ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2007, p. 3354–3360 Vol. 51, No. 9
0066-4804/07/$08.00+0 doi:10.1128/AAC.00339-07
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New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate[▽]

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Received 13 March 2007/Returned for modification 6 May 2007/Accepted 24 May 2007

Plasmid-mediated Qnr and AAC(6′)-Ib-cr have been recognized as new molecular mechanisms affecting fluoroquinolone (FQ) resistance. C316, an *Escherichia coli* strain demonstrating resistance to various FQs, was isolated in Japan. Resistance to FQs was augmented in an *E. coli* CSH2 transconjugant, but PCR failed to detect *qnr* genes, suggesting the presence of novel plasmid-mediated FQ resistance mechanisms. Susceptibility tests, DNA manipulation, and analyses of the gene and its product were performed to characterize the genetic determinant. A novel FQ-resistant gene, *qepA*, was identified in a plasmid, pHPA, of *E. coli* C316, and both *qepA* and *rmtB* genes were mediated by a probable transposable element flanked by two copies of IS*26*. Levels of resistance to norfloxacin, ciprofloxacin, and enrofloxacin were significantly elevated in *E. coli* transformants harboring *qepA* under AcrB-TolC-deficient conditions. QepA showed considerable similarities to transporters belonging to the 14-transmembrane-segment family of environmental actinomycetes. The effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on accumulation of norfloxacin was assayed in a *qepA*-harboring *E. coli* transformant. The intracellular accumulation of norfloxacin was decreased in a *qepA*-expressing *E. coli* transformant, but this phenomenon was canceled by CCCP. The augmented FQ resistance level acquired by the probable intergeneric transfer of a gene encoding a major facilitator superfamily-type efflux pump from some environmental microbes to *E. coli* was first identified. Surveillance of the *qepA*-harboring clinical isolates should be encouraged to minimize further dissemination of the kind of plasmid-dependent FQ resistance determinants among pathogenic microbes.

Fluoroquinolones (FQs) are synthetic chemical agents and among the most commonly prescribed antimicrobials because of their broad-spectrum antimicrobial activity. Extensive clinical and agricultural use of FQs has led to high rates of resistance to these agents among pathogenic microbes (6, 9). The most common mechanism for resistance to FQs among pathogenic microbes is the mutation of chromosomal genes encoding DNA gyrase and/or topoisomerase IV (12, 13). Changes in the expression of efflux pumps and porin proteins are also a common FQ resistance mechanism in bacteria (1), but no plasmid-mediated FQ efflux pump has been documented to date. Four chromosome-dependent efflux systems responsible for FQ resistance have so far been reported, e.g., the resistance nodulation division family, AcrAB-TolC in *Escherichia coli* (7, 28) and MexAB-OprM in *Pseudomonas aeruginosa* (24); the major facilitator superfamily (MFS), NorA of *Staphylococcus aureus* (34); the multidrug and toxic compound extrusion family, NorM of *Vibrio parahaemolyticus* (19); and the ATP-binding cassette family, VcaM of non-O1 *Vibrio cholerae* (14).

Two molecular groups have been identified as plasmid-mediated FQ resistance mechanisms to date. Qnr peptides, QnrA (30), QnrB (15), and QnrS (11), were identified from *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *E. coli*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter cloacae*, *Shigella flexneri*, and a *Salmonella* sp. (8, 16, 21, 31), and these peptides interfere with DNA gyrase by mimicking DNA (18). AAC(6′)-Ib-cr was also found to possess an N-acetylating activity of the piperazinyl substituent of ciprofloxacin and norfloxacin (26), and this enzyme has been reported to be geographically widespread (22, 25).

In the present study, we identified a novel FQ resistance mechanism, QepA, as a plasmid-mediated efflux pump found in an *E. coli* clinical isolate from Japan.

(These findings have been reported at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, 2006, San Francisco, CA [33].)

MATERIALS AND METHODS

Bacterial strains, susceptibility testing, and DNA manipulation. *E. coli* strain C316, which displayed a multiple-resistance profile to aminoglycosides, FQs, and broad-spectrum β-lactams, except for ceftazidime and imipenem, was isolated from the urine of an inpatient at a medical facility in Hyogo Prefecture, Japan, in March 2002. The strains and plasmids used in this study are listed in Table 1. The bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antimicrobial agents.

