resistance in Enterobacteriaceae isolates that do not produce 16S rRNA MTases. Our study shows that Enterobacteriaceae clinical isolates have continuously acquired either amikacin acetyltransferases or 16S rRNA MTases to enable amikacin resistance.

Materials and methods

Bacterial strains

Fourteen amikacin-resistant (MIC ≥64 mg/L) Enterobacteriaceae clinical isolates (3 *Escherichia coli*, 4 *Klebsiella pneumoniae*, 2 *Proteus mirabilis* and 5 *Serratia marcescens*) were used in this study.

Susceptibility testing

MICs were determined with the agar dilution method.[12] *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains were used as quality controls.

Detection of amikacin resistance genes by PCR

Detection of aminoglycoside resistance 16S rRNA MTase genes was performed as described previously.[13] The presence of *aac(6′)-Ia* and *aac(6′)-Ib*, which are prevalent among Enterobacteriaceae as determining factors of amikacin resistance, was determined by PCR using the primers listed in Table S1 (available as Supplementary data at *JAC* Online).

Conjugation and transformation

E. coli CSH2 (rifampicin^r) was used as the recipient for conjugation. The conjugants were selected on LB agar plates containing 2 mg/L amikacin and 100 mg/L rifampicin.[14] Plasmids of the *S. marcescens* NUBL-11663 strain were extracted and electroporated into *E. coli* DH10B. The transformants were selected on LB agar plates supplemented with 2 mg/L amikacin.

Cloning of aac(6′)-Ian

The p11663 plasmid was extracted from the *E. coli* DH10B transformant using the QIAGEN Plasmid Midi Kit (Qiagen) and partially digested with Sau3AI. The digested fragments were ligated to the pBC-SK+ vector previously digested with BamHI, dephosphorylated and then introduced into *E. coli* DH5α. The transformants were selected on LB agar plates supplemented with 30 mg/L chloramphenicol and 2 mg/L amikacin.

Complete nucleotide sequence of p11663

Plasmids were extracted from an *E. coli* DH10B transformant carrying p11663 as described above and subjected to electrophoresis with a CHEF-DR III system (Bio-Rad). The extracted plasmids were digested with S1 nuclease (Takara) and subjected to electrophoresis. The DNA band corresponding to p11663 was extracted using a Wizard SV Gel and PCR Clean-Up System (Promega). The sequence of p11663 was generated using paired-end libraries with 100 bp inserts on an Illumina Hiseq 2000 system. A velvet *de novo* assembler (velvet_1.2.03) was used to generate contigs from the reads. The gaps were closed by PCR and cloning experiments and followed by sequencing. The nucleotide sequences were analysed by GeneMark.hmm for Prokaryotes (http://exon.gatech.edu/GeneMark/gmhmmp.cgi) and BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR cloning of bla_{TLA-3}

The region including bla_{TLA-3} and its putative promoter was amplified with the primers listed in Table S1, cloned into pBC-SK+ and introduced into *E. coli* DH5α. The transformants were selected on LB agar plates supplemented with 2 mg/L ceftazidime and 30 mg/L chloramphenicol.

Expression and purification of recombinant AAC(6′)-Ian

The *aac(6′)-Ian* gene was amplified with the primers listed in Table S1 and cloned into a pET22b(+) vector. The resultant plasmid (pET-AAC) was introduced into *E. coli* BL21(DE3)pLysS. The cells were cultured in LB broth containing 100 mg/L ampicillin and 30 mg/L chloramphenicol at 37°C. When the OD at 610 nm reached 0.4, IPTG was added and the culture was further continued. The cells were collected, resuspended in PBS (pH 7.4) and disrupted with a French press. After ultracentrifugation, the supernatant was bound with 2 mL of Ni Sepharose™ 6 Fast Flow (GE Healthcare) and eluted with PBS containing 500 mM imidazole. The eluted protein was buffer-exchanged to PBS containing 20 mM imidazole and loaded onto a HisTrap™ HP column (GE Healthcare). The protein was eluted with a linear gradient of 20–500 mM imidazole. The collected protein was dialysed against 20 mM Bis-Tris buffer (pH 6.0) containing 50 mM NaCl, loaded onto a Mono Q column (GE Healthcare) and eluted with a linear gradient of 50–500 mM NaCl. The eluted protein was concentrated, loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl. The eluted protein was stored at −80°C until use.

Thin-layer chromatography (TLC) analysis of aminoglycoside acetylation by recombinant AAC(6′)-Ian

Acetylation of aminoglycosides using recombinant AAC(6′)-Ian was performed as described previously.[9] Briefly, each aminoglycoside solution (2 mM) was mixed with 2 mM acetyl coenzyme A (acetyl-CoA) and recombinant AAC(6′)-Ian (2.5 μg) in 50 μL of PBS (pH 7.4) and then incubated at 37°C for 16 h. Each mixture was applied to a TLC Silica Gel 60 F254 (Merck) and then developed with 5% phosphate potassium solution. Ninhydrin (Wako) was overlaid on silica gels to detect aminoglycoside derivatives.

HPLC

HPLC was also performed to identify the acetylation of aminoglycosides. AAC(6′)-Iad, the positive control for the AAC(6′) group enzyme, was purified as described previously.[7] The purified AAC(6′)-Iad or AAC(6′)-Ian (5 μg) was added to 250 μL reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.6), 15 mM $MgCl_2$, 4 mM acetyl-CoA and 6 mM arbekacin or 10 mM neomycin. The incubation was performed at 37°C for 30 min and equal volumes of 2-propanol and the derivatization reagent were added.[15] After incubation for 10 min at 60°C, the sample was loaded onto a Chemcobond 5-ODS-H column (4.6×100 mm; Chemco Scientific) equipped with an Agilent 1100 HPLC system (Agilent Technologies). The mobile phase was the same as that described previously.[7]

Nucleotide sequence accession number

The complete nucleotide sequence of p11663 presented in this study has been deposited in GenBank under accession no. AP014611.

Results and discussion

Detection of AAC(6′) group enzyme gene in amikacin-resistant Enterobacteriaceae

Fourteen amikacin-resistant Enterobacteriaceae without 16S rRNA MTase genes are listed in Table 1. For these isolates, the presence of *aac(6′)-Ia* and *aac(6′)-Ib* was first explored by PCR and the results are summarized in Table 1. Nine strains were found to carry *aac(6′)-Ia* or *aac(6′)-Ib*, but five strains (one *E. coli*, three *S. marcescens* and one *P. mirabilis*) gave negative results when evaluating resistance genes by PCR and these strains were predicted to have unknown mechanisms underlying amikacin resistance such as a new AAC(6′) group enzyme. *S. marcescens*

Table 1. Profiles of bacterial strains used in this study

	Amikacin MIC (mg/L)	*aac(6′)-Ia*	*aac(6′)-Ib*
E. coli NUBL-11655	64		
E. coli NUBL-11656	128		+
E. coli NUBL-11657	>256	+	
K. pneumoniae NUBL-11658	64		+
K. pneumoniae NUBL-1533	64		+
K. pneumoniae NUBL-4605	64		+
K. pneumoniae NUBL-4622	64		+
P. mirabilis NUBL-11659	64	+	
P. mirabilis NUBL-11660	128		
S. marcescens NUBL-2	64		
S. marcescens NUBL-11661	64	+	
S. marcescens NUBL-11662	64		
S. marcescens NUBL-11663	128		
S. marcescens NUBL-11664	>256		+

strain NUBL-11663 showed a high level of resistance to amikacin (MIC 128 mg/L). We therefore decided to focus on clarifying the molecular mechanism underlying the amikacin resistance of the *S. marcescens* NUBL-11663 strain.

Transfer of amikacin resistance and cloning resistance determinants

The amikacin-resistant phenotype of the *S. marcescens* NUBL-11663 strain was successfully co-transferred with β-lactam resistance to *E. coli* strain CSH2 by conjugation. Introduction of plasmids extracted from the NUBL-11663 strain by electroporation also conferred amikacin resistance to *E. coli* DH10B (Table 2). These results indicate that the amikacin resistance determinant was located on the plasmids. We extracted plasmids from the *E. coli* DH10B transformant and identified one plasmid, named p11663, with a size of ~170 kb (Figure S1).

Next, we attempted to isolate the amikacin resistance determinant located on p11663 by a cloning experiment and obtained one plasmid (pBC-amk) carrying a 1.2 kb insert by selection using amikacin. This 1.2 kb insert had one 573 bp ORF, encoding a protein consisting of 190 amino acids. A homology database search revealed that this protein has a coenzyme A-binding pocket, assigned to be a putative GCN5-like *N*-acetyltransferase, and has 97% amino acid identity to aminoglycoside 6′-*N*-acetyltransferase, AAC(6′), of a whole genome-determined *Acinetobacter baumannii* AYE strain (GenBank accession no. CU459141),[16] although the function of AAC(6′) of *A. baumannii* AYE has not been elucidated yet. Thus, the gene we isolated was predicted to encode an AAC(6′) conferring amikacin resistance and termed as AAC(6′)-Ian. AAC(6′)-Ian has low amino acid identity (<25%) to the functionally determined AAC(6′) group enzymes shown in Figure 1.

