T: jack of all trades. *J Bacteriol* **188**, 8272–8282.

Song, W., Lee, H., Lee, K., Jeong, S. H., Bae, I. K., Kim, J. S. & Kwak, H. S. (2009). CTX-M-14 and CTX-M-15 enzymes are the dominant type of extended-spectrum β -lactamase in clinical isolates of *Escherichia coli* from Korea. *J Med Microbiol* **58**, 261–266.

Sorum, H., L'Abbe-Lund, T. M., Solberg, A. & Wold, A. (2003). Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrob Agents Chemother* **47**, 1285–1290.

Stokes, H. W., Tomaras, C., Parsons, Y. & Hall, R. M. (1993). The partial 3'-conserved segment duplications in the integrons In6 from pSa and In7 from pDGO100 have a common origin. *Plasmid* **30**, 39–50.

Toleman, M. A., Bennett, P. M. & Walsh, T. R. (2006). *ISCR* elements: novel gene-capturing systems of the 21st century? *Microbiol Mol Biol Rev* **70**, 296–316.

Verdet, C., Arlet, G., Barnaud, G., Lagrange, P. H. & Philippon, A. (2000). A novel integron in *Salmonella enterica* serovar Enteritidis, carrying the *bla*_{DHA-1} gene and its regulator gene *ampR*, originated from *Morganella morganii*. *Antimicrob Agents Chemother* **44**, 222–225.