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Transparency declarations

None to declare.

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Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens

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Sir,

High-level aminoglycoside resistance mediated by the production of 16S rRNA methylase has been increasingly reported among various Gram-negative pathogens. Six 16S rRNA methylase genes have been previously identified: *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*.[1] However, in food animals, only two studies have described the presence of the *armA* and *rmtB* genes in pigs, respectively.[2,3] In China, aminoglycoside antibiotics are widely used for the prevention and control of *Escherichia coli* infections of chickens. However, it remained unknown if 16S rRNA methylase genes were present in *E. coli* isolated from chickens.

Between March and May 2008, a total of 120 individual *E. coli* strains were isolated from the livers ($n=154$) of diseased and dead chickens in four farms (Farms A–D) in Henan Province, China. Among them, 12 isolates exhibited high-level resistance to the aminoglycoside antibiotic amikacin (MICs > 512 mg/L). These isolates were screened by PCR for the six known types of 16S rRNA methylase genes. The genes that produced positive results were further confirmed by DNA sequencing of the amplicons. In addition, phenotypic and genotypic tests for extended-spectrum β-lactamases in 16S rRNA methylase-positive isolates were performed according to CLSI recommendations and using PCR assays as previously described.[4,5] Overall, the *armA* and *rmtB* genes were detected in 3 and 9, respectively, of the 12 isolates that had high-level amikacin resistance. No positive amplicons were found to be present for the other four 16S rRNA methylase genes (Table 1). Multiplex PCR was performed to determine whether the 12 isolates were commensals (A and B1) or were associated with phylogroups exhibiting extraintestinal virulence (B2 and D).[6] Five of them were found to be associated with extraintestinal virulence (Table 1).

Genetic relationships of the *E. coli* isolates that produced 16S rRNA methylase were assessed by PFGE after digestion with XbaI. The PFGE patterns differentiated these samples ($n=12$) into seven major pulsotypes, indicating that both horizontal and vertical transfer could have played an important role in the dissemination of the 16S rRNA methylase genes (Table 1).

The 16S rRNA methylase resistance determinants from *armA*- or *rmtB*-positive isolates were transferred to *E. coli* J53Az^R and DH10B by conjugation and electroporation, respectively, to investigate whether the two determinants were localized on plasmids and whether transfer of these genes increased the resistance of the recipient *E. coli* to antimicrobials. Southern hybridization of digested plasmid DNA from the isolates and their transformants/transconjugants was performed with digoxigenin-labelled probes specific for *armA* and *rmtB*, respectively. As shown in Table 1, *armA* and *rmtB* genes could transfer among *armA*- and *rmtB*-positive isolates by conjugation and electroporation. Also, increases in MICs of multiple classes of antimicrobials were found for both the transconjugants and transformants, suggesting that co-transferred resistance to other antimicrobial agents could occur on these plasmids. Hybridization with the *rmtB*-specific probe indicated that *rmtB* genes from three isolates (isolates 63, 3 and 73) were localized on two differently sized digested fragments. Interestingly, hybridization with the *armA*-specific probe revealed that one isolate (isolate 14) contained two copies of the

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Practical Disk Diffusion Test for Detecting Group B Streptococcus with Reduced Penicillin Susceptibility▽

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Although group B streptococcus (GBS) has been considered to be uniformly susceptible to β-lactams, the presence of GBS with reduced penicillin susceptibility (PRGBS) was recently confirmed genetically. We developed a feasible and reliable method for screening PRGBS in clinical microbiology laboratories using a combination of ceftibuten, oxacillin, and ceftizoxime disks.

Streptcoccus agalactiae (group B streptococcus [GBS]) is a leading cause of neonatal sepsis and meningitis and is also an important pathogen for elderly people and those suffering from underlying medical disorders (1, 5, 7, 11). GBS results in the highest mortality and morbidity if it causes invasive infections in neonates, including very-low-birth-weight infants (6, 10, 12). About 5% of GBS-infected infants die, and if they survive, they often suffer from severe neurological sequelae, such as mental retardation and vision and/or auditory disabilities (2), but development of GBS vaccines is still under investigation (8). Penicillins are the first-line agents in the treatment of GBS infections because all clinical GBS isolates have been considered to be uniformly susceptible to β-lactams, including penicillins (2, 3). However, we have recently identified and molecularly characterized several clinical GBS isolates demonstrating reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (*pbp2x*) gene (9), and similar PRGBS isolates were recently reported in the United States (4). PRGBS isolates were indeed confirmed to be nonsusceptible to penicillin G (PCG) by the agar dilution method, but this PCG nonsusceptibility was not apparent even if the PCG disk diffusion method was performed in accordance with the recommendations of the CLSI (Clinical and Laboratory Standards Institute) (3). Here we developed, therefore, a feasible and practical new disk test method for discriminating PRGBS from the clinically isolated GBS using three disks containing ceftibuten, oxacillin, and ceftizoxime, respectively.

Forty-eight clinical isolates were identified as GBS using a streptococcus grouping kit (Slidex Strepto [bioMerieux, Marcy l'Etoile, France] and Streptex [Mitsubishi Chemical Medience Corporation, Tokyo, Japan]). *Streptococcus agalactiae* ATCC BAA-611 and ATCC 12403 were used as the reference strains in bacteriological identification. The MICs of PCG for two ATCC standard strains and clinical isolates were determined by the agar dilution method recommended by the CLSI. *Streptococcus pneumoniae* 49619 was used for validation of the MIC measurements. The disk diffusion method was performed as recommended by the CLSI in evaluation of the applicability of each β-lactam disk for discrimination of PRGBS strains from penicillin-susceptible strains.

At first, the MICs of PCG for 2 ATCC strains and 48 clinical GBS isolates were measured by the CLSI standard agar dilution methods, and 34 strains, including 2 reference strains, proved susceptible to PCG (MIC, ≤0.12 μg/ml), whereas the remaining 16 clinical isolates were nonsusceptible to PCG (MIC, >0.12 μg/ml) (Fig. 1A and Table 1). The highest PCG MIC for such PCG-nonsusceptible GBS strains was 1 μg/ml.

In the next step, the nucleotide sequences of the PBP2X genes of all GBS strains used in this investigation were determined, and all 34 penicillin-susceptible GBS (PSGBS) strains were found to harbor neither V405A nor Q557E substitutions in PBP2X, which are conserved among PBP2Xs of almost all PRGBS strains. Among the 16 PRGBS isolates, 15 isolates harbored the PRGBS-specific mutations in PBP2X genes that cause V405A and/or Q557E substitutions in PBP2X, but no such substitution was found in one PRGBS strain, B7, which harbored multiple substitutions in PBP2X other than V405A and Q557E (Table 1), as reported previously (9).

To validate the PCG disk for screening PRGBS, we applied the standard disk diffusion method for GBS with PCG disks in accordance with the CLSI recommendation for streptococci other than pneumococci. For each isolate, the PCG MIC determined by the agar dilution method and the diameter of the growth-inhibitory zone measured by the standard disk diffusion method were plotted on a scatter diagram (Fig. 1A). In all isolates tested, the growth-inhibitory zones around the PCG disk were >24 mm (CLSI susceptibility criteria). Thus, it seemed very difficult to exactly discriminate all of the PRGBS from PSGBS by the CLSI standard disk diffusion method using only a PCG disk.

We previously reported that PRGBS showed reduced susceptibility not only to PCG but also to oxacillin and ceftizoxime (9). Thus, we examined the applicability of 44 β-lactam disks commercially available in Japan for detection of PRGBS (Table 2) and found that the ceftibuten disk was also applicable for screening PRGBS.