E. coli KAM32 (14), a highly susceptible antimicrobial laboratory strain, was transformed with *qepA*-carrying plasmids for a precise assay of the MICs of antimicrobials and chemical agents. Transformants were selected on LB agar plates containing chloramphenicol (20 μg/ml) and norfloxacin (0.025 μg/ml). The susceptibilities to antimicrobials, dyes, and other drugs were tested by the agar dilution method according to the procedure recommended by the NCCLS (presently CLSI) document M7-A6 (20).

Transconjugation analysis was performed with *E. coli* CSH2 as the recipient by the filter mating method. Transconjugants were selected on LB agar plates supplemented with rifampin (50 μg/ml) and kanamycin (25 μg/ml), because the R plasmid carried a kanamycin resistance determinant, *rmtB*. Plasmid DNA from a transconjugant was purified by the method of Kado and Liu (17). Transformation of *E. coli* DH10B with the plasmid DNA of the *E. coli* CSH2 transconjugant was performed by conventional electroporation techniques. Transformants were selected on LB agar containing kanamycin (25 μg/ml).

Basic recombinant DNA techniques described by Sambrook et al. (27) were

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▽ Published ahead of print on 4 June 2007.

TABLE 1. Bacterial strains and plasmids used in this study

E. coli strain	Plasmid	Characteristic(s)
C316	pHPA	Multidrug-resistant clinical isolate harboring an R plasmid, pHPA, from urine (Hyogo, Japan); GyrA mutations at Leu83 and Asn87
CSH2	None	Host strain of conjugation; resistant to rifampin and nalidixic acid
DH10B	None	Δ(*ara-leu*)*7696 araD139 galE15 galK16* Δ(*lac*)*X74 rpsL nupG* [ϕ80Δ(*lacZ*)M15]
KAM32	None	Highly susceptible antimicrobial laboratory strain (Δ*acrB ydhE hsd*) derived from *E. coli* K-12[a]
	pHPA	Transformant harboring the R plasmid, pHPA, derived from a wild strain *E. coli*, C316
	pSTV28	Transformant harboring a chloramphenicol-resistant cloning vector, pSTV28
	pSTVqepA	Transformant harboring a recombinant plasmid that carries a 3.2-kbp SacI-SalI fragment containing a *qepA* gene ligated to pSTV28
	pSTVΔqepA	Transformant harboring a recombinant plasmid carrying a disrupted *qepA* gene by EZ-Tn*5* <KAN-2> insertion

[a] See references 14 and 19.

employed in gene manipulations. The FQ resistance plasmid, pHPA, was prepared from *E. coli* DH10B and digested with SacI and SalI. The resultant fragments were ligated into a cloning vector, pSTV28 (Takara Bio, Inc., Otsu, Japan), that was restricted with the same enzymes, and the resultant recombinant plasmid that affects FQ resistance was named pSTVqepA. The nucleotide sequence of the insert of pSTVqepA was determined on both strands using BigDye Terminator cycle sequencing ready reaction kits and a DNA sequence analyzer, model 3100 (Applied Biosystems, Foster City, CA). The alignments of nucleotide and amino acid sequences were performed with Sequencher version 4.2.2 (Hitachi Software Engineering Co., Ltd., Yokohama, Japan) and GENETYX-MAC, version 12.2.3 (Software Development Co., Ltd., Tokyo, Japan).

Using the in vitro mutagenesis system of the EZ-Tn*5* <KAN-2> insertion kit (Epicenter, Madison, WI), a kanamycin-resistant transposon was inserted into the FQ resistance gene, *qepA*, for its inactivation, and the resultant plasmid was named pSTVΔqepA. Disruption of the *qepA* gene in pSTVΔqepA was checked by sequencing analysis using specific primers for the transposon ends.