Antimicrobial susceptibility testing

The MICs of various aminoglycosides for the parent *S. marcescens* NUBL-11663 and *E. coli* DH10B transformant strains are shown in

Table 2. Susceptibility testing

	MIC (mg/L)																			
	Aminoglycosides													β-lactams						
Bacterial strains	APR	ABK	AMK	TOB	KAN	GEN	ISP	SIS	NET	STR	SPT	HGM	NEO	RSM	PRM	CAZ	CAZ+CLA[a]	CTX	FEP	CMZ	MEM
S. marcescens NUBL-11663 (p11663)	4	32	128	128	>256	>256	128	256	128	256	>256	16	8	>256	4	128	2	32	8	128	0.25
E. coli DH10B (p11663)	2	32	64	128	>256	256	128	128	128	>256	256	16	4	>256	4	256	1	4	0.5	1	0.031
E. coli DH10B[b]	1	0.5	0.25	0.5	0.5	0.5	1	0.5	0.5	>256	16	8	0.5	2	0.5	0.5	0.25	0.063	0.031	1	≤0.016
E. coli DH5α (pBC-amk)	2	8	32	16	128	0.25	32	8	32	1	8	16	4	>256	2	ND	ND	ND	ND	ND	ND
E. coli DH5α (pBC-TLA-3)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	32	0.25	1	0.125	1	≤0.016
E. coli DH5α (pBC-SK+)	1	0.25	0.5	0.25	0.5	0.5	0.25	0.5	0.25	1	8	8	0.5	1	1	0.125	0.125	≤0.016	≤0.016	1	≤0.016

APR, apramycin; ABK, arbekacin; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; GEN, gentamicin; ISP, isepamicin; SIS, sisomicin; NET, netilmicin; STR, streptomycin; SPT, spectinomycin; HGM, hygromycin B; NEO, neomycin; RSM, ribostamycin; PRM, paromomycin; CAZ, ceftazidime; CLA, clavulanic acid; CTX, cefotaxime; FEP, cefepime; CMZ, cefmetazole; MEM, meropenem; ND, not determined.

[a]The concentration of clavulanic acid was fixed at 4 mg/L.

[b]*E. coli* DH10B naturally shows resistance to streptomycin.

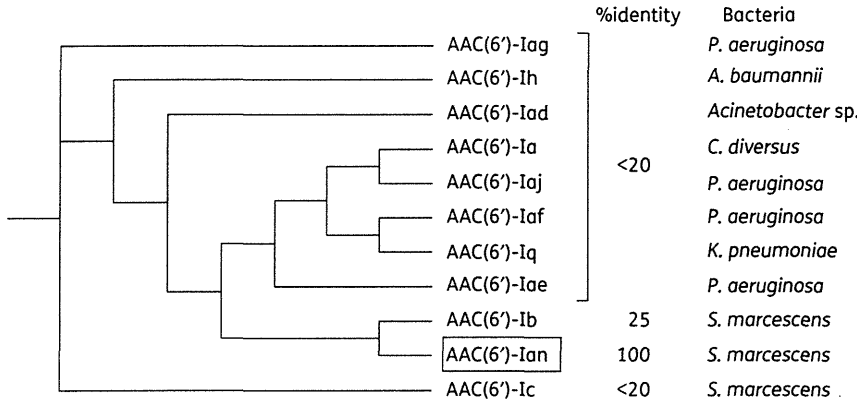

Figure 1. Tree view depicting the identity of AAC(6')-Ian with other AAC(6') enzymes. The tree was constructed using the ClustalW/X version 2.1 program (http://clustal.org). GenBank accession numbers: AAC(6')-Ian, this study; AAC(6')-Ia, M18967; AAC(6')-Ib, M23634; AAC(6')-Ic, M94066; AAC(6')-Ih, L29044; AAC(6')-Iq, AF047556; AAC(6')-Iad, AB119105; AAC(6')-Iae, AB104852; AAC(6')-Iaf, AB462903; AAC(6')-Iag, AB472901; and AAC(6')-Iaj, AB709942.

Table 2. A parent *S. marcescens* NUBL-11663 strain showed resistance to a variety of aminoglycosides, 4,6-disubstituted 2-deoxystreptamines (DOS), 4,5-disubstituted 2-DOS, streptomycin and spectinomycin. The *E. coli* DH10B transformant showed an aminoglycoside resistance profile very similar to that of the parent strain. In *E. coli* DH5α carrying *aac(6')-Ian*, increased MICs were observed for 4,6-disubstituted 2-DOS except for gentamicin and 4,5-disubstituted 2-DOS, but not for the other aminoglycosides. This aminoglycoside resistance phenotype conferred by the production of AAC(6')-Ian was quite similar to that conferred by other aminoglycoside AAC(6') group enzymes.[7–11] The *E. coli* DH10B transformant harbouring p11663 demonstrated gentamicin and spectinomycin resistance that was not observed in *E. coli* DH5α carrying *aac(6')-Ian*, indicating that p11663 carried additional aminoglycoside resistance determinants other than *aac(6')-Ian* (discussed below). Regardless, these findings clearly show that the production of AAC(6')-Ian was mainly responsible for the amikacin resistance in the *S. marcescens* NUBL-11663 strain.

Complete nucleotide sequence of p11663 and genetic environment of aac(6')-Ian

The circular map of p11663 is shown in Figure 2(a). Plasmid p11663 had a length of 169829 bp and belonged to incompatibility group A/C2 with a broad host range. The average G+C content of p11663 was 52.0%. Plasmid comparative analysis revealed that the backbone of p11663 exhibited the highest similarity to that of plasmid pP91278 from a *Photobacterium damselae* subsp. *piscicida* isolate from the USA (GenBank accession no. AB277724).[17]

The genetic environments of the newly identified *aac(6')-Ian* are shown in Figure 2(b). The backbone elements upstream of *aac(6')-Ian* correspond partially to those found in the pHH1107 plasmid (GenBank accession no. FJ012881), which is a low-GC plasmid recovered from soil.[18] Heuer *et al.*[18] suggested that the original host of these low-GC antibiotic plasmids may be *Acinetobacter* species. The IS*1106*-like element, which was located at the 5′-end of *aac(6')-Ian*, appeared to be inserted into a genetic region similar to the backbone sequence of the pHH1107 plasmid because a duplicated 8 bp sequence (CTGGCGAA) was found on both sides of the IS*1106*-like element, which is a hallmark of a previous insertion event. The *aac(6')-Ian* gene and IS*1106*-like element were flanked by two copies of a unique region (sequence 1) including an ISCR-like element. The genetic region including the *aac(6')-Ian* gene and its upstream 16 bp and downstream 151 bp had 94% nucleotide identity to those located in the chromosomal DNA of the *A. baumannii* AYE strain (GenBank accession no. CT025832) (Figure 2b). The *aac(6')* gene of *A. baumannii* AYE was flanked by two copies of ISCR-like elements that were quite different from those surrounding *aac(6')-Ian*. A similar AAC(6') enzyme was found in *A. baumannii* (NCBI Reference Sequence WP_000960976), although its enzymatic function has not been evaluated; thus, *aac(6')-Ian* mediated by p11663 of the *S. marcescens* NUBL-11663 strain and *aac(6')* found in *A. baumannii* strains may have evolved from a common ancestor.

Other antibiotic resistance genes in p11663

Another notable antibiotic resistance gene found in p11663 was bla_{TLA-3}, a new variant of the TLA-type ESBL gene. TLA-type β-lactamase was identified for the first time in Asian countries including Japan. TLA-3 has 93% and 52% amino acid identity to TLA-1, found in plasmids of Enterobacteriaceae clinical isolates exclusively from Latin America (Figure S2),[19–21] and TLA-2, found in the plasmid pRSB101 recovered from a wastewater treatment plant,[22] respectively. The introduction of a recombinant plasmid carrying bla_{TLA-3} (pBC-TLA-3) conferred resistance to ceftazidime, cefotaxime and cefepime, but not to cefmetazole and meropenem (Table 2). The resistance to ceftazidime was reduced in the presence of clavulanic acid. These resistant phenotypes, the preferred β-lactam substrate and the high sensitivity to the β-lactamase inhibitor of TLA-3 correspond well to the characteristics of typical ESBLs belonging to class A β-lactamases.[23]

The surrounding genetic organization of bla_{TLA-3} is largely different from that of bla_{TLA-1} of pRZA92 (GenBank accession