To evaluate the sensitivity and specificity of the disk diffu-

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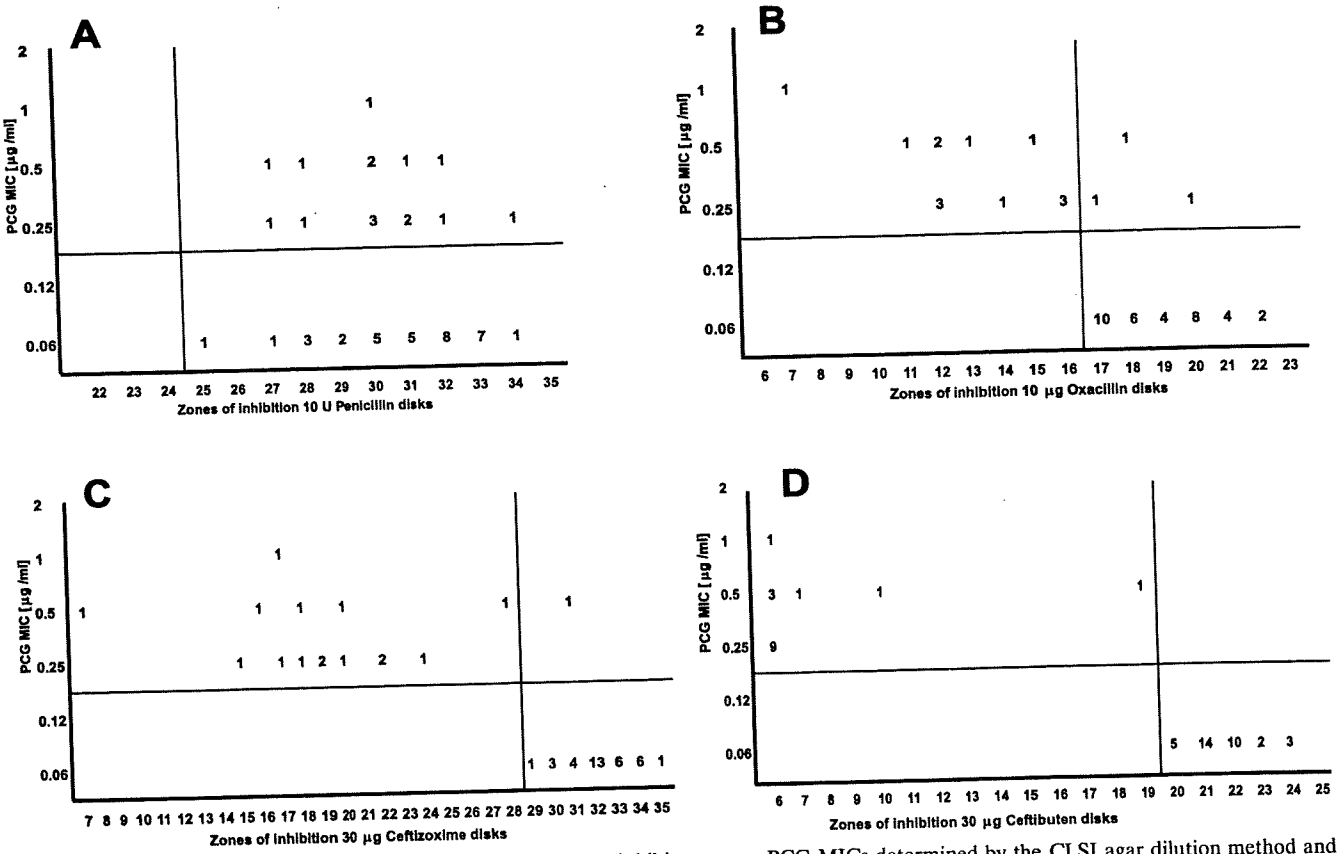

FIG. 1. Scatter diagram of MICs and the sizes of the growth-inhibitory zone. PCG MICs determined by the CLSI agar dilution method and diameters of the growth-inhibitory zone measured by CLSI-recommended standard disk diffusion method are plotted using a PCG disk (A). MICs determined by the agar dilution method and the diameters of growth inhibitory zone measured by the CLSI-recommended standard disk diffusion method are plotted using an oxacillin disk (B), ceftizoxime disk (C), and ceftibuten disk (D). The numbers in the scatter diagram indicate the number of clinical isolates in each intersection.

sion method, we performed the standard disk diffusion method of CLSI using oxacillin, ceftizoxime, and ceftibuten disks (Fig. 1B, C, and D). Because the CLSI has not determined the cutoff values for susceptible criteria in these three disks, we set the provisional cutoff values for "reduced susceptible" criteria in the three disks using the smallest-diameter values of susceptible strains: e.g., oxacillin, <17 mm; ceftizoxime, <29 mm; and ceftibuten, <20 mm. Under this condition, the sensitivities of

TABLE 1. MICs of PCG for 16 strains of PRGBS, amino acid substitutions in PBP2X of PRGBS, and the diameters of growth-inhibitory zones around oxacillin, ceftizoxime, and ceftibuten disks

Strain	MIC (μg/ml) of PCG	Amino acid substitutions in PBP2X	Diam (mm) of growth-inhibitory zone with:		
			Oxacillin	Ceftizoxime	Ceftibuten
B1	0.5	M349I, I377V, F399I, P445S, T555S, Q557E	13	7	6
B6	0.25	E411K, T555S, Q557E	12	15	6
B7	0.25	I377V, T394A, G398A	16	22	6
B8	0.25	I377V, F395L, P396T, V405A, R433H, H438Y, G648A	16	17	6
B10	0.5	G526R, Q557E, S726L	15	16	7
B12	0.25	G526R, Q557E, S726L	14	19	6
B40	0.5	P396S, G526R, Q557E, S726L	11	20	6
B60	0.25	I377V, T394A, G398A, Q557E	16	22	6
B68	0.5	A514V, Q557E	18	28	10
B502	0.5	I377V, F395L, V405A, R433H, H438Y, G648A	12	18	6
B503	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	20	24	6
B513	1	A400V, V405A, Q557E	7	17	6
B514	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	12	19	6
B516	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	17	18	6
M16	0.5	A514V, Q557E	12	31	19
M19	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	12	20	6

TABLE 2. Antibiotic concentrations of 44 Kirby-Bauer disks used in the applicability check for detecting PRGBS

Antibiotic	Concn
PCG	10 U
Oxacillin	1 μg
Ampicillin	10 μg
Amoxicillin	25 μg
Aspoxicillin	100 μg
Piperacillin	100 μg
Cephalothin	30 μg
Cefazolin	30 μg
Cefamandole	30 μg
Cefotiam	30 μg
Cefoperazone	75 μg
Cefuroxime	30 μg
Cefotaxime	30 μg
Ceftizoxime	30 μg
Cefmenoxime	30 μg
Cefpiramide	75 μg
Ceftazidime	30 μg
Ceftriaxone	30 μg
Cefodizime	30 μg
Cefpirome	30 μg
Cefepime	30 μg
Cefozopran	30 μg
Cefsulodin	30 μg
Cefoxitin	30 μg
Cefmetazole	30 μg
Cefotetan	30 μg
Cefbuperazone	75 μg
Cefminox	30 μg
Cephalexin	30 μg
Cefaclor	30 μg
Cefixime	5 μg
Ceftibuten	30 μg
Cefdinir	5 μg
Cefpodoxime	10 μg
Cefteram	10 μg
Cefcapene	5 μg
Cefditoren	5 μg
Moxalactam	30 μg
Flomoxef	30 μg
Imipenem	10 μg
Panipenem	10 μg
Meropenem	10 μg
Aztreonam	30 μg
Carumonam	30 μg

these disks were 13/16 (81%), 15/16 (94%), and 16/16 (100%), respectively, and the respective specificities were 34/37 (92%), 34/35 (97%), and 34/34 (100%). Thus, the sensitivity and specificity of the CLSI standard disk method using oxacillin, ceftizoxime, and ceftibuten disks were confirmed to be fully applicable for discrimination of PRGBS from the clinical GBS isolates. Moreover, all of the clinical PRGBS isolates were detected by at least one of the three disks. Therefore, the combination of the three disks could be expected to successfully distinguish all of the PRGBS from PSGBS (Table 3) in the routine work of clinical microbiology laboratories.

Indeed, one PRGBS isolate showed a 19-mm growth-inhibitory zone around the ceftibuten disk (Fig. 1D and Table 1), and this strain might well be misclassified as PSGBS. However, the results from the other disk containing oxacillin would complement exact detection of PRGBS. The PRGBS detection method developed in this investigation using ceftibuten, as well as oxacillin and ceftizoxime disks, would promise high specificity and sensitivity without necessitating any expensive or special equipment. This method, therefore, could come into wide use for detection of PRGBS in the daily antimicrobial susceptibility testing of GBS after its validation by multiple reference laboratories. Due to the lack of exact detection test methods for PRGBS, no confirmed case of penicillin treatment failure has been reported in GBS infections to date, and no clinical significance of PRGBS has so far been evaluated. Indeed, the PRGBS isolates were identified by measurement of MICs using the agar dilution method without regard for penicillin treatment failure. Moreover, it seems very difficult without case-controlled analyses to conclude whether the antibiotic treatment failure in the GBS infection cases is mainly due to bacterial penicillin nonsusceptibility or depends on a deteriorated ability to defend against microbial infections of the host. The new test method reported here offers a promising, easy, and reliable way to detect PRGBS and thus promote case analyses of infections with PRGBS in the future.

TABLE 3. Specificity and sensitivity of each β-lactam disk used in the disk diffusion methods

Disk	No. of isolates/total (%)[a]	
	Specificity	Sensitivity
Penicillin	34/50 (68)	0/16 (0)
Oxacillin	34/37 (92)	13/16 (81)
Ceftizoxime	34/35 (97)	15/16 (94)
Ceftibuten	34/34 (100)	16/16 (100)
Combination	34/34 (100)	16/16 (100)

[a] Denominators of specificity values are the numbers of isolates with a growth-inhibitory zone diameter around each disk above the tentative cutoff value. Denominators of sensitivity values are the numbers of PRGBS isolates determined by PCG MICs.