Functional assay of gene product. The assay for norfloxacin accumulation in bacterial cells was performed according to Huda et al. (14) and Morita et al. (19). *E. coli* KAM32(pSTV28), *E. coli* KAM32(pSTVqepA), and *E. coli* KAM32 (pSTVΔqepA) cells were grown in the LB broth supplemented with 30 μg/ml of chloramphenicol. The cells were harvested at the late logarithmic phase and washed twice with 0.2 M MOPS (morpholinepropanesulfonic acid)-Tris buffer (pH 7.0), and the bacterial pellet was resuspended in the same buffer and adjusted to 50 μg of bacterial cells (wet weight) per ml for the assay. Norfloxacin (100 μM) was added to the assay mixture, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100 μM) was added to the same mixture 10 min later. The first sample containing no CCCP was obtained 10 min after the addition of norfloxacin, and the second sample containing CCCP was taken 30 min after the addition of norfloxacin. Samples were centrifuged at 10,000 $\times g$ for 1 min at 4°C. The pellet was suspended in 1 ml of 100 mM glycine-HCl (pH 3.0), and the suspension was shaken overnight at room temperature and centrifuged at 10,000 $\times g$ for 5 min at room temperature. The supernatant was diluted twofold with 100 mM glycine-HCl (pH 3.0), and fluorescence was measured with excitation at 370 nm and emission at 450 nm with a Hitachi F2000 fluorescence spectrophotometer (Hitachi High-Technologies Co., Ltd., Tokyo, Japan).

Statistical analyses. The statistical analyses were done with SPSS 14.0J for Windows (SPSS Japan, Inc., Tokyo, Japan). Student's *t* test was used for analyses

TABLE 2. Results of susceptibility tests

Antimicrobial agent or chemical	MIC (μg/ml) for:						Fold increase in MIC for *E. coli* KAM32(pSTVqepA) vs. *E. coli* KAM32(pSTV28)
	E. coli C316	*E. coli* KAM32	*E. coli* KAM32(pHPA)	*E. coli* KAM32(pSTV28)	*E. coli* KAM32(pSTVqepA)	*E. coli* KAM32(pSTVΔqepA)	
Ampicillin	>128	2	>128	2	2	2	1
Erythromycin	>128	2	>128	1	2	1	2
Kanamycin	>128	1	>128	1	1	>128	1
Tetracycline	>128	0.25	0.25	0.25	0.25	0.125	1
Nalidixic acid	>128	1	1	1	2	1	2
Norfloxacin	>128	0.016	0.25	0.016	1	0.016	64
Enrofloxacin	>128	0.008	0.25	0.008	0.25	0.008	32
Tosufloxacin	>128	0.002	0.032	0.002	0.032	0.002	16
Levofloxacin	32	0.008	0.016	0.004	0.016	0.004	4
Ciprofloxacin	>128	0.004	0.064	0.004	0.125	0.004	32
Lomefloxacin	>128	0.032	0.064	0.032	0.064	0.032	2
Pazufloxacin	16	0.004	0.016	0.004	0.016	0.004	4
Sparfloxacin	16	0.001	0.002	0.001	0.002	0.001	2
Moxifloxacin	32	0.002	0.016	0.002	0.016	0.002	8
Gatifloxacin	32	0.004	0.016	0.002	0.016	0.004	8
CCCP	16	16	16	16	16	16	1
Acriflavine	>128	2	4	2	4	2	2
Rhodamine 6G	>128	4	4	4	4	4	1
Crystal violet	16	2	2	2	2	2	1
Sodium dodecyl sulfate	>128	128	128	128	128	128	1
Ethidium bromide	>128	4	8	4	8	4	2
Deoxycholate	>1,024	1,024	512	512	512	512	1
Benzalkonium	64	4	4	4	4	4	1

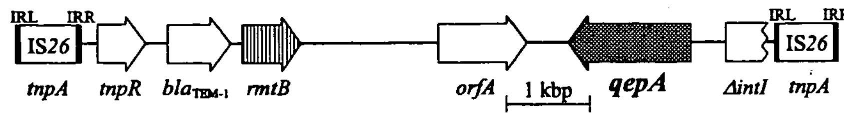

FIG. 1. Organization of the element mediating *qepA*. *qepA* is located in a 10-kb region flanked by two copies of IS*26*, and this probable transposable element also contains *rmtB*, together with *tnpR*, bla_{TEM-1}, and several probable open reading frames. IRL, 5′-GGCACTGTTGCAAA-3′; IRR, 5′-TTTGCAACAGTGCC-3′.

of norfloxacin accumulation in both *qepA*-expressing and -nonexpressing clones with or without CCCP.