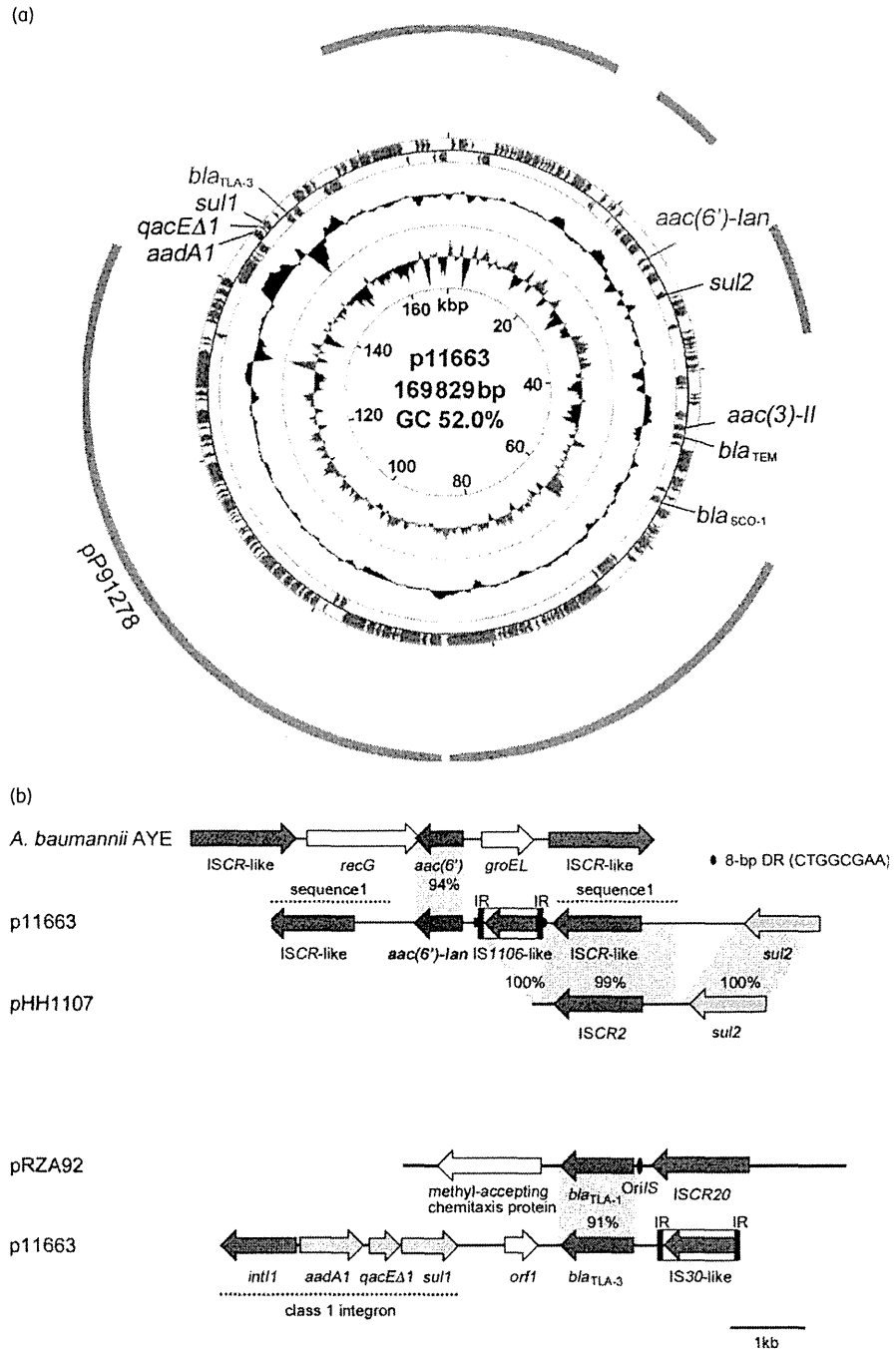

Figure 2. (a) Circular map of plasmid p11663. Each ORF is shown with an arrow to indicate the direction of transcription. The black inner circle shows the G+C content plotted against the average G+C content of 52.0% and green and purple circles represent GC skew information. The outside grey circle indicates the region genetically similar to pP91278 (GenBank accession no. AB277724). (b) Schematic map of genetic environments of *aac(6')-Ian* and bla_{TLA-3}. ORFs are shown as arrows indicating the direction of transcription. The *aac(6')* and bla_{TLA} genes are coloured in red and the other genes involved in antibiotic resistance are coloured in faint orange. The genes related to DNA recombination and transposition are coloured in green. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

no. AF148067) from the *E. coli* strain R170 (Figure 2b).[20] Only the 1.2 kb sequence containing bla_{TLA-3} and its upstream and downstream region showed 91% nucleotide identity to the sequences covering bla_{TLA-1} and its surrounding region.

In addition, bla_{TEM} (which encodes a TEM-type β-lactamase with F100L substitution relative to TEM-208), bla_{SCO-1} (carbenicillinase gene) and *aac(3)-II* were present in the p11663 plasmid (Figure 2a). The *aac(3)-II* gene is a gentamicin resistance determinant in the *S. marcescens* NUBL-11663 strain.

Overexpression and purification of histidine-tagged AAC(6′)-Ian

E. coli BL21(DE3)pLysS and the pET22b(+) expression vector were used for overexpression and purification of C-terminus histidine-tagged AAC(6′)-Ian. *E. coli* BL21(DE3)pLysS carrying pET22b(+) was susceptible to amikacin (MIC ≤0.25 mg/L), whereas *E. coli* BL21(DE3)pLysS carrying pET-AAC showed resistance to amikacin (MIC 32 mg/L), indicating that the production of histidine-tagged AAC(6′)-Ian was functional and responsible for amikacin resistance in *E. coli* BL21(DE3)pLysS (data not shown).

Acetylation of aminoglycosides by AAC(6′)-Ian

TLC analysis using a variety of aminoglycosides as a substrate was performed to determine the biochemical characteristics of AAC(6′)-Ian. AAC(6′)-Ian was predicted to be an acetyltransferase because it contained the acetyl-CoA-binding motif; thus, acetyl-CoA was first used as a cofactor in an *in vitro* reaction. The results of the TLC analysis are shown in Figure 3. AAC(6′)-Ian acetylated 4,6- and 4,5-disubstituted 2-DOS aminoglycosides, arbekacin, amikacin, tobramycin, kanamycin, gentamicin, isepamicin, sisomicin, netilmicin, neomycin and ribostamycin, which possess an amino group at the 6′-position, although the modifications of gentamicin were only partial because it was a mixture of derivatives such as gentamicin C1a, C1 and C2 and gentamicin C1 has no amino group at the 6′-position that can be modified. Apramycin and paromomycin with a hydroxyl group at the 6′-position were not modified by AAC(6′)-Ian. All aminoglycosides except gentamicin, whose MICs were increased by the production of AAC(6′)-Ian in *E. coli*, were acetylated by the recombinant AAC(6′)-Ian.

The results of the HPLC analysis are shown in Table 3. The retention times, which corresponded to the peak of *o*-phthalaldehyde derivatives of arbekacin and neomycin modified by AAC(6′)-Ian in the presence of acetyl-CoA, were the same as those for AAC(6′)-Iad, which was previously characterized and found to modify the 6′-position of aminoglycosides.[7] These results also indicate that AAC(6′)-Ian acetylates the amino group at the 6′-position of aminoglycosides using an acetyl-CoA as a cofactor.

AAC(6′)-Ian modified arbekacin, one of the potent aminoglycosides against Gram-positive and Gram-negative bacteria (Table 2). The effect of acetylation at the 6′-position of arbekacin is controversial, as it was considered to result in nearly no loss of the antimicrobial effect of arbekacin in some previous reports.[8,24] However, the production of AAC(6′)-Ian increased the MIC of arbekacin from 0.25 to 8 mg/L, i.e. to the moderate resistance level in *E. coli*. Enzymes similar to AAC(6′) such as AAC(6′)-Iad, AAC(6′)-Iaj and AAC(6′)-Iag that confer moderate resistance to arbekacin have been identified in *Acinetobacter* sp. and *P. aeruginosa*[7,10,11]

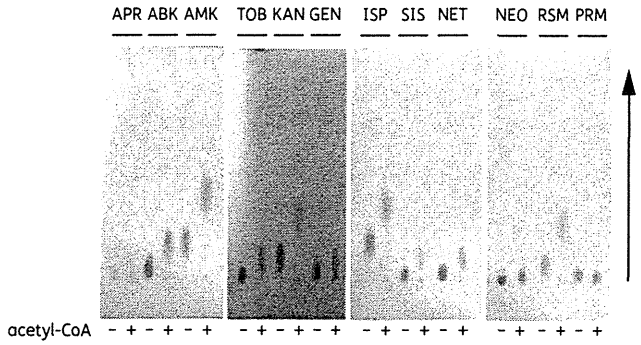

Figure 3. Detection of various acetylated aminoglycosides by TLC. Purified AAC(6′)-Ian and aminoglycosides were mixed with (+) or without (−) acetyl-CoA and developed in the direction indicated by the arrow. The gentamicin used in this study included gentamicin C1, C1a and C2 derivatives. APR, apramycin; ABK, arbekacin; AMK, amikacin; TOB, tobramycin; KAN, kanamycin; GEN, gentamicin; ISP, isepamicin; SIS, sisomicin; NET, netilmicin; NEO, neomycin; RSM, ribostamycin; PRM, paromomycin.