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Nationwide epidemiological study revealed the dissemination of meticillin-resistant *Staphylococcus aureus* carrying a specific set of virulence-associated genes in Japanese hospitals

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To study comprehensive toxin profiles and the chromosomal diversity of current Japanese hospital-associated meticillin-resistant *Staphylococcus aureus* (HA-MRSA) strains, we conducted PCR-based identification of 28 toxin genes, and staphylococcal cassette chromosome *mec* (SCC*mec*) typing and PFGE analysis of 208 MRSA strains isolated from 100 hospitals throughout Japan. Of the tested HA-MRSA strains, 80.3 % were *tst*-positive. The most frequent toxin gene profile was characterized by the carriage of 13 genes, *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld* and *hlg-2*. Ninety of the 208 strains had this profile, which was named pattern A. Among the 118 non-pattern A strains, 100 had similar toxin gene profiles, the concordance rates to pattern A of which were more than 80 %. Consequently, 91.3 % of the examined HA-MRSA strains carried similar toxin profiles, although PFGE patterns showed a wide variation. These strains belonged to SCC*mec* type II, *agr* II and coagulase type II. We concluded that, unlike MRSA from many other countries, most of the Japanese HA-MRSA strains belonged to, or were related to, a specific group carrying the set of 13 toxin genes, irrespective of chromosomal diversity. In addition, among the 13 toxin genes, the coexistence rates of *tst*, *sec* and *sel*, and those of *seg*, *sei*, *sem*, *sen* and *seo*, were higher than for the other toxin genes. High coexistence rates of *tst*, *sec* and *sel* genes suggested the presence of the pathogenicity island SaPIn1 in these strains.

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INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA) still remains the most important hospital-associated pathogen in many countries. The rate of MRSA incidence among *S. aureus* isolates has been approximately 70 % in most Japanese hospitals over the past 20 years (Kimura *et al.*, 1992; Diekema *et al.*, 2001). The exotoxin gene profiles of MRSA have been used as molecular epidemiological markers. Moreover, the profile of exotoxin genes in a strain can be a potential factor of the virulence aspect of the strain.

Abbreviations: HA-MRSA, hospital-associated MRSA; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

Molecular epidemiological studies of MRSA have also been conducted by using methods such as PFGE, multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. By using PFGE, Oliveira *et al.* (2002) identified five major MRSA clones. Among these clones, USA100 (New York/Japan) was characterized as a clonal complex 5 : ST5-MRSA-II by MLST and SCC*mec* typing (Enright *et al.*, 2002; McDougal *et al.*, 2003). The USA100 clone includes the largest group of isolates from throughout the United States (McDougal *et al.*, 2003). These were characterized as enterotoxin gene cluster (*egc*) locus-positive and *tst*-negative (Diep *et al.*, 2006), whereas previous studies suggested that Japanese MRSA isolates which were supposed to belong to the

USA100 clone (New York/Japan clone) were *tst*-positive and *egc* locus-positive (Piao *et al.*, 2005; Zaraket *et al.*, 2007; Nishi *et al.*, 2002; Hu *et al.*, 2008), although strains from only a limited area were analysed in these studies. MLST- and PFGE-based epidemiological classification therefore is likely insufficient for the characterization of isolates regarding their virulence aspects. An investigation of toxin profiles would provide fundamental information about the possible virulence of MRSA strains. The toxin gene profiles of Japanese MRSA isolates determined in the previous studies were based on local, not nationwide, surveillance. Therefore, the results of these studies did not necessarily represent the overall toxin characteristics of Japanese MRSA isolates.

In this study, we investigated the genotypic characteristics using a PCR method with reference to 28 virulence-associated exotoxin genes and carried out chromosomal PFGE analysis of MRSA strains which were uniformly collected from hospitals located throughout Japan.

METHODS

Bacterial isolates. Two hundred and eight Japanese MRSA strains were analysed in this study. These strains were randomly collected from 100 different hospitals located throughout Japan during 2002–2004 by a nationwide surveillance study conducted by the National Institute of Infectious Diseases, Japan. In order to investigate the general features of the toxin gene carriage of Japanese MRSA strains, the 100 hospitals were selected to cover as much as possible of the area of Japan (Fig. 1). In addition, only one to four strains were collected from each hospital. Further, these strains were isolated from various clinical specimens from epidemiologically unrelated patients, which included carriers and infected patients. The specimens included blood (n=34), intracatheter (n=2), sputum (n=78), tracheal tube (n=1), pharyngeal mucosa (n=16), nasal mucosa (n=13), faeces (n=10), urine (n=4), otorrhoea (n=10), skin (n=3), pus (n=28), soft tissue (n=1), cerebrospinal fluid (n=1), pleural effusion (n=1), ascites (n=1), vaginal discharge (n=1) and others (n=4).

The strains were identified as MRSA in each hospital. In our laboratory, all collected strains were reconfirmed as MRSA by conventional biological tests and by the presence of the *mecA* gene using PCR with specific primers (Bignardi *et al.*, 1996).

Template preparation and PCR procedure. Template DNA of *S. aureus* was prepared as described previously (Louie *et al.*, 2000). Primers that were previously described for the *tst, sea, seb, sec, sed, see, seg, seh, sei, sej, sem, sen, seo, edinA, lukPV, lukED, lukM, hla, hlb, hld, hlg* and *hlg-2* genes were used (Jarraud *et al.*, 2002). Primers for other genes were designed in this study from the sequences deposited in GenBank (NCBI) as shown in Table 1. Strains used for positive controls are listed in Table 2. The presence of the toxin genes in these positive controls of clinical origin was confirmed by PCR followed by DNA sequencing, and, when necessary, gene products were identified by proteomic analysis (Nakano *et al.*, 2002a).

PCR was performed in a 10 µl reaction mixture containing 0.4 µl template DNA, 1 µl 10 × *Taq* buffer, 0.8 µl dNTP mixture, 0.5 µM of each primer and 0.25 U *Taq* polymerase (Takara Bio). The following amplification cycles were repeated 30 times: denaturation for 30 s at 94 °C, annealing of primers for 30 s at suitable temperature for each amplification, and extension for 60 s at 72 °C. Amplified products were analysed by 1.4 % agarose gel electrophoresis in 1 × TAE buffer at 135 V for 20 min. The gel was stained with ethidium bromide and then exposed to UV light to visualize the amplified products. When the bands were not clear, we repeated the experiment a few times to confirm the reproducibility.

Fig. 1. Distribution map of 100 Japanese hospitals. Filled circles represent the locations of hospitals where the 208 MRSA strains were isolated.

Typing of SCC*mec*, *agr* and coagulase. SCC*mec* typing was performed by PCR using previously published primers that identified the class of *mec* gene complex and the type of cassette chromosome recombinase (*ccr*) gene (Ito *et al.*, 2001). The *agr* class was determined by PCR using specific primers as previously described (Gilot *et al.*, 2002). Coagulase typing was performed with a staphylococcal coagulase typing antiserum kit (Denka Seiken).

Chromosomal DNA fingerprinting by PFGE. *Sma*I macrorestriction patterns of chromosomal DNA were obtained by use of the CHEF-DR III System (Bio-Rad) with the following settings: voltage, 6 V cm^{-1}; temperature, 14 °C; initial time, 1 s; final time, 40 s; and duration, 20 h. Chromosomal patterns were examined visually, and digitally analysed with Finger Print II (Bio-Rad) to generate a dendrogram based on Dice coefficients. PFGE patterns were grouped according to the criterion of Tenover *et al.* (1995) and using a dendrogram similarity of more than 80 % to assign strain relatedness (McDougal *et al.*, 2003).

RESULTS AND DISCUSSION

Carriage profile of superantigenic toxin genes in Japanese hospital-associated MRSA (HA-MRSA)

The toxin genes were detected with the aforementioned published primers and the primers that we newly designed

Table 1. Primers newly designed in this study

Gene	Primer	Sequence (5′–3′)	Annealing temperature (°C)	Product length (bp)
sek	*sek*-f	AACTTGATGTTTTTGGTATT	56.0	237
	sek-r	AAATGTTGTATTCTTCTTGA		
sel	*sel*-f	TGATACATTGTATGCTGAAT	56.0	427
	sel-r	GTAAAATAAATCATACGTGT		
eta	*eta*-f	CTATTTACTGTAGGAGCTAG	47.5	741
	eta-r	ATTTATTTGATGCTCTCTAT		
etb	*etb*-f	ATACACACATTACGGATAAT	50.5	629
	etb-r	CAAAGTGTCTCCAAAAGTAT		
edinB	*edinB*-f	CATAAATACTCCTCTAAG	54.0	444
	edinB-r	GCATATTCTGTCCCTCTA		
edinC	*edinC*-f	TATTAAGCATTCATTCAA	44.0	626
	edinC-r	GTAGTCTGTTCCTCTA		

in this study based on the DNA sequence databases. As shown in Table 3, the positive carriage rates of *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen* and *seo* were 76.4–94.2 %. The carriage rates of these eight genes were much higher than those of *sea* (9.6 %) and *seb* (21.2 %). The other superantigenic toxin genes, *sed*, *see*, *seh*, *sej* and *sek*, showed low carriage rates of less than 2.4 %. Two hundred and four out of 208 strains carried at least one of the 15 superantigenic toxin genes tested in this study, which corresponds to a carriage rate of 98.1 %.

Carriage profile of other toxin genes

The exfoliative toxin genes *eta* and *etb* and the epidermal cell differentiation inhibitor genes *edinA*, *edinB* and *edinC* were rarely detected. Only two strains were *eta*-positive. These two strains carried none of the superantigenic toxin genes. Panton–Valentine leukocidin component genes *lukPV* were not detected in any of the strains. The *lukED* genes showed a high carriage rate of 96.6 %. Haemolysin genes *hla*, *hlb*, *hld* and *hlg-2* variant were also frequently detected. The γ-haemolysin gene variant *hlg-2* was dominant in Japanese MRSA strains.