Nucleotide sequence accession number. The nucleotide sequence of the *qepA* gene and its flanking regions appears in the EMBL/GenBank/DDBJ (E/G/D) databases under accession number AB263754.

RESULTS

Transfer of FQ resistance and antimicrobial susceptibility. FQ resistance was successfully transferred from *E. coli* C316 to *E. coli* CSH2 at a frequency of 10^{-5} to 10^{-6} cells per recipient cell by conjugation, and the MIC of norfloxacin for the CSH2 transconjugant was elevated above 0.125 μg/ml. Strain CSH2 shows resistance to nalidixic acid by *gyrA* mutation; therefore, the exact norfloxacin MIC was measured in *E. coli* DH10B. The norfloxacin MIC for DH10B was elevated from <0.008 μg/ml to 0.25 μg/ml by introduction of the plasmid pHPA, carrying *qepA*. For the *E. coli* KAM32 transformants that harbor pSTVqepA carrying a 3.2-kbp SacI-SalI fragment of pHPA, 32- to 64-fold-higher MICs of three FQs, norfloxacin, ciprofloxacin, and enrofloxacin, were observed and compared to those for both host strains of *E. coli* KAM32 with or without the vector plasmid (Table 2). The MICs of the other antimicrobial agents and various chemicals, including erythromycin, acriflavine, and ethidium bromide, were not changed when the 3.2-kbp SacI-SalI fragment of pHPA was introduced with pSTVqepA in *E. coli* KAM32. The *E. coli* KAM32 transcon-