Table 3. Results of HPLC analysis

	Retention time (min)	
Agent(s)	AAC(6′)-Ian	AAC(6′)-Iad
Arbekacin	12.4	12.5
Arbekacin + acetyl-CoA	8.0	8.0
Neomycin	7.5	7.6
Neomycin + acetyl-CoA	3.9	3.9

and this peculiar effect against arbekacin was generally attributed to lower K_m and/or higher k_{cat} values against arbekacin. AAC(6′)-Ian, in addition to AAC(6′)-Iad, AAC(6′)-Iaj and AAC(6′)-Iag, would have high catalytic activity against arbekacin. AAC(6′)-group enzymes can commonly modify the 6′-position of aminoglycosides, but their substrate specificity for aminoglycosides may be highly variable considering their large diversity in amino acid constitution (Figure 1).

In addition, it is also possible that the expression of *aac(6′)-Ian* may be enhanced through the original promoter regions or insertion of an upstream IS*1106* element, resulting in an increase in the MIC of arbekacin. Further genetic and enzymatic characterization is necessary to explain the elevation of the MIC of arbekacin conferred by *aac(6′)-Ian*.

Conclusions

We report here a novel plasmid-mediated amikacin acetyltransferase, AAC(6′)-Ian, and ESBL, TLA-3, from a *S. marcescens* clinical isolate. Our results suggest that Enterobacteriaceae are still acquiring new variants of AAC(6′) group enzymes as the mechanisms for resistance to aminoglycosides including amikacin. In Enterobacteriaceae, the prevalence of amikacin resistance is still

low (<1%) in Japan (http://www.nih-janis.jp/report/kensa.html), compared with the resistance rate for other classes of antibiotics such as third-generation cephalosporins and fluoroquinolones. Thus, it appears that the potency of amikacin is still sufficient to make it effective for the treatment of infectious diseases caused by Enterobacteriaceae. However, increased use of amikacin would promote the spread of resistance determinants; thus, continuous monitoring is necessary to prevent and control the further spread of resistance determinants such as amikacin acetyltransferases including AAC(6')-Ian to allow aminoglycosides to continue to be used for treatment of bacterial infectious diseases.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Have group A streptococci with reduced penicillin susceptibility emerged?

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Keywords: GAS, *Streptococcus pyogenes*, β-lactam non-susceptibility, penicillin-binding proteins, PBPs, group B streptococci with reduced penicillin susceptibility

Sir,

Clinical isolates of group A *Streptococcus* (GAS) continue to be regarded as fully susceptible to β-lactams, including penicillin, which is the first-line drug treatment for GAS infections.[1] Although there have been reports of the emergence of GAS with reduced susceptibility to penicillin in Japan[2] and India,[3] these findings remain to be confirmed elsewhere. Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) has some microbiological similarities to GAS, and GBS clinical isolates with reduced penicillin susceptibility (PRGBS) have been reported in Japan[4,5] and North America.[6–8] PRGBS clinical isolates acquire reduced penicillin susceptibility through amino acid substitutions adjacent to the conserved active-site motifs of PBP2X.[4–10] Therefore, we hypothesized that GAS clinical isolates might acquire reduced β-lactam susceptibility by mechanisms similar to those that function in PRGBS. In the present study, we analysed 256 clinical isolates of GAS to monitor whether GAS with reduced susceptibility to penicillin has emerged in Japan.

MICs of penicillin, oxacillin, ceftizoxime, cefaclor and ceftibuten for 256 GAS clinical isolates derived from geographically diverse areas in Japan between 2010 and 2012 were determined by the agar dilution method as recommended by the CLSI. These five antimicrobial agents were chosen because PRGBS clinical isolates showed reduced susceptibility to their effects.[4,9,10] The distributions of β-lactam MICs for the 256 clinical isolates showed a single peak for each β-lactam and no clinical isolate showed an MIC of penicillin exceeding the criteria for 'susceptible' (all ≤0.12 mg/L) established by the CLSI (Table 1).

The nucleotide sequences of genes for PBP and 16S rRNA of eight clinical isolates showing relatively high β-lactam MICs were analysed (Tables S1 to S4, available as Supplementary data at *JAC* Online). Of the eight clinical isolates tested, three and five, respectively, were identified as *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) with the streptococcal group A antigen. Among the eight clinical isolates no mutations expected to contribute to reduced β-lactam susceptibility were found in any of the PBP genes examined.

The emergence of GAS with reduced penicillin susceptibility in Japan is very unlikely at present. Some genetic lineages of SDSE possessing streptococcal group A antigen may tend to show relatively high β-lactam MICs without having specific amino acid substitutions in their PBPs.

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Transparency declarations

Conflicts of interest: none to declare.

The manuscript was edited by Editage, a language-editing company.

Supplementary data

Supplementary data, including Materials and methods, Results and Tables S1–S4, are available at *JAC* Online (http://jac.oxfordjournals.org/).

Table 1. Distribution of MICs of β-lactams for clinical isolates of GAS ($n = 256$)

	MIC (mg/L)										
Antibacterial agent	≤0.002	0.004	0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2
Penicillin	1	1	41	212	1						
Oxacillin					23	224	9				
Cefaclor						12	161	79	4		
Ceftizoxime			11	224	21						
Ceftibuten							2	71	177	5	1

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AU1 ▶

Genetic Profiles of Fluoroquinolone-Nonsusceptible *Klebsiella pneumoniae* Among Cephalosporin-Resistant *K. pneumoniae*

AU2 ▶

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The rate of fluoroquinolone (FQ) resistance among the cephalosporin-resistant *Klebsiella pneumoniae* is considerably high, however, their genetic profiles have not been well investigated. We selected 61 ciprofloxacin-nonsusceptible isolates from 102 *K. pneumoniae* isolates judged to be "resistant" to some cephalosporins during 2009 and 2012 throughout Japan. Pulsed-field gel electrophoresis excluded clonal isolates, and 29 isolates were subjected to multilocus sequence typing (MLST), detection of the amino acid substitutions in the quinolone resistance determining regions (QRDRs) of GyrA and ParC, β-lactamase typing, and identification of plasmid-mediated quinolone resistance (PMQR) genes. PCR-based replicon typing was performed, after PMQR gene transfer. Four major sequence types (STs) or clonal complexes (CCs), that is, ST37, CC17 (consisting of ST17 and ST20), ST11, and CC528 (consisting of ST528 and ST1130), were found, and they accounted for 48.2% of the isolates tested. Amino acid substitutions in the QRDRs and the presence of PMQR genes were identified in 20 (68.9%) and 18 (62.0%) isolates, respectively. The replicon type of three PMQR-carrying plasmids was IncN, but others were nontypable. Fifteen (83.3%) of the 18 PMQR-harboring isolates coharbored bla_{CTX-M} and/or bla_{DHA-1}. Ciprofloxacin-nonsusceptible *K. pneumoniae* clinical isolates demonstrating cephalosporin resistance often belong to the global epidemic lineages and possess PMQR and/or QRDR substitutions.

Introduction

AU3 ▶

NALIDIXIC ACID IS THE first synthetic quinolone approved for clinical use in the 1960s. Ofloxacin, a fluoroquinolone (FQ), became clinically available in Japan in 1985. Since then, various kinds of FQs have been developed and used widely because they have broad-spectrum antimicrobial activities against both gram-positive and gram-negative bacteria. Before 2000, FQ resistance was rare especially among the family *Enterobacteriaceae*. However, rapid increase of resistance to FQs has occurred worldwide, especially in *Escherichia coli* due to the clonal spread of several genetic lineages such as *E. coli* O25b:H4-ST131. The rate of quinolone resistance in clinical *E. coli* isolates was reported to be 50.6% in Hong Kong[20] and about 25% of the healthy people living in Barcelona have quinolone-resistant *E. coli* in their intestines.[11] A rapid increase in FQ-resistant *E. coli* has also been reported in the United Kingdom.[21] The main mechanism of quinolone resistance in *E. coli* is amino acid substitutions in the quinolone resistance determining regions (QRDRs) of DNA gyrase (GyrA) and DNA topoisomerase IV (ParC), which are essential for DNA replications and are the targets of FQs, together with enhanced function of efflux pump systems such as AcrAB-TolC.[31] Moreover, after the late 1990s, several types of plasmid-mediated quinolone resistance (PMQR), for example, Qnr peptides, AAC(6′)-Ib-cr, and QepA, newly emerged, although they conferred a low-level FQ resistance that is sometimes below breakpoints for resistant of the Clinical and Laboratory Standards Institute (CLSI).

Klebsiella pneumoniae is one of the most important pathogens causing nosocomial and community-acquired infections and considered as the usual bacterial species of ESBL producers among the family *Enterobacteriaceae*. Moreover, the strains producing carbapenemases such as KPC, NDM, and OXA-48 have so far been found mainly from *K. pneumoniae* isolated worldwide, including European, American, and Asian countries. Those carbapenemase-producing isolates usually demonstrate FQ resistance[22] through acquisition of amino acid substitutions in QRDRs and enhanced function of efflux systems.[1] Thus, the spread of multidrug-resistant *K. pneumoniae* is becoming an ongoing global threat to public health. Fortunately, the rate of FQ resistance among *K. pneumoniae* clinical

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isolates overall is still low at present in Japan. According to the report published in 2012 by the Japan Nosocomial Infections Surveillance (JANIS), the rate of levofloxacin-nonsusceptible isolates in *K. pneumoniae* was 2.4%, which was much lower than that (above 34%) in *E. coli* (www.nih-janis.jp/report/kensa.html) on the basis of the CLSI's breakpoints at 2007 (M100-S17), although increased isolation of FQ-resistant *K. pneumoniae* has been accompanied with the increase of carbapenemase-producing *K. pneumoniae* worldwide, including the European countries and the United States. Multilocus sequence typing (MLST), an analysis of nucleotide sequence-based methods adequate for characterizing the genetic relationships of bacterial isolates in a broad perspective has become popular and has revealed that specific STs have some relationships with specific resistance genes, for example, *E. coli* ST131 with bla_{CTX-M} and *K. pneumoniae* ST258 with bla_{KPC-2}.[10,35] Thus, we speculated that the clonal spreads of some *K. pneumoniae* clinical isolates belonging to specific genetic lineages may well contribute to the prevalence of FQ resistance among the cephalosporin-resistant *K. pneumoniae*, because FQ resistance was more frequently found among cephalosporin-resistant *K. pneumoniae* than among cephalosporin-susceptible ones. Therefore, we studied the genetic profiles of ciprofloxacin-nonsusceptible isolates among the cephalosporin-resistant *K. pneumoniae* clinical isolates from 24 different hospitals located in 11 different prefectures in Japan.

Materials and Methods

Preliminary study

Before we investigated the genetic lineages of FQ-resistant *K. pneumoniae* to exclude clonal spread among *K. pneumoniae* in Japan, we chose 22 of the 176 *K. pneumoniae* isolates susceptible to both FQs and cephalosporins recovered from separate regions in Japan and investigated their STs. As a result, no apparent ST clusters were found among them.

Bacterial isolates

Among 16,526 clinical *K. pneumoniae* isolates sent to a private microbiology laboratory from 2009 to 2012, we collected 102 *K. pneumoniae* isolates from 24 separate hospitals located in 11 prefectures of Japan, which were first judged as "resistant" to cefotaxime, ceftazidime, cefozopran, cefepime, and/or cefpirome by the MicroScan WalkAway system (Siemens Healthcare Diagnostics, Inc.) in a private microbiology laboratory. Of the 102 isolates, 61 were selected because MICs of ciprofloxacin and/or levofloxacin were above the breakpoint of intermediate by the agar dilution method recommended by the CLSI (M100-S20).[15] For the multiple isolates recovered from the same hospital, pulsed-field gel electrophoresis (PFGE) was performed to exclude repetitious isolations of the same clone. Consequently, 29 ciprofloxacin-nonsusceptible *K. pneumoniae* clinical isolates were selected and subjected to this study.

Antimicrobial susceptibility

The antimicrobial susceptibility test was performed by the agar dilution method according to the protocol recommended by the CLSI (M07-A8)[14] for the following antimicrobial agents obtained from the indicated sources: cefotaxime (CTX), imipenem (IPM), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), colistin (CST), fosfomycin (FOF), Wako Pure Chemical Industries, Ltd.; ceftazidime (CAZ), levofloxacin (LVX) Tokyo Chemical Industry Co. Ltd.; cefepime (FEP), Santa Cruz Biotechnology, Inc.; cefminox (CMNX), Meiji Seika Pharma Co., Ltd.; and tigecycline (TGC), Pfizer Japan, Inc. Glucose-6-phosphatase (25 mg/L) (Sigma-Aldrich Co. LLC.) was added to the media for the susceptibility test against FOF.

Multilocus sequence typing

MLST was performed on the 29 isolates using the protocol for *K. pneumoniae* provided by the Institute Pasteur website (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). New alleles and STs were submitted to the MLST website and new ST numbers were assigned. In the present study, clonal complex (CC) was defined as the group of STs that are related to each other by a single locus variant (SLV) level.

Screening of genes for β-lactamase and PMQR

PCR was performed on 29 isolates to detect the following genes: bla_{TEM}, bla_{SHV},[36] $bla_{CTX-M-1\ group}$, $bla_{CTX-M-2\ group}$, $bla_{CTX-M-9\ group}$,[29] bla_{ACC},[25] bla_{FOX}, bla_{MOX}, bla_{DHA}, bla_{CIT}, bla_{EBC},[27] *qnrA*, *qnrB*, *qnrS*,[6] *qnrC*,[34] *qnrD*,[7] *qepA*,[37] and *aac-(6′)-Ib* genes with the primers previously described. The obtained PCR amplicons of bla_{TEM}/bla_{SHV} and *aac-(6′)-Ib* genes were further subjected to nucleotide sequencing to determine whether or not they encode ESBLs and *aac-(6′)-Ib-cr*, respectively, and bla_{CTX-M} genes were also sequenced with the appropriate primers.[8,30]

Analysis of alterations in QRDRs

The amino acid substitutions in QRDRs of GyrA and ParC were deduced by nucleotide sequencing of *gyrA* and *parC* genes with the primers described previously.[4,24] The nucleotide mutations were identified based on the available nucleotide sequences of *gyrA* and *parC* of *K. pneumoniae* ATCC 13833.

Transfer of quinolone resistance genes and PCR-based replicon typing

The broth-mating conjugation experiment was carried out with *E. coli* J53 as the recipient strain, and transconjugants were selected on the Luria-Bertani (LB) agar plates containing sodium azide (150 mg/L) and ciprofloxacin (0.05 mg/L). In the isolates from which no transconjugant was obtained by the above-described conjugation procedure, transformation with purified plasmids was performed. The plasmid DNA was extracted by the method of Kado and Lui.[18] *E. coli* DH10B was used as the recipient strain, and transformants were selected on the plates containing ciprofloxacin (0.05 mg/L). For the isolates from which no transconjugants or transformants were obtained despite repeated attempts using LB agar plates containing ciprofloxacin as described above, we performed an alternative method to obtain transconjugants and/or transformants, depending on the fact that both PMQR genes and ESBL genes are frequently cocarried by the same plasmid. For this purpose, ampicillin-resistant transconjugants

and/or transformants were first selected by the agar plates supplemented with ampicillin (100 mg/L). We also used *E. coli* CSH2 and J53 for the recipient in a conjugation experiment using an agar plate supplemented with rifampicin (150 mg/L) and ampicillin (100 mg/L). The presence of ESBL and PMQR genes was checked by PCR on several colonies grown on each plate, and then it was confirmed by nucleotide sequencing whether or not the objective genes were transferred. PCR-based replicon typing (PBRT) was performed on the obtained transconjugants and/or transformants.[5]

Results

Bacterial isolates

First, we tried to collect quinolone-resistant *K. pneumoniae*, although they are still rare in Japan at present. Then, we screened quinolone-resistant *K. pneumoniae* isolates among the *K. pneumoniae*, which acquired resistance to cephalosporins, because the rate of quinolone resistance tended to be high among the cephalosporin-resistant *K. pneumoniae* isolates. Isolate Nos. 40, 53, and 56 were susceptible to cefotaxime and ceftazidime by the agar dilution method, even though they were first judged as resistant to cefotaxime, ceftazidime, cefozopran, cefepime, and/or cefpirome by the MicroScan WalkAway system. The 61 ciprofloxacin-nonsusceptible isolates selected were recovered from January 5, 2009, to October 3, 2012, from 24 hospitals located in 11 prefectures in Japan. Four, 2, and 2 isolates demonstrating different PFGE profiles from each other were recovered from hospitals S-1, A-1, and TK-3, respectively. In seven hospitals, more than two isolates belonging to the same clonal lineage in each hospital were found by PFGE. The isolates recovered from the seven hospitals belonged to separate sequence types (STs) (Table 1). Interestingly, 22 isolates belonging to the same clonal lineage were recovered from one hospital (hospital I-1), suggesting a probable nosocomial outbreak of FQ-resistant *K. pneumoniae* in hospital I-1. We selected a single isolate from the multiple isolates in these seven hospitals for further investigation.

T1 ▶

Antimicrobial susceptibility

The range of ciprofloxacin MICs for each isolate varied. The fourth-generation cephalosporin and cefepime showed relatively low MICs for the isolates compared with the MICs of cefotaxime and ceftazidime, although some isolates demonstrated intermediate or resistant profiles against cefepime. Most isolates were susceptible to imipenem, however, in some isolates, such as isolates 1 and 3, intermediate or resistant profiles to imipenem (MICs, 2–4 mg/L) were found. This was due to the harboring of bla_{IMP} or bla_{NDM} in the isolates 1 and 3, respectively, as detected later by PCR. A high MIC of fosfomycin (>256 mg/L) was found for two isolates. The MIC of tigecycline was >16 mg/L for three isolates. Colistin, which has not yet been approved for clinical use in Japan, seemed very effective for all isolates (Table 2). ◀T2

TABLE 1. SEVEN HOSPITALS FROM WHICH MORE THAN TWO FQ-RESISTANT *K. PNEUMONIAE* ISOLATES BELONGING TO THE SAME CLONE WERE RECOVERED

Hospital	*Isolate No. appeared in Tables 2–4*	*The number of isolates showing the same PFGE pattern in each hospital*	*ST*
I-1	10	22	14
S-1	1	5	528
W-2	4	3	37
TK-3	14	3	1127
TK-1	21	3	12
C-1	59	2	17
W-1	62	2	280

A single isolate was recovered in remaining 16 hospitals.

Multilocus sequence typing

The results of MLST analysis are shown in Table 3. The most frequent ST was ST37 (5 of 29 isolates; 17%), followed by ST17 (4 of 29 isolates; 14%). One ST20, a SLV from ST17, was found; thus, CC17 consisted of five isolates. Two isolates belonging to ST17 were recovered from the hospital S-1, and PFGE revealed that those isolates were probably related by the criteria described by Tenover *et al.*[32] (data not shown). However, MICs of ciprofloxacin and levofloxacin for these two isolates were different, as shown in Table 3. Among the 29 isolates tested, two of them belonged to ST11 (7%). ST1130 was a SLV from ST528, and consequently, CC528 comprised two isolates (7%). ST37, ST17, ST20, and ST11 formed a large genetic complex CC37, and ST528 and ST1130 also formed another genetic complex CC528, as shown in Fig. 1, although CC17 and CC528 may have some genetic relatedness on the minimal spanning tree, as observed in Fig. 2. ◀T3 ◀F1 ◀F2

ESBL and PMQR genes

As shown in Table 3, 15 of 29 isolates harbored genes for CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, or CTX-M-65. Eleven isolates were positive for bla_{TEM}, but none of them were ESBL producers; 10 of them were positive for bla_{TEM-1} and one was positive for bla_{TEM-1b}. Most isolates had bla_{SHV}, but only three of them harbored bla_{SHV-12}. As plasmid-mediated AmpC, bla_{DHA-1} was found in 6 isolates. Eighteen isolates had PMQR genes, the most frequent one being *qnrS*, followed by *qnrB*, *aac-(6′)-Ib-cr*, and *qnrA*. Four of five *aac(6′)-Ib-cr*-positive isolates coharbored *qnrB*. The genes *qnrC*, *qnrD*, and *qepA* were not detected in the present study. *fosA3* was found in the fosfomycin highly resistant (MIC, >256 mg/L) strain No. 9 (Table 2).

QRDR substitutions

Nine isolates (isolate Nos. 46, 49, 14, 28, 29, 52, 53, 62, and 65 shown in Table 3) did not have any amino acid substitutions in QRDRs, but they harbored at least one gene for PMQR and showed relatively low FQ MICs (≤32 mg/L) compared to the isolates having QRDR alterations. All the isolates belonging to ST37 and ST11 had more than two amino acid substitutions in QRDRs and showed higher quinolone resistance levels than those of other STs. Among the isolates belonging to singletons, four isolates (isolate Nos. 3, 11, 40, and 51) had more than two amino acid substitutions in the QRDRs of GyrA and/or ParC, and they

TABLE 2. ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF 29 ISOLATES TESTED

	MIC (mg/L)											
Isolate No.	*CTX*	*CAZ*	*FEP*	*CMNX*	*IPM*	*AMK*	*GEN*	*CIP*	*LVX*	*CST*	*FOF*	*TGC (Etest)*[a]
4	64	32	16	256	<0.125	1	2	>128	64	0.25	8	4
9	128	8	4	1	<0.125	>256	>256	>128	64	0.5	>256	4
33	16	256	0.5	32	0.5	>256	>256	>128	128	0.125	8	>16 (3)
34	128	256	0.25	32	0.5	>256	>256	>128	128	0.125	16	1
41	32	256	4	32	0.5	>256	>256	>128	128	0.125	128	2
18	8	64	1	1	0.25	16	64	2	2	0.25	64	1
24	16	32	2	2	<0.125	8	32	16	16	0.25	32	2
46	32	4	8	1	<0.125	1	32	16	16	0.125	16	2
59	16	8	8	2	<0.125	1	64	32	32	0.5	32	>16 (4)
49	8	64	4	1	0.25	4	0.5	8	8	0.125	8	8
48	128	16	16	2	<0.125	2	0.5	>128	>128	0.063	16	2
60	256	>256	2	>256	1	8	0.5	>128	128	0.25	>256	2
1[b]	32	32	4	256	2	1	1	64	64	0.25	32	2
56	0.25	2	<0.125	1	<0.125	0.5	0.25	128	128	0.5	8	8
3[b]	>256	>256	64	256	4	>256	>256	>128	64	0.25	16	4
8	64	8	16	2	<0.125	1	0.25	16	16	0.25	8	2
10	64	8	8	1	<0.125	1	0.5	8	8	0.25	8	4
11	16	128	0.25	32	0.25	>256	>256	>128	128	0.125	8	16 (3)
14	64	64	8	2	<0.125	4	4	16	16	0.25	16	1
21	8	32	1	1	<0.125	8	0.5	8	8	0.5	8	4
28	64	4	16	2	<0.125	1	1	16	16	0.125	16	4
29	64	4	8	0.5	0.25	0.5	0.25	16	16	1	8	16 (3)
40	<0.125	<0.125	<0.125	1	0.25	1	0.25	64	32	0.125	8	2
51	64	16	4	2	<0.125	1	0.5	128	32	0.5	8	4
52	32	2	4	2	<0.125	1	0.25	32	16	0.5	8	>16 (4)
53	<0.125	0.5	<0.125	0.5	<0.125	0.5	64	16	32	0.5	16	16 (3)
54	16	2	8	1	0.5	0.5	0.25	16	16	0.5	4	8
62	>256	128	32	2	0.25	4	256	4	1	0.125	32	8
65	256	64	32	1	0.25	2	64	2	1	0.25	16	16 (2)

Isolate No. is listed by the order as shown in Table 3.
MIC ranges measured: CTX, CAZ, FEP, CMNX, IPM, AMK, GEN, and FOF; 0.125 through 256.
CIP and LVX; 1 through 128, CST; 0.016 through 8, TGC; 0.016 through 16
MIC breakpoints for Enterobacteriaceae suggested by CLSI M100-S20, 2010 (S, I, R): CTX; (≦1, 2, ≧4), CAZ; (≦4, 8, ≧16), FEP; (≦8, 16, ≧32), IPM; (≦4, 8, ≧16), GEN; (≦4, 8, ≧16), AMK; (≦16, 32, ≧64), CIP; (≦1, 2, ≧4), LVX; (≦2, 4, ≧8).
[a]Etest was performed for the isolates for which high TGC MICs (≧16 mg/L) were found by the CLSI agar dilution method.
[b]Genes for NDM-1 and IMP-1 were detected by PCR in isolate numbers 1 and 3, respectively.
CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CMNX, cefminox; IPM, imipenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; LVX, levofloxacin; CST, colistin; FOF, fosfomycin; TGC, tigecycline.

also demonstrated considerably high resistance levels to FQs (Table 3).

Transfer of PMQR genes and PBRT

T4 ▶ Transconjugants or transformants were obtained in 11 of 18 isolates that harbored PMQR (Table 4). Seven of them were selected directly by ciprofloxacin and four were indirectly selected by ampicillin. Genes transferred by conjugation and/or transformation by electroporation are also shown in Table 4, and ciprofloxacin and levofloxacin MICs for transconjugants and transformants with the PMQR gene are indicated in Table 4. PBRT was performed in all of the 11 transconjugants or transformants and only three were identified as IncN, but the others could not be determined by the PBRT.

Discussion

FQs have broad-spectrum antimicrobial activities against both gram-positive and gram-negative bacteria and have been used widely since the 1980s. In some parts of Europe like Greece, however, the rate of FQ resistance in *K. pneumoniae* has become very high, and this would be attributable to the high prevalence of carbapenemase-producing *K. pneumoniae* belonging to some genetic lineages like ST11.[33] On the other hand, there remain some regions, like Japan, where the prevalence of FQ-resistant *K. pneumoniae* is still much lower than that in the endemic areas of carbapenemase-producing *K. pneumoniae* (CPE). *E. coli* O25b:H4-ST131 has played a major role in worldwide dissemination of FQ-resistant *E. coli*, which have acquired genes for CTX-M-type ESBLs and multiple virulence factors.[16,17,23,28] However, there are a few molecular epidemiological studies focused on FQ-resistant *K. pneumoniae*. Indeed, cephalosporin-resistant *K. pneumoniae* isolates, including ESBL-producing ones, tend to demonstrate resistance to FQs, but their genetic profiles still remain unclear. Thus, this is, to our knowledge, the first study on the genetic profiles of ciprofloxacin-nonsusceptible *K. pneumoniae* showing cephalosporin nonsusceptibility, conducted in the CPE nonepidemic areas.

TABLE 3. MOLECULAR EPIDEMIOLOGICAL CHARACTERISTICS OF 29 *K. PNEUMONIAE* ISOLATES TESTED

Isolate No.	*Source*	*Hospital of isolation (prefecture)*	*ST*	*CC*	*QRDR substitution*				*MIC(mg/L)*		*PMQR gene*	*ESBL and/or AmpC*
					gyrA(GyrA)		*parC(ParC)*					
					TCC(88Ser)	*GAC(87Asp)*	*TCG(80Ser)*	*GAA(84Glu)*	*CPFX*	*LVFX*		
4	Sputum	W-2 (Wakayama)	37	37	TTC(Phe)	AAC(Asn)	CGC(Arg)	WT[a]	>128	64		CTX-M-2
9	Stool	N-3 (Nagano)	37		TTC(Phe)	GAA(Gln)	CGC(Arg)	WT	>128	64		CTX-M-65
33	Pus	S-2 (Saitama)	37		ATC(Ile)	WT	CGC(Arg)	WT	>128	128	*qnrB*	DHA
34	Urine	N-1 (Nagano)	37		ATC(Ile)	WT	CGC(Arg)	WT	>128	128	*qnrB*	DHA
41	Vaginal swab	N-2 (Nagano)	37		ATC(Ile)	WT	ATG(Met)	WT	>128	128	*qnrB, aac(6')-Ib-cr*	SHV-12, DHA
18	Pus	S-1 (Saitama)	17	17 (37)	TAC(Tyr)	WT	WT	WT	2	2		
24	Urine	S-1 (Saitama)	17		TAC(Tyr)	WT	WT	WT	16	16		
46	Nasal cavity	TK-5 (Tokyo)	17		WT	WT	WT	WT	16	16	*qnrS*	CTX-M-14
59	Sputum	C-1 (Chiba)	17		TAC(Tyr)	WT	WT	WT	32	32	*qnrS*	CTX-M-14
49	Urine	TK-3 (Tokyo)	20		WT	WT	WT	WT	8	8	*qnrA*	SHV-12
48	Urine	TK-6 (Tokyo)	11	11 (37)	ATC(Ile)	WT	ATC(Ile)	WT	>128	>128	*qnrB, aac(6')-Ib-cr*	CTX-M-14
60	Urine	TK-4 (Tokyo)	11		ATC(Ile)	WT	ATC(Ile)	WT	>128	128	*qnrB, aac(6')-Ib-cr*	DHA
1	Sputum	S-1 (Saitama)	528	528	TTC(Phe)	WT	WT	WT	64	64		
56	Pus	S-1 (Saitama)	1,130		TTC(Phe)	WT	WT	WT	128	128	*qnrS*	

(*continued*)

TABLE 3. (CONTINUED)

Isolate No.	Source	Hospital of isolation (prefecture)	ST	CC	QRDR substitution				MIC(mg/L)		PMQR gene	ESBL and/or AmpC
					gyrA(GyrA)		parC(ParC)					
					TCC(88Ser)	GAC(87Asp)	TCG(80Ser)	GAA(84Glu)	CPFX	LVFX		
3	Urine	S-3 (Saitama)	42	Singleton	TAC(Tyr)	TAC(Tyr)	WT	WT	>128	64		DHA
8	Sputum	TK-1 (Tokyo)	1,126		TAC(Tyr)	WT	WT	WT	16	16		CTX-M-3
10	Pus	I-1 (Ibaragi)	14		TAC(Tyr)	WT	WT	WT	8	8		CTX-M-3
11	Sputum	K-2 (Kanagawa)	147		ATC(Ile)	WT	ATT(Ile)	WT	>128	128	*qnrB*	DHA
14	Sputum	TK-3 (Tokyo)	1,127		WT	WT	WT	WT	16	16	*qnrA*	CTX-M-9, SHV-12
21	Sputum	T-1 (Tochigi)	12		TTC(Phe)	WT	WT	WT	8	8		
28	Sputum	A-1 (Aichi)	1,133		WT	WT	WT	WT	16	16	*qnrS*	CTX-M-2
29	Sputum	A-1 (Aichi)	1,134		WT	WT	WT	WT	16	16	*qnrS*	CTX-M-2
40	Throat swab	I-3 (Ibaragi)	1,128		ATC(Ile)	WT	AGA(Arg)	WT	64	32	*qnrS*	
51	Sputum	G-1 (Gifu)	1,129		ATC(Ile)	WT	ATC(Ile)	WT	128	32		CTX-M-15
52	Urine	TK-7 (Tokyo)	23		WT	WT	WT	WT	32	16	*qnrB, aac(6')-Ib-cr*	CTX-M-14
53	Sputum	C-4 (Chiba)	661		WT	WT	WT	WT	16	32	*qnrS*	
54	Urine	IS-2 (Ishikawa)	268		TAC(Tyr)	WT	WT	WT	16	16		CTX-M-14
62	Urine	W-1 (Wakayama)	280		WT	WT	WT	WT	4	1	*aac(6')-Ib-cr*	CTX-M-15
65	Sputum	IS-1 (Ishikawa)	70		WT	WT	WT	WT	2	1	*qnrS*	CTX-M-14

[a]No mutation was seen on the basis of *gyrA* and *parC* of *K. pneumoniae* ATCC 13833.

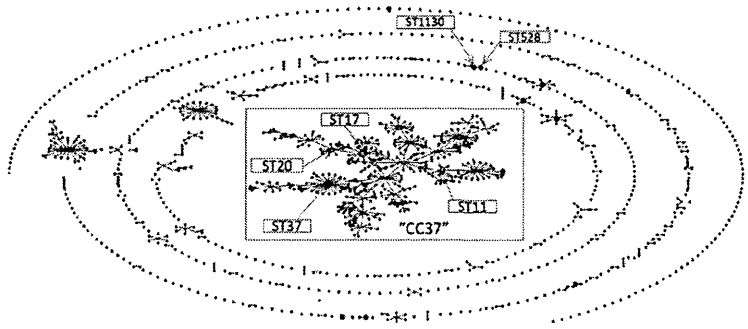

FIG. 1. Population snapshot by eBURST analysis (http://eburst.mlst.net) showing clusters of linked and unlinked STs in the entire *K. pneumoniae* MLST database. www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html/. The dominant STs found in the present study are highlighted in black box with arrowed lines. Most of them are within CC37 (inside the square), which includes many international multidrug-resistant STs. A color version of this figure is available in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/mdr). ◀SF1

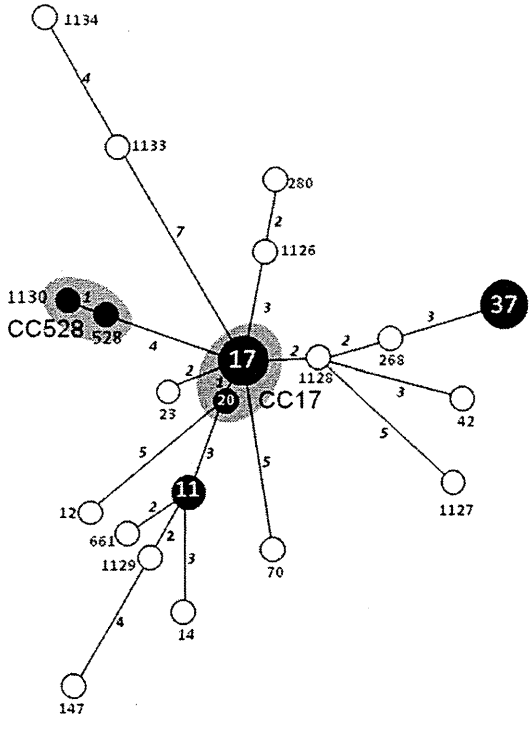

FIG. 2. Minimal spanning tree of the 29 fluoroquinolone-nonsusceptible *K. pneumoniae* isolates in Japan. The *gray* zones represent the same clonal complex (six common alleles). The area of *circles* is equivalent to the number of isolates. The length of lines represents the number of alleles that are found in common between the STs connected to the lines, and the italic numbers next to the connecting lines correspond to the number of allelic differences. *Black circles* represent the dominant CCs and STs found in the present study.

The MLST analyses revealed that STs of ciprofloxacin-nonsusceptible *K. pneumoniae* isolated from Japan mainly consisted of two clusters: ST37 ($n=5$) and clonal complex 17 (CC17) ($n=5$), including ST17 and ST20, a SLV from ST17. Three major STs, that is, ST37, ST17, and ST11, related to FQ-resistant *K. pneumoniae*, have often been found in the isolates acquiring various antimicrobial resistance genes, and they are included in the CC37, in which KPC-producing ST258[10] was also included.[35] CC17, including ST17 and its SLV ST20, was one of the major STs associated with ESBL production in Canada,[26] and ST37 was often found among the isolates producing various β-lactamases, for example, plasmid-mediated AmpC,[13] KPC,[2,19] and NDM.[12] Although only two isolates were identified as ST11 in the present study, it is the most widely distributed one among these three STs, especially related to carbapenemases like KPC, NDM, and OXA-48. From examining 39 *K. pneumoniae* producing NDM-1 carbapenemase from India, the United Kingdom, and Sweden, ST11 was the unique type commonly found in all three countries.[12] Furthermore, ST11 was one of the major STs among ciprofloxacin-resistant *K. pneumoniae* isolates acquiring $bla_{CTX\text{-}M\text{-}15}$ that has spread in Hungary,[9] and the OXA-48-producing *K. pneumoniae* that caused a nosocomial outbreak in Greece also belonged to ST11.[33] Thus, the CC37 isolates that harbor bla_{KPC}, bla_{NDM}, or $bla_{OXA\text{-}48}$ genes had been spreading worldwide, including the European countries and the United States. However, carbapenemase producers are still very rare in Japan despite the considerable presence of CC37 isolates among the CIP-nonsusceptible *K. pneumoniae* isolates, as shown in Fig. 1. Since CC37 isolates tend to easily acquire various plasmids mediating a variety of drug resistance genes and become endemic, the trends of CC37 isolates should be monitored intensively to block their further spread, especially in the areas where carbapenem-resistant *K. pneumoniae* has not become endemic as yet, like Japan.

Multiple amino acid substitutions in QRDRs are needed to acquire high-level resistance to FQs, and the FQ MICs for the isolates possessing double or more amino acid substitutions in QRDRs were higher than those for the isolates

TABLE 4. MIC FOR *E. COLI* TRANSFORMANTS AND TRANSCONJUGANTS POSSESSING PMQR GENES AND INC TYPES OF PLASMIDS ENCODING PMQR AND/OR β-LACTAMASE GENES

Strain No.	*E. coli recipient*	*antimicrobial used for selection of tf or tc (tf or tc)*	*PMQR gene detected in the tf or tc*	*MIC (mg/L) for tf or tc*		*ESBL and/or AmpC genes cotransferred with PMQR*	*Inc type of plasmid carrying PMQR and/or β-lactamase genes*
				CIP	*LVX*		
34	DH10B	C (tf)	*qnrB*	0.128	0.128	DHA	ND[a]
41	DH10B	A (tf)	*qnrB*, *aac(6′)-Ib-cr*	0.016	0.016	SHV-12, DHA	ND
46	CSH2	A (tc)	*qnrS*	0.256	0.512	CTX-M-14	ND
49	CSH2	C (tc)	*qnrA*	0.256	0.512	SHV-12	ND
48	DH10B	A (tf)	*qnrB*	0.016	0.016	TEM-1 (non-ESBL)	ND
56	DH10B	C (tf)	*qnrS*	0.128	0.256		N
11	CSH2	A (tc)	*qnrB*	0.256	0.512	DHA	ND
28	DH10B	C (tf)	*qnrS*	0.256	0.256	CTX-M-2	N
40	DH10B	C (tf)	*qnrS*	0.256	0.256		ND
52	DH10B	C (tf)	*qnrB*, *aac(6′)-Ib-cr*	0.064	0.032		N
53	J53	C (tc)	*qnrS*	0.256	0.512		ND
E. coli DH10B				0.002	0.008		
E. coli J53				0.016	0.032		
E. coli CSH2				0.128	0.064		

tf, transformant; tc, transconjugant; A, ampicillin; C, ciprofloxacin.
[a]ND, Inc type was not determined by the PBRT.

without or with a single substitution, as found in the present study (Table 3). Interestingly, the isolates belonging to ST17 had one or no amino acid substitution in QRDRs, whereas all isolates belonging to ST37 and ST11 had double or more amino acid substitutions in QRDRs and augmented ciprofloxacin and levofloxacin MICs were found for these isolates. To the best of our knowledge, the relationship between MLST and QRDR alterations has not been well explicated in *K. pneumoniae* to date. Certain genetic lineages belonging to specific STs might have a tendency to accumulate multiple amino acid substitutions in QRDRs during frequent FQ use. It seems notable that 9 ciprofloxacin-nonsusceptible isolates investigated in the present study had no amino acid substitutions in their QRDRs, but all of them had at least one PMQR gene. Since the 9 isolates showed considerably high resistance levels to FQs by possessing wild-type GyrA and ParC, unidentified mechanisms other than PMQR acquisition, like altered permeability and augmented energy-dependent efflux pump systems such as AcrAB efflux system,[3] have been suggested among the 9 isolates.

Our study design has two limitations to discuss the genetic lineage of FQ-nonsusceptible *K. pneumoniae* in Japan. The first is that the investigated ciprofloxacin-nonsusceptible ones were selected among cephalosporin-resistant ones. The second is that the number of objective isolates does not seem to be enough. Before we investigated the genetic lineages of FQ-resistant *K. pneumoniae*, we chose 22 out of 176 *K. pneumoniae* isolates susceptible to both FQs and cephalosporins recovered from separate regions in Japan and investigated their STs as a preliminary study. As a result, no apparent ST clusters were found among them, and FQ-resistant *K. pneumoniae* was rarely found among the cephalosporin-susceptible ones. The fact that FQ-resistant ones are still very rare in Japan would depend on the scarce prevalence of carbapenemase-producing isolates. So, we select the objective isolates among the cephalosporin-resistant ones, in consideration of the fact that ESBL-producing isolates are often resistant to FQs. We believe that three STs (ST37, CC17, and ST11) are common among the ciprofloxacin-nonsusceptible *K. pneumoniae* isolates showing resistance to some cephalosporins, although these STs may well overlap the STs of ESBL producers.

In conclusion, we characterized 29 ciprofloxacin-nonsusceptible *K. pneumoniae* clinical isolates among the cephalosporin-resistant ones obtained from 24 hospitals located in 11 different prefectures of Japan; and 48.2% of the 29 isolates tested converged on four STs or CCs; ST37, CC17, ST11, and CC528. *K. pneumoniae* ST37, CC17, and ST11 are also known as the international epidemic lineages, which frequently produce KPC-, OXA-48-, or NDM-type carbapenemases worldwide. The remaining STs were singletons, however, some of them were novel ones newly identified in the present study. The trend of FQ-nonsusceptible *K. pneumoniae* isolates, particularly ST37, CC17, and ST11, should be carefully monitored hereafter to block their further spread.

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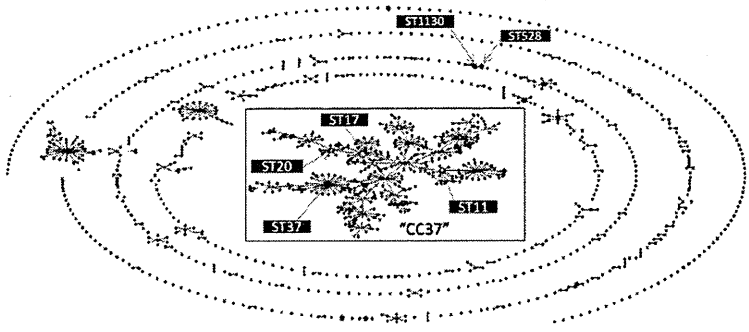
Supplementary Data



SUPPLEMENTARY FIG. S1. Population snapshot by eBURST analysis (http://eburst.mlst.net) showing clusters of linked and unlinked STs in the entire *K. pneumoniae* MLST database. www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html/. The dominant STs found in this study are boxed with arrowed lines. Most of them are within CC37 (inside the square), which includes many international multidrug-resistant STs. Blue circles represent primary founders, and the primary founder of a group is defined as the ST that differs from the largest number of other STs at only a single locus. Yellow circle represents subgroup founders, and they are STs with at least two assigned descendent SLVs. Pink circles represent the STs found in this study, and the dominant STs in this study are highlighted in the black box with arrowed lines. Most of them are within CC37 (inside the *square*), which includes many international multidrug-resistant STs.

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AU1: Please note that gene symbols in any article should be formatted as per the gene nomenclature. Thus, please make sure that gene symbols, if any in this article, are italicized.
AU2: Please review all authors' surnames for accurate indexing citations.
AU3: Please review your article for extremely long paragraphs and divide them where appropriate.
AU4: Please mention the authors' degrees.