Toxin patterns of HA-MRSA

Fifty-eight different toxin patterns were identified in the 208 HA-MRSA strains. The major patterns are shown in Table 4. The most frequent pattern, pattern A, was characterized by the carriage of the following 13 genes: *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld* and *hlg-2*. Ninety strains showed this pattern, which corresponds to 43.3 % of all tested strains. The next most frequent major patterns were patterns B, C, and D. Only one out of 28 genes was different in these three patterns compared to pattern A. Therefore, these patterns showed high concordance rates of 96.4 % to pattern A. The minor toxin patterns also showed high concordance rates. One hundred strains had concordance rates of more than 82.1 % to pattern A, which corresponds to 84.7 % of the MRSA strains other than of pattern A; i.e. 190 strains (91.3 %) carried quite similar toxin profiles.

Table 2. Strains used for positive controls

Strain	Gene	Source
MRSA N315	*tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld*, *hlg-2*	Kuroda *et al.* (2001)
S. aureus ATCC 27664	*see*	Sergeev *et al.* (2004)
S. aureus ATCC 49775	*lukPV*, *hlg*	Lina *et al.* (1999)
S. aureus ATCC 31890	*lukM*	Kaneko *et al.* (1997)
MRSA MF330	*sea*, *seb*, *seh*, *sek*	Clinical isolate*
MRSA GG28	*sed*, *sej*	Clinical isolate*
MRSA MH	*eta*	Clinical isolate†
MRSA NA1	*etb*, *edinC*	Clinical isolate†
MRSA C9	*edinA*	Clinical isolate‡
MRSA B11	*edinB*	Clinical isolate‡

*Food poisoning.
†Staphylococcal scalded skin syndrome.
‡Skin region of atopic dermatitis.

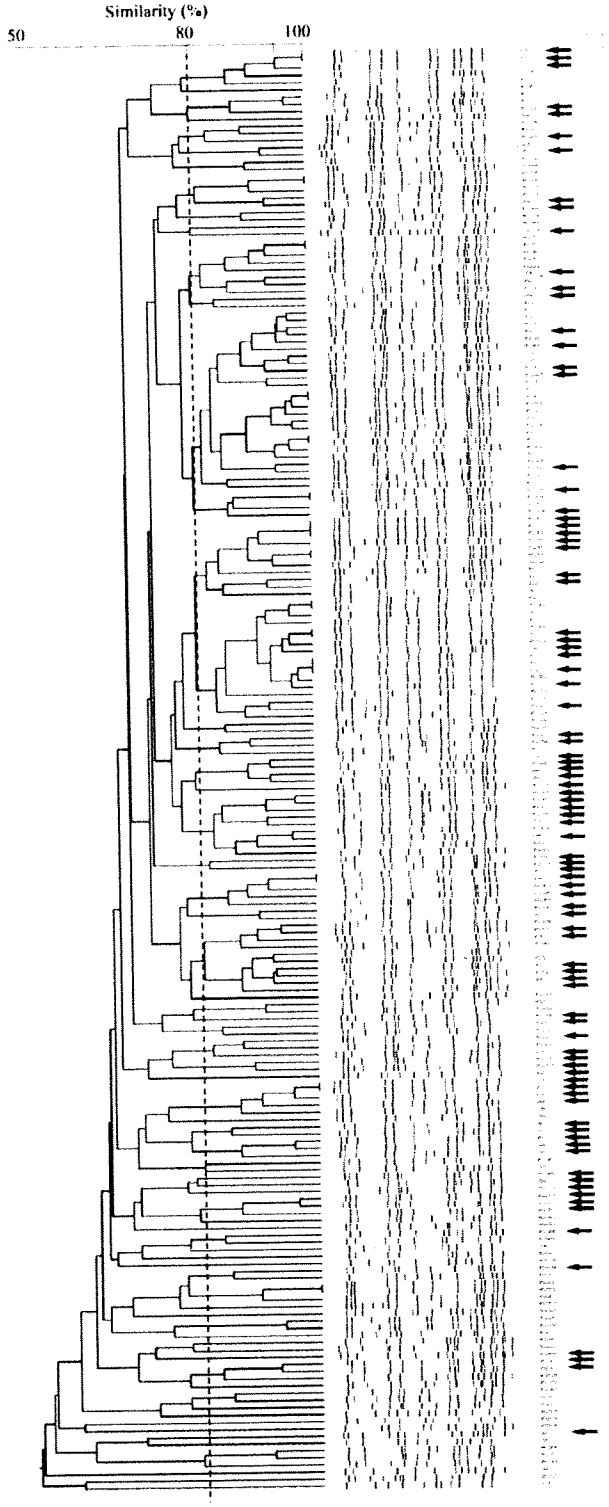

Fig. 2. Dendrogram of PFGE results based on the Dice coefficients and schematic representation of the *Sma*I restriction pulsotype of Japanese MRSA strains. Arrows represent strains belonging to the most frequent toxin gene pattern A group. Strain names are available from the corresponding author.

Table 3. Identification of toxin genes in Japanese MRSA strains

Pathogenic factor	Gene	MRSA (n=208)	
		n	%
Toxic shock syndrome toxin 1	*tst*	167	80.3
Enterotoxins	*sea*	20	9.6
	seb	44	21.2
	sec	168	80.8
	sed	2	1.0
	see	0	0.0
	seg	186	89.4
	seh	3	1.4
	sei	189	90.9
	sej	3	1.4
	sek	5	2.4
	sel	159	76.4
	sem	196	94.2
	sen	184	88.5
	seo	195	93.8
More than one of the superantigens		204	98.1
Exfoliative toxins	*eta*	2	1.0
	etb	0	0.0
Epidermal cell differentiation inhibitors	*edinA*	0	0.0
	edinB	0	0.0
	edinC	0	0.0
Leukocidins	*lukPV*	0	0.0
	lukED	201	96.6
	lukM	0	0.0
Haemolysins	*hla*	202	97.1
	hlb	176	84.6
	hld	203	97.6
	hlg	4	1.9
	hlg-2	202	97.1

SCC*mec* type, *agr* class and coagulase type of strains with major toxin patterns

The types of SCC*mec*, *agr* and coagulase were determined for ten strains with major toxin patterns which were selected randomly. Of ten strains, three were from toxin pattern A, two from pattern B, two from pattern C, one from pattern D, one from pattern E, and one from pattern F. We found that all strains were SCC*mec* type II, *agr* class II and coagulase type II. Irrespective of differences in toxin pattern, these strains belonged to a single group of SCC*mec*, *agr* and coagulase types. The SCC*mec* region and most of the toxin genes analysed in this study were supposed to be extrinsic in origin (Kuroda *et al.*, 2001; Novick, 2003). It is therefore likely that the acquisition of the SCC*mec* region and the toxin genes were genetically independent events.

PFGE profiles

PFGE typing was performed for all of the 208 HA-MRSA strains. Of these, 202 were successfully analysed (Fig. 2).

Table 4. Major patterns among the 58 toxin patterns in Japanese MRSA strains

+, Gene is present; blank cells, gene is absent.

Gene	Pattern [no. (%) of strains]					
	A [90 (43.3)]	B [21 (10.1)]	C [12 (5.8)]	D [7 (3.4)]	E [7 (3.4)]	F [6 (2.9)]
tst	+	+	+	+		+
sea				+		+
seb		+			+	
sec	+	+	+	+		+
sed						
see						
seg	+	+	+	+	+	+
seh						
sei	+	+	+	+	+	+
sej						
sek						
sel	+	+	+	+		+
sem	+	+	+	+	+	+
sen	+	+	+	+	+	+
seo	+	+	+	+	+	+
eta						
etb						
edinA						
edinB						
edinC						
lukPV						
lukED	+	+	+	+	+	+
lukM						
hla	+	+	+	+	+	+
hlb	+	+		+	+	
hld	+	+	+	+	+	+
hlg						
hlg-2	+	+	+	+	+	+
	Concordance rate with pattern A (%)					
	–	96.4	96.4	96.4	85.7	92.9

The PFGE profiles of the 202 strains were classified into 74 PFGE types using a cut-off of 80 % similarity (McDougal *et al.*, 2003) as well as the criterion of Tenover *et al.* (1995). Among the 202 strains, 89 shown with arrows in Fig. 2 belonged to the toxin gene pattern A group and these strains were classified into 39 PFGE types, corresponding to 52.7 % of all the identified PFGE types. This indicated that the strains carrying the set of pattern A toxin genes differentiated without changing stable carriage of these toxin genes or rather that extrinsic genetic elements such as phages and plasmids which carried the toxin genes were disseminated by horizontal transmission among various HA-MRSA strains in Japan.

In a previous study, Piao *et al.* (2005) determined toxin patterns of Japanese MRSA isolated from a hospital in Tokyo. Similar studies were also conducted in other hospitals (Zaraket *et al.*, 2007; Nishi *et al.*, 2002; Hu *et al.*, 2008). These four studies commonly reported the high carriage rate of *tst*, *sec* and the *egc* locus (*seg*, *sei*, *sem*, *sen* and *seo*), although MRSA strains for the analysis in each study were collected from hospitals within a narrow geographical area. Our study also presented similar but more conclusive results for strains collected throughout Japan. The toxin patterns that we have determined therefore could represent those of MRSA isolates from throughout Japan having various genetic backgrounds as determined by PFGE.

Coexistence of toxin genes

As shown in Table 4, while most of the major patterns had high concordance rates with pattern A, pattern E showed somewhat lower concordance. This was due to a lack of *tst*, *sec* and *sel* genes. We also observed the same for the minor toxin patterns, which are not shown in Table 4. Regardless of whether they were major or minor, the toxin patterns showing relatively lower concordance rates often lacked *tst*, *sec* and *sel* genes at the same time. Similarly, there were some strains which lacked a set of *seg*, *sei*, *sem*, *sen* and *seo* genes. In order to evaluate the coexistence of toxin genes

Table 5. Rates of coexistence among the 13 genes

Cells marked with dark-grey shading contain genes relating to SaPIn1. Cells marked with light-grey shading contain genes relating to SaPIn3. Unmarked cells contain genes that do not relate to these pathogenicity islands.

tst		*sec*		*sel*		*seg*		*sei*		*sem*		*sen*		*seo*		*lukED*		*hla*		*hlb*		*hld*		*hlg-2*	
Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%
sel	92.3	*sel*	92.8	*sec*	92.8	*sei*	96.6	*seg*	96.6	*seo*	97.6	*seo*	93.8	*sem*	97.6	*hlg-2*	96.6	*hld*	95.7	*hld*	83.2	*hla*	95.7	*lukED*	96.6
sec	90.9	*tst*	90.9	*tst*	92.3	*sem*	94.2	*sem*	96.6	*sem*	96.6	*sem*	93.3	*sei*	95.2	*hld*	95.2	*lukED*	94.7	*sem*	82.7	*lukED*	95.2	*hld*	94.7
sei	84.6	*sei*	88.9	*seg*	84.1	*seo*	93.8	*seo*	95.2	*seg*	94.2	*sei*	92.8	*seg*	93.8	*hla*	94.7	*hlg-2*	94.2	*hla*	82.7	*hlg-2*	94.7	*hla*	94.2
seg	83.2	*seg*	88.5	*sei*	83.7	*lukED*	92.8	*sen*	92.8	*sen*	93.3	*seg*	92.3	*sen*	93.8	*seg*	92.8	*seo*	91.8	*hlg-2*	81.7	*seo*	92.3	*sem*	91.3
seo	82.7	*seo*	86.1	*seo*	81.7	*sen*	92.3	*lukED*	92.3	*hld*	91.8	*lukED*	88.9	*hld*	92.3	*sei*	92.3	*sem*	91.3	*seo*	81.3	*sem*	91.8	*sei*	90.9
sem	82.2	*sem*	85.6	*sem*	80.3	*hlg-2*	90.4	*hlg-2*	90.9	*hla*	91.3	*hlg-2*	87.5	*hla*	91.8	*seo*	91.3	*sei*	89.9	*lukED*	81.3	*sei*	89.4	*seo*	90.9
lukED	81.7	*sen*	85.6	*sen*	80.3	*hld*	88.9	*hla*	89.9	*hlg-2*	91.3	*hld*	87.0	*lukED*	91.3	*sem*	90.9	*seg*	88.5	*seg*	79.8	*seg*	88.9	*seg*	90.4
sen	81.3	*lukED*	82.2	*lukED*	78.8	*sec*	88.5	*hld*	89.4	*lukED*	90.9	*hla*	86.5	*hlg-2*	90.9	*sen*	88.9	*sen*	86.5	*sei*	79.3	*sen*	87.0	*sen*	87.5
hlg-2	81.3	*hld*	81.3	*hlg-2*	77.4	*hla*	88.5	*sec*	88.9	*sec*	85.6	*sec*	85.6	*sec*	86.1	*sec*	82.2	*hla*	82.7	*sem*	78.8	*hlb*	83.2	*hlb*	81.7
hla	80.3	*hla*	80.8	*hld*	76.9	*sel*	84.1	*tst*	84.6	*hlb*	82.7	*tst*	81.3	*tst*	82.7	*tst*	81.7	*sec*	80.8	*sec*	73.1	*sec*	81.3	*tst*	81.3
hld	78.8	*hlg-2*	80.8	*hla*	76.4	*tst*	83.2	*sel*	83.7	*tst*	82.2	*sel*	80.3	*sel*	81.7	*hlb*	81.3	*tst*	80.3	*tst*	73.6	*tst*	78.8	*sec*	80.8
hlb	71.6	*hlb*	73.1	*hlb*	69.7	*hlb*	79.8	*hlb*	79.3	*sel*	80.3	*hlb*	78.8	*hlb*	81.3	*sel*	78.8	*sel*	76.4	*sel*	69.7	*sel*	76.9	*sel*	77.8

quantitatively, we calculated the coexistence rates between 13 genes in each strain (Table 5). The 13 genes were *tst, sec, seg, sei, sel, sem, sen, seo, lukED, hla, hlb, hld* and *hlg-2*, which belonged to pattern A. As already mentioned above, these 13 genes showed high carriage rates among the examined strains, resulting in high coexistence rates with each other. However, as shown in Table 5, if we arrange them in order of the coexistence rate, *tst, sec, sel* genes showed the highest coexistence rates between each other (marked with dark grey). Likewise, *seg, sei, sem, sen* and *seo* genes showed higher coexistence rates (marked with light grey). Consequently, our results indicate that the set of three genes *tst, sec* and *sel*, and the set of five genes *seg, sei, sem, sen* and *seo*, highly coexist in Japanese HA-MRSA. According to the genome analysis of an early Japanese hospital isolate, MRSA N315, two major pathogenicity islands, SaPIn1 and SaPIn3, were identified on the chromosome (Kuroda *et al.*, 2001). SaPIn1 was characterized as the carrier of *tst, sec* and *sel* genes and SaPIn3 was the carrier of the *egc* locus and *lukED* genes. In the *egc* locus of N315, *seg, sei, sem, sen* and *seo* genes were identified. In our study, we confirmed that the toxin pattern of MRSA N315 belongs to the most frequent pattern A. In addition, our results showed that the correlation of existence between *tst, sec* and *sel*, and also between *seg, sei, sem, sen* and *seo*, was higher than for the other toxin genes, which suggested that a majority of Japanese HA-MRSA strains probably carried pathogenicity islands SaPIn1 and SaPIn3.

Characterization of Japanese MRSA isolates regarding their virulence aspects

The pathogenicity of MRSA depends, at least partially, on the toxin repertoire of each MRSA strain. Therefore, the manifestations of MRSA infection may vary in different countries. The most remarkable difference between the toxin patterns of Japanese strains and those of MRSA strains isolated in European countries and North America was high carriage and high coexistence rates of *tst, sec* and *sel* genes in the Japanese strains. These three genes have been rarely observed in European and North American MRSA strains. We reported in a previous study that MRSA strains isolated from patients with neonatal toxic shock syndrome-like exanthematous disease (NTED) produced a large amount of TSST-1 and SEC (Nakano *et al.*, 2002b). Moreover, MRSA strains associated with enterocolitis, which was sometimes accompanied by toxic shock syndrome and multiorgan failure, were also TSST-1- and SEC-positive (Watanabe *et al.*, 2001). The patients with NTED have been reported only in Japan (Takahashi *et al.*, 1998; Takahashi, 2003). MRSA-associated enterocolitis has been occasionally observed in Japanese patients receiving surgical operations, while it has rarely been reported in other countries. It is therefore likely that Japanese MRSA isolates that were *tst*- and *sec*-positive were involved in the aetiology of these infections. As for the Japanese HA-MRSA strains, most of the *S. aureus* isolates from

European countries also carried an identical *egc* locus (Jarraud *et al.*, 2001). The typical *egc* locus encodes five intact enterotoxins, for which superantigenic activities have been proven (Jarraud *et al.*, 2001). These enterotoxins, however, are unlikely to be involved in the pathogenesis of severe infections, since their expression level is very low (Omoe *et al.*, 2002) and, moreover, the prevalence of the *egc* locus in *S. aureus* decreases significantly with severity of infection (Ferry *et al.*, 2005).

The conclusive result in our study was that the most of the Japanese HA-MRSA isolates had specific toxin patterns which were characterized by a high carriage rate of *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld* and *hlg-2* genes, although their PFGE patterns showed a wide variation. Compared with strains isolated in European countries and North America, the most remarkable difference was that Japanese MRSA isolates carried *tst*, *sec* and *sel* genes at high frequencies.

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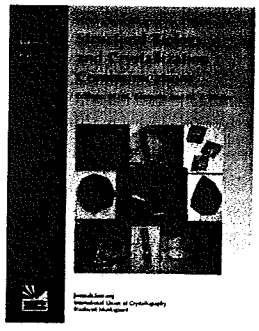

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Structure of AmpC β-lactamase ($AmpC^D$) from an *Escherichia coli* clinical isolate with a tripeptide deletion (Gly286-Ser287-Asp288) in the H10 helix

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The X-ray crystal structure of AmpC β-lactamase ($AmpC^D$) with a tripeptide deletion (Gly286-Ser287-Asp288) produced by *Escherichia coli* HKY28, a ceftazidime-resistant strain, was determined at a resolution of 1.7 Å. The structure of $AmpC^D$ suggests that the tripeptide deletion at positions 286–288 located in the H10 helix causes a structural change of the Asn289–Asn294 region from the α-helix present in the native AmpC β-lactamase of *E. coli* to a loop structure, which results in a widening of the substrate-binding site.

1. Introduction

AmpC β-lactamase belongs to the molecular class C β-lactamases (Ambler, 1980; Babic *et al.*, 2006) and is clinically as important as class A β-lactamases (Rice & Bonomo, 2000) as it hydrolyzes a broad range of β-lactam antibiotics, including the extended-spectrum cephalosporins such as ceftazidime, cefotaxime, cefepime and cefpirome. In addition, AmpC β-lactamase is generally not susceptible to inhibition by clavulanic acid, although tazobactam sometimes inhibits this enzyme. Therefore, the spread of AmpC β-lactamase is a serious threat to antibiotic chemotherapy for infectious diseases. Recently, many AmpC variants with extended-spectrum activity have been clinically isolated from various bacterial pathogens such as *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes* and *Serratia marcescens*. One of the main reasons for the alteration of substrate specificity in these variants is thought to be structural modification of the protein (Nordmann & Mammeri, 2007; Nukaga *et al.*, 2004; Vakulenko *et al.*, 2002; Trépanier *et al.*, 1999) such as amino-acid replacement (Raimondi *et al.*, 2001; Trépanier *et al.*, 1999; Vakulenko *et al.*, 2002), insertion (Mammeri *et al.*, 2007; Nukaga *et al.*, 1998; Crichlow *et al.*, 1999) and deletion (Mammeri *et al.*, 2004; Doi *et al.*, 2004; Barnaud *et al.*, 2001).

In 1994, Arakawa and coworkers reported a chromosomal AmpC β-lactamase produced by an *E. coli* clinical isolate from a urine specimen in Japan, HKY28 (Doi *et al.*, 2004). From a comparison of the amino-acid sequence of AmpC of *E. coli* HKY28 (denoted $AmpC^D$) with that of *E. coli* K-12 (Jaurin & Grundstrom, 1981), $AmpC^D$ contained three amino-acid deletions at positions 286, 287 and 288, corresponding to Gly, Ser and Asp residues, respectively, located on the H10 helix. With respect to substrate specificity, $AmpC^D$ conferred resistance to ceftazidime with a minimal inhibitory concentration (MIC) of 32 μg ml^{-1}, although *E. coli* rarely acquires resistance to this drug. Moreover, the hydrolytic activity of β-lactam antibiotics by $AmpC^D$ was suppressed by the clinically available β-lactamase inhibitors sulbactam and tazobactam and to some extent by clavulanic acid.

To elucidate the structural changes in the vicinity of the substrate-binding site resulting from the tripeptide deletion, we carried out a crystallographic analysis of $AmpC^D$ β-lactamase. In this paper, we report the crystal structure of $AmpC^D$ β-lactamase at a resolution of 1.7 Å and a comparison with the structure of AmpC β-lactamase of *E. coli* (denoted native AmpC; PDB code 1ke4) at a resolution of 1.72 Å.

2. Materials and methods

2.1. Expression and purification

E. coli HKY28 was isolated from a culture of urine from an inpatient in Japan in 1994. The *amp*C gene of *E. coli* HKY28 was cloned between the *Eco*RI and *Bam*HI sites of the expression vector pBCKS+ (Stratagene) to yield pBE28W, which was transformed into *E. coli* CS14-2 (Doi *et al.*, 2004). For protein purification, the plasmid was re-extracted using a Wizard Plus SV Minipreps DNA-purification system (Promega) from the strain *E. coli* CS14-2 pBCKS+/AmpC^{D}, which was retransformed into competent *E. coli* JM109 cells.

E. coli JM109 harbouring pBE28W was cultured at 310 K for 24 h in 10 l LB broth supplemented with 30 µg ml^{-1} chloramphenicol. Cells were harvested by centrifugation at 5000*g* for 15 min at 277 K. The pellets (about 50 g wet weight) were washed by resuspension in 50 ml 20 m*M* bis-tris–HCl buffer pH 6.5 with repeat centrifugation. The supernatant was discarded. The pellets were resuspended in 50 ml of the same buffer, disrupted by sonication for 5 min and centrifuged at 100 000*g* for 75 min at 275 K. The supernatant was loaded onto an SP Sepharose Fast Flow column (GE Healthcare) and the proteins were eluted with a linear gradient of 0–0.5 *M* NaCl. The fractions were analyzed by SDS–PAGE and by their ability to turn over nitrocefin. The fractions containing the desired activity were pooled and concentrated to a volume of 10 ml using Ultracel YM-10 (Millipore). The buffer was exchanged from 20 m*M* bis-tris–HCl pH 6.5 to 20 m*M* bis-tris–HCl pH 6.5, 1 *M* ammonium sulfate. The buffer-exchanged protein was loaded onto a Sephacryl SR-100 HR column (GE Healthcare) and was eluted with 20 m*M* bis-tris–HCl pH 6.5, 1 *M* ammonium sulfate, 0.3 *M* NaCl. Fractions containing AmpC^{D} β-lactamase were pooled and concentrated to a volume of 10 ml. The protein was then again reloaded onto a Phenyl Sepharose 6 Fast Flow column (low sub; GE Healthcare) and eluted with a linear gradient of 1.0–0.5 *M* ammonium sulfate. The enzyme was further concentrated to a volume of 2 ml using both Ultracel YM-10 (Millipore) and Amicon Ultra-15 (Millipore). The enzyme was more than 95% pure as judged by SDS–PAGE. For crystallization of purified AmpC^{D}, the buffer was exchanged to 20 m*M* HEPES–NaOH pH 7.5 using Amicon Ultra (Millipore).

2.2. Crystallization

Initial screening for AmpC^{D} crystallization conditions was performed at 293 K by the hanging-drop method (Luft & DeTitta, 1992) using the screening kits Crystal Screen and Crystal Screen 2 (Hampton Research). In the initial crystallization procedure, drops were prepared by mixing 1 µl protein solution (15 mg ml^{-1}) with 1 µl reservoir solution and were equilibrated against 350 µl reservoir solution. Crystals of AmpC^{D} were first obtained in one month from condition No. 40 [20%(*w*/*v*) PEG 4000, 20%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate tribasic dihydrate pH 5.6] of Crystal Screen. Improved crystals were subsequently obtained by refining the successful conditions using the hanging-drop method in 24-well VDX plates (Hampton Research): 1 µl concentrated protein solution (10 mg ml^{-1}) in 20 m*M* HEPES–NaOH pH 7.5 was combined with 1 µl reservoir solution containing 20%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6. This protein drop was suspended over 350 µl reservoir solution containing 20%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6 at 293 K. Crystals formed after 10 d. The crystals were flash-frozen in nitrogen gas at 100 K after cryoprotection by brief exposure to reservoir solution containing 40%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6.

Table 1
Crystallographic data-collection and refinement statistics for AmpC^{D}.

Values in parentheses are for the highest resolution shell.

Data collection	
Resolution (Å)	50.0–1.70 (1.76–1.70)
Wavelength (Å)	1.07
Unit-cell parameters (Å, °)	a = 47.1, b = 47.4, c = 81.5, α = 82.6, β = 80.9, γ = 65.4
Space group	$P1$
Redundancy	2.2 (1.7)
Completeness (%)	94.3 (82.8)
R_{merge}†	0.062 (0.267)
No. of observed reflections	141726 (9810)
No. of unique reflections	65343 (5771)
$\langle I/\sigma(I)\rangle$	18.1 (2.6)
Refinement statistics	
σ cutoff	0
Resolution (Å)	39.2–1.70 (1.74–1.70)
No. of reflections used	62030 (3855)
B factors (Å^2)	
Average	16.9
Protein	15.6
Water	28.6
No. of non-H atoms‡	
Protein	5796
Water	611
R.m.s.d. from ideal§	
Bond lengths (Å)	0.012
Angles (°)	1.4
$R_{working}$¶	0.163 (0.228)
R_{free}††	0.206 (0.306)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity for reflection hkl and $\langle I(hkl)\rangle$ is the average intensity calculated for reflection hkl from replicate data. ‡ Per asymmetric unit. § R.m.s.d.: root-mean-square-deviation. ¶ $R_{working} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. †† $R_{free} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$ for 5% of the data not used at any stage of structural refinement.

2.3. Data collection and refinement

The data set used for structure determination was collected at SPring-8 to a resolution of 1.7 Å at a wavelength of λ = 1.07 Å. The data were integrated, merged and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The refined structure of native AmpC of *E. coli* at a resolution of 2.0 Å (PDB code 2bls; Usher *et al.*, 1998) was used as the search model for molecular replacement using *AMoRe* (Navaza, 1994), a component of the *CCP*4 program suite v.6.0.0 (Collaborative Computational Project, Number 4, 1994). Refinement was interspersed with model building using *REFMAC* v.5.2.0019 (Murshudov *et al.*, 1997), a component of the *CCP*4 program suite v.6.0.0 (Collaborative Computational Project, Number 4, 1994), and *Coot* v.0.1.2 (Emsley & Cowtan, 2004). The quality of the model was inspected using the program *PROCHECK* (Laskowski *et al.*, 1993). Figures were generated using *PyMOL* (http://pymol.sourceforge.net/) and *MolFeat* v.3.5. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB code 2zj9). The structure of AmpC^{D} was refined to a final R factor of 16.3% and a free R factor of 20.6% and the root-mean-square-deviation (r.m.s.d.) values from the ideal bond distances and angles are 0.012 Å and 1.4°, respectively. Data-collection and refinement statistics are listed in Table 1.

3. Results and discussion

The final refined structural model contains two AmpC^{D} molecules per asymmetric unit, consisting of residues Ala4–Gln361 for molecules *A* and *B*, with the exception of the deleted residues 286–288. A Ramachandran plot shows that 92.2% of the residues are in the most favoured regions, with a further 7.8% in additionally allowed regions. The r.m.s.d. value between the C$^{\alpha}$ atoms of the two monomers is

0.23 Å. As expected, each AmpC^{D} monomer adopts a mixed α/β structure of nine antiparallel β-sheets with a helical domain on one side and a mixed α/β domain on the other (Fig. 1*a*), as found in native AmpC of *E. coli* (Usher *et al.*, 1998; Powers & Shoichet, 2002; Fig. 1*b*) and other AmpC β-lactamases from *Citrobacter freundii* (Oefner *et al.*, 1990) and *Enterobacter cloacae* (Lobkovsky *et al.*, 1993).

For simplicity, only one molecule (molecule *B*) will be considered in the present discussion. The structures of AmpC^{D} and native AmpC of *E. coli*, which has 98% sequence homology (PDB code 1ke4; 1.72 Å resolution; Powers & Shoichet, 2002), were superimposed. The r.m.s.d. for the C$^{\alpha}$ atoms between Ala4 and Gln361, excluding the residues Gly286, Ser287 and Asp288 located on the H10 helix, is 0.72 Å. Upon comparison of the two overall structures, a significant difference was observed in the vicinity of the deleted resides Gly286-Ser287-Asp288. In the AmpC^{D} structure (Fig. 2*b*), the deletion causes a structural change in the 289–293 segment, corresponding to Asn289–Leu293, following the tripeptide deletion, which changes from the α-helix structure present in native AmpC (Fig. 2*a*) to a more extended loop. As a result, relative to the structure of native AmpC, the C$^{\alpha}$ atoms of residues Asn285 and Asn289, which are before and after the tripeptide deletion, move by approximately 3 Å away from the catalytically important O$^{\gamma}$ atom of Ser64, indicating that the

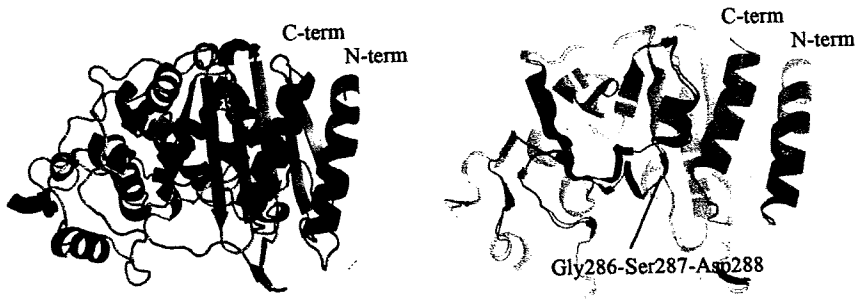

Figure 1
(*a*) Overall structure of AmpC^{D} β-lactamase. Only molecule *B* is depicted. α-Helices, β-strands and loops are shown in red, green and cyan, respectively. (*b*) Slabbed view of overall structure superposition of native AmpC β-lactamase (red; PDB code 1ke4) and AmpC^{D} β-lactamase (blue). In each structure, only molecule *B* is depicted.

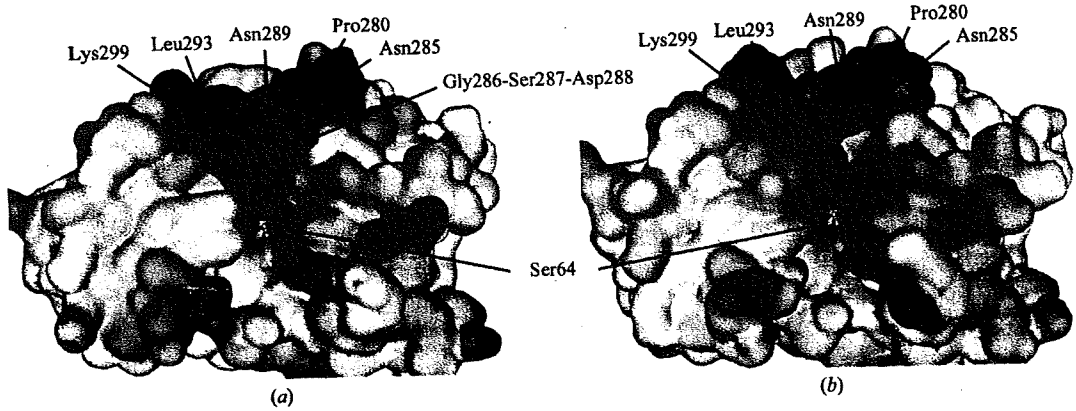

Figure 2
Electrostatic potential representations of (*a*) native AmpC β-lactamase and (*b*) AmpC^{D} β-lactamase. The catalytic residue Ser64 is represented as a ball-and-stick model (the O$^{\gamma}$ atom of Ser64 is coloured red). The Pro280–Lys299 region in native AmpC β-lactamase and AmpC^{D} β-lactamase is represented by a ribbon model coloured green on a transparent surface. In native AmpC β-lactamase, residues Gly286–Asp288 in the Pro280–Lys299 region are coloured violet.

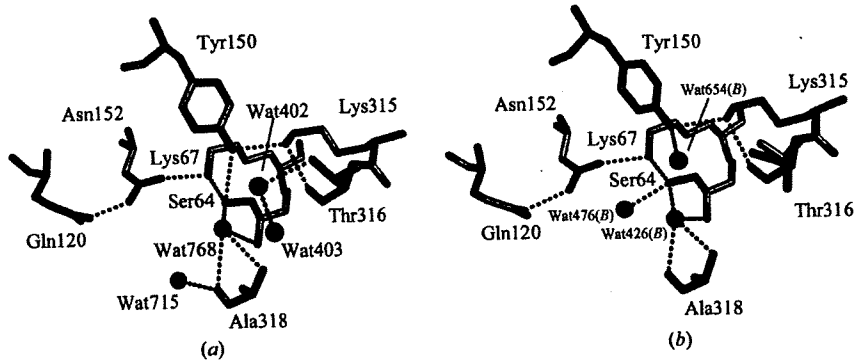

Figure 3
Substrate-binding site of (*a*) native AmpC β-lactamase determined to 1.72 Å resolution and (*b*) AmpC^{D} β-lactamase. In each structure, only molecule *B* is depicted. C, O and N atoms of the enzyme are shown in grey, red and blue, respectively. Water molecules cited in the text are labelled and are represented by red spheres. Dashed lines indicate hydrogen bonds with distances between 2.5 and 3.2 Å.

substrate-binding site of AmpC^D is wider than that of native AmpC. The crystal structure of AmpC^D also supports the previous results of molecular-modelling studies on AmpC^D with ceftazidime (Doi *et al.*, 2004); the tripeptide deletion in AmpC^D provides a more open site that can accommodate the R2 side chain of ceftazidime.

In the substrate-binding site of AmpC β-lactamases, residues Ser64, Lys67, Tyr150, Asn152, Lys315, Thr316 and the main chain of Ala318 are thought to be important in the hydrolysis reaction of β-lactam antibiotics (Lobkovsky *et al.*, 1993; Oefner *et al.*, 1990; Monnaie, Dubus & Frère, 1994; Monnaie, Dubus, Cooke *et al.*, 1994; Dubus *et al.*, 1994, 1995, 1996; Lobkovsky *et al.*, 1994). An overlay of these seven residues between AmpC^D and native AmpC shows a close fit, with the exceptions of the side chain of Tyr150 (the phenyl ring is rotated 19° around the $C^\beta-C^\gamma$ bond relative to that in molecule *B* of native AmpC). Thus, the structures of the key catalytic residues in the substrate-binding site are not dramatically affected, but, as mentioned above, the collapse of the α-helix on the tripeptide deletion in the H10 helix appears to give rise to an expansion of the substrate-binding site and is therefore believed to be the primary reason for the altered selectivity profile exhibited by AmpC^D relative to that of native AmpC (Doi *et al.*, 2004).

In the structure of native AmpC (Fig. 3*a*), four water molecules (Wat402, Wat403, Wat715 and Wat768) were observed in the substrate-binding site, where Wat402 was presumed to be the deacylating water (Powers & Shoichet, 2002) which hydrogen bonds to Thr316 $O^{\gamma1}$ and Wat403. Wat715 is hydrogen bonded to the main-chain carbonyl O atom of Ala318 and Wat768 interacts with Ser64 and Ala318. In the substrate-binding site of AmpC^D, three water molecules [Wat426(*B*), Wat476(*B*) and Wat654(*B*) in molecule *B*] were located in the active site (Fig. 3*b*). As in native AmpC, a water molecule [Wat426(*B*) in the AmpC^D structure] is bound in the site which stabilizes the tetrahedral intermediate of the lactamase reaction (Usher *et al.*, 1998; Murphy & Pratt, 1988) and is hydrogen bonded to the main-chain N atoms of Ser64 and Ala318, Ser64 O^γ and the main-chain carbonyl O atom of Ala318.

In conclusion, we have determined the crystal structure of AmpC^D of *E. coli* with a tripeptide deletion (Gly286-Ser287-Asp288) in the H10 helix and revealed the structural changes associated with the tripeptide deletion. However, further crystallographic studies on AmpC^D in complexes with the hydrolyzed products of substrates and with inhibitors are required to further understand the structural correlations with enzyme activity and the altered selectivity profile.

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Accumulation of Plasmid-Mediated Fluoroquinolone Resistance Genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* Co-producing RmtB and Class A β-lactamase LAP-1

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Abstract. A new plasmid-mediated fluoroquinolone efflux pump gene, *qepA*, is known to be associated with the *rmtB* gene, which confers high-level resistance to aminoglycosides. We investigated the *qepA* gene in 573 AmpC-producing *Enterobacteriaceae* including one *Citrobacter freundii* known to harbor *rmtB*. Of them, two clonally unrelated *E. aerogenes* harbored *qepA*. Both isolates co-harbored *rmtB*, *qnrS1*, *qepA*, and bla_{LAP-1} on an IncFI type plasmid. The *qepA* was flanked by two copies of IS*26* containing IS*CR3C*, *tnpA*, *tnpR*, bla_{TEM}, and *rmtB*. The *qnrS1* and bla_{LAP-1} were located upstream of *qepA*. All the resistance determinants (*qepA*, *qnrS1*, *rmtB*, and bla_{LAP-1}) were co-transferred to *E. coli* J53 by filter mating from both isolates. Although the prevalence of *qepA* is currently low, considering the presence of IS*CR3C* and the possibility of co-selection and co-transferability of plasmids, more active surveillance for these multi-drug resistant bacteria and prudent use of antimicrobials are needed.

Keywords: *qepA*, *qnrS1*, *rmtB*, bla_{LAP-1}, IS*CR3C*, gene co-transferance

Introduction

Resistance to quinolones in bacteria belonging to the family *Enterobacteriaceae* most commonly arises stepwise as a result of chromosomal mutations in *gyrA* and/or *parC* genes encoding DNA gyrase and topoisomerase IV, respectively. Recently, plasmid-encoded quinolone resistance determinants such as *qnr* and *aac(6')-Ib-cr* have been shown to be distributed worldwide [1,2]. A new plasmid-mediated fluoroquinolone efflux pump, QepA, was identified in three *Escherichia coli* isolates from Japan [2,3] and Belgium [1]. In addition, the *qepA* gene was associated with the *rmtB* gene, which confers high-level resistance to aminoglycosides.

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We earlier detected *rmtB* in only one clinical isolate of *Citrobacter freundii* of the 413 AmpC-producing *Enterobacteriaceae* screened [4]. Therefore, we investigated the incidence of *qepA* among AmpC-producing *Enterobacteriaceae* including the strain previously reported to harbor *rmtB*.

Materials and Methods

A total of 572 consecutive, non-duplicate isolates, including *Enterobacter cloacae* (168), *Enterobacter aerogenes* (146), *C. freundii* (109), and *Serratia marcescens* (149), were collected from clinical specimens at 12 clinical laboratories in Korea between March and July 2006. One *C. freundii* isolated in 2003, which harbored *rmtB*, was included.

The MICs of ciprofloxacin and nalidixic acid were determined by the agar dilution method according to the CLSI (formerly NCCLS) guidelines [5]. For 223 isolates (69 *E. cloacae*, 28 *E. aerogenes*, 43 *C. freundii*, and 83 *S. marcescens*) showing ciprofloxacin MICs ≥0.25 μg/ml and/or nalidixic acid MICs ≥16 μg/ml, and the *C. freundii* isolate harboring *rmtB*, the presence of *qepA* was screened by PCR as described

previously [2]. A *qepA*-positive plasmid (pSTVqepA) [2] was used as a positive control.

Conjugation experiments were performed with the *qepA*-positive isolates as donors and azide-resistant *E. coli* J53 as a recipient strain by filter mating. Transconjugants were selected on LB agar plates supplemented with sodium azide (150 μg/ml) and ciprofloxacin (0.25 μg/ml). From the transconjugants, plasmid DNAs were extracted, and PCRs for *qepA*, *rmtB*, *qnrS1*, and bla_{LAP-1} were performed using primers reported previously (Table 1). MICs for donors, transconjugants, and recipient were measured by agar dilution method in accordance with the CLSI guidelines [5]. The antimicrobials tested were ciprofloxacin, nalidixic acid, piperacillin, cefazolin, cefuroxime, ceftazidime, cefepime, and amikacin.

For the isolates harboring *qepA*, the known associated genes (IS*26*, bla_{TEM-1}, *rmtB*) [2], and other plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrS1*, and *aac(6')-Ib-cr*) were also investigated with PCR as described previously [6,7]. From the plasmids recovered from the transconjugants, the sequences of DNAs adjacent to *qepA* and *qnrS1* were determined with a series of PCR primers (listed in Table 1) on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analyses and comparison with known sequences were performed with the BLAST program at the National Center for Biotechnology Information.

From the plasmid DNAs obtained from transconjugants, the sequences of DNA adjacent to *qepA* and *qnrS1* were determined with a series of outward-facing primers starting from both sides of the *qep* gene on plasmid DNA purified from transconjugants. The incompatibility group was determined by PCR-based replicon typing [8]. For determination of plasmid size, the plasmid DNAs from the transconjugants and the reference strain (*E. coli* NCTC50192) were subjected to electrophoresis on 0.7% agarose gel.

Results and Discussion

The *qepA* gene was detected in two *E. aerogenes* isolates (YS10 and YS11) from two separate patients in one hospital. The two isolates had been isolated from bile and sputum, respectively. To investigate the clonality of the isolates, PFGE analysis was carried out according to the manufacturer's protocol (Bio-Rad, Inc., Hercules, CA, USA), and the two isolates were found not to be related according to the criteria of Tenover et al [9] (data not shown). Both isolates harbored *qepA*, *qnrS1*, *rmtB*, bla_{TEM-1}, bla_{LAP-1}, and *aac(6')-Ib* [2]. Sequence analysis revealed that *qepA* was flanked by two copies of IS*26* containing IS*CR3C* recently described by Cattoir et al [10], *tnpA*, *tnpR*, bla_{TEM}, and *rmtB*, as was observed in *E. coli* C316 (AB103506) [2].

The *qnrS1* and bla_{LAP-1} were located upstream of *qepA*, separated by 12,806-bp and 15,060-bp, respectively, that included truncated *Tn2*, IS*26*, and CS12 fimbrial gene cluster of *E. coli*, which was also identified at downstream of *qnrS1* in *K. pneumoniae* NK745 [11] and *Salmonella enterica* serovar Infantis [12] (Fig. 1). By filter mating, all the above resistance determinants except *aac(6')-Ib* were transferred to *E. coli* J53 and were carried on a IncFI type plasmid of about 120-kb. The MICs of ciprofloxacin, nalidixic acid, amikacin, and β-lactams for donors, transconjugants, and *E. coli* J53 are summarized in Table 2.

QepA, the most recently identified plasmid-mediated fluoroquinolone efflux pump, showed considerable similarity in amino acid sequence to the MFS type efflux pumps of *Actinomycetales (Nocardia farcinica, Streptomyces globisporus,* and *Streptomyces clavuligerus*) [2] and soil bacteria, and this might be related to the agricultural use of fluoroquinolones, as well as their use in humans. The finding that *qepA* is usually linked with *rmtB*, which confers multiple resistance to aminoglycosides, reminds us that aminoglycosides are derived from *Actinomycetales* (*Streptomyces* spp and *Micromonospora* spp) and that there is a potential for co-selection of the QepA determinant by the use of aminoglycosides. In addition, the fact that *rmtB*-harboring *C. freundii* did not contain *qepA* indicates that *rmtB* is not necessarily associated with *qepA*. This finding supports the hypothesis that the uptake of *qepA* from soil bacteria by *rmtB*-producing *E. coli*, which is frequently isolated from pigs exposed to aminoglycosides, with further dissemination via food, may be the way in which this new resistance trait evolved [13].

Both of the *E. aerogenes* isolates characterized in this study harbored *aac(6')-Ib* in addition to *qnrS1*. Although the gene was not mutated into *aac(6')-Ib-cr* in these isolates, this could happen during persistent exposure to fluoroquinolones and reminds us of a recent Korean report of a *K. pneumoniae* isolate harboring both *qnrS1* and *aac(6')-Ib-cr* [14]. Accumulation of *qnr*, *aac(6')-Ib-cr*, and *qepA* in bacterial cells is more likely a result of plasmid conjugation or natural transformation.

In this study, the presence of *qepA* was first identified in *E. aerogenes* isolates and was linked with the high-level aminoglycoside resistance gene, *rmtB*, as reported previously in *E. coli* [1]. It is also