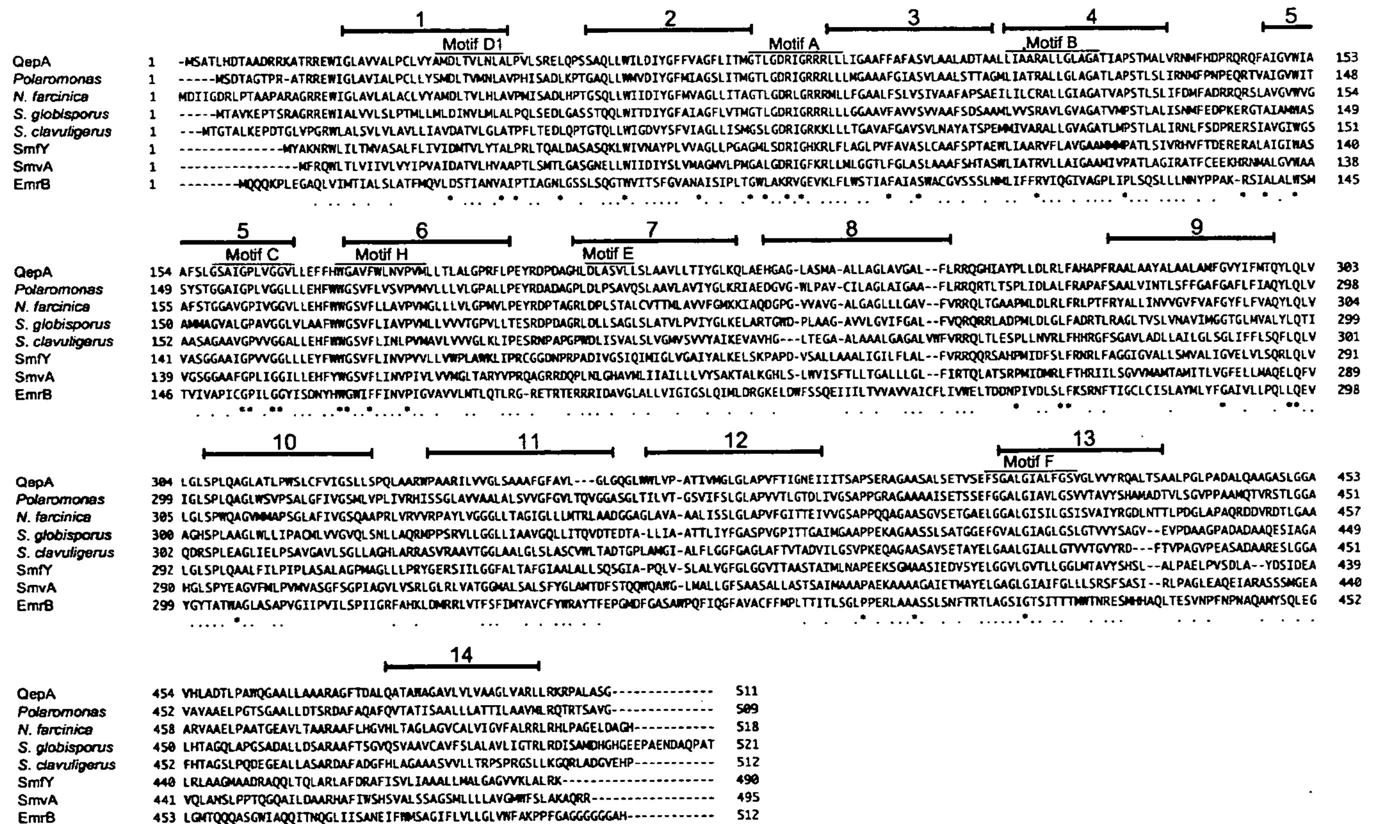

FIG. 2. Comparison of amino acid sequences of 14-TMS MFS-type efflux pumps with QepA. Fourteen portions of probable 14-TMS motifs and seven specific motifs, A, B, C, D1, E, F, and H, are shown by horizontal lines above the alignments. Asterisks and dots indicate identical and similar amino acid residues, respectively. Dashes represent gaps. *Polaromonas*, a putative efflux pump of the *Polaromonas* sp. strain JS666 chromosome (NCBI Protein Data Bank accession number ABE43318); *N. farcinica*, the putative arabinose efflux permease AraJ of the *Nocardia farcinica* chromosome (NCBI Protein Data Bank accession number BAD57397); *S. globisporus*, SgcB of *Streptomyces globisporus* (E/G/D accession number AY048670); *S. clavuligerus*, the putative efflux pump EpeA of the *Streptomyces clavuligerus* chromosome (E/G/D accession number AJ302083); SmfY, a *Serratia marcescens* chromosomal efflux pump protein (E/G/D accession number AB251607); SmvA, a *Salmonella enterica* chromosomal efflux pump protein (E/G/D accession number P37594); and EmrB, an *Escherichia coli* chromosomal efflux pump protein (E/G/D accession number AAC75733).

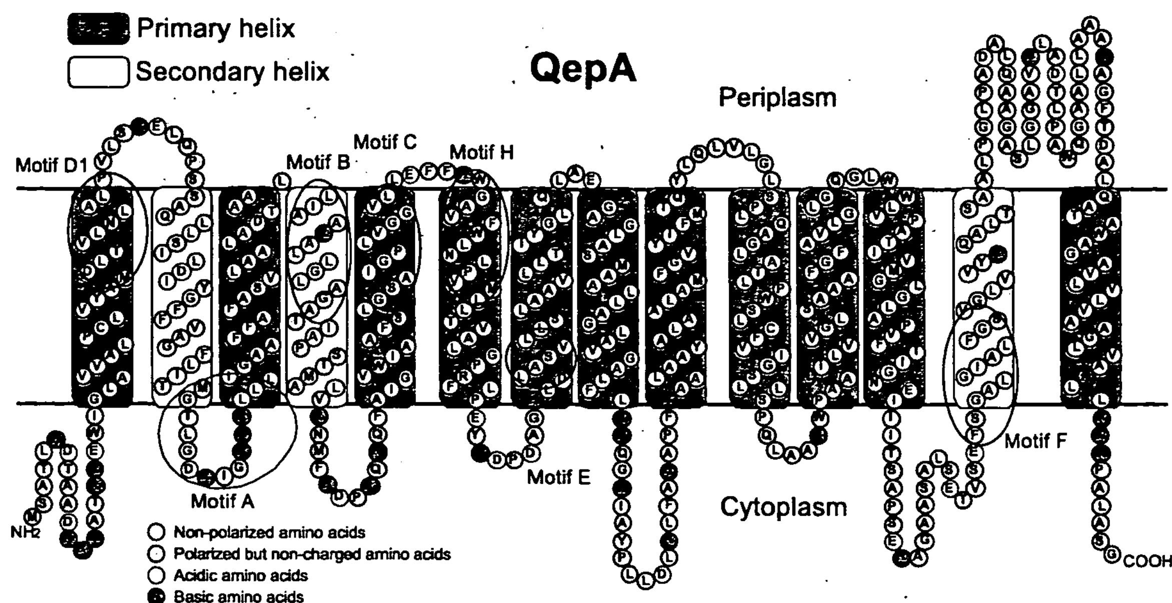

FIG. 3. Predicted secondary structure of QepA. The secondary transmembrane structure of QepA (511 amino acid residues) was calculated and illustrated by the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/). Fourteen probable α-helix TMS and seven specific motifs are indicated. Classes of amino acid residues are differentiated by four colors.

jugant harboring pHPA showed high-level resistance to ampicillin, erythromycin, and kanamycin (Table 2). These resistance profiles were later found to depend on carriage by pHPA of a CTX-M-type β-lactamase gene ($bla_{CTX-M-12}$), a macrolide phosphotransferase gene (*mphA*), and a 16S rRNA methylase gene (*rmtB*) (data not shown). No positive band was observed in the strain harboring pHPA by a PCR analysis for detecting plasmid-mediated FQ resistance *qnr* genes (data not shown). Therefore, pHPA was considered to harbor a new plasmid-mediated FQ resistance determinant. The nucleotide sequence in the *ori* region of pHPA was very similar to that of R100, suggesting that it belongs to the group of IncFII plasmids.

Characteristics of FQ resistance determinant and its product. Sequence analysis of the 3.2-kbp SacI-SalI pHPA fragment cloned into pSTVqepA revealed a 1,536-bp open reading frame with a high G+C content (72%) that was named *qepA*. Considerable similarities between the codon usage patterns of *qepA* and genes for the MFS-type efflux pumps of *Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus* were observed; however, no apparent similarities between the manners of codon usage of *qepA* and those of gram-negative bacteria, such as *rmrA* (E/G/D accession number AF233286), *smvA* (E/G/D accession number D26057), and *smfY* (E/G/D accession number AB251607) (data not shown) were seen. The *qepA*-harboring transferable plasmid, pHPA, also carried a 16S rRNA methylase gene, *rmtB* (5) (E/G/D accession number AB103506), which shows a considerable similarity to the 16S rRNA methylases essential for self-protection of actinomycetes from the hazardous effects of their own aminoglycosides (4, 29). The *qepA* gene is located in an ~10-kb region, flanked by two copies of IS*26* containing *tnpA*, and this region also contained several open reading frames, including *tnpR*, bla_{TEM-1}, and *rmtB*, as shown in Fig. 1.

The *qepA* gene encoded a putative protein, QepA, of 511 amino acids. The predicted amino acid sequence of QepA was subjected to a calculation with the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/) and was found to have probably 14 transmembrane segments (TMS) (Fig. 2 and 3).

Structural characteristics of QepA. The deduced amino acid sequence of QepA showed considerable similarity to probable membrane transporters of members of the order *Actinomycetales*, such as *Nocardia farcinica* (51%; NCBI protein database accession number BAD57397), *Streptomyces globisporus* (49%; E/G/D accession number AY048670), and *Streptomyces clavuligerus* (46%; E/G/D accession number AJ302083). A lower sequence homology (less than 38%) was seen with the major gram-negative bacterial 14-TMS family of MFS-type efflux pumps, such as EmrB (E/G/D accession number AAC75733), SmvA (E/G/D accession number P37594), and SmfY (E/G/D accession number AB251607), and with putative MFS-type efflux pumps of *Actinomycetales*. The phylogenetic tree in Fig. 4, which was calculated with the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-e.html), also suggested that QepA belongs to the 14-TMS family transporters of gram-positive *Actinomycetales* but not those of gram-negative bacteria.

Efflux of norfloxacin by QepA. The accumulation of norfloxacin in *qepA*-harboring *E. coli* KAM32(pSTVqepA) was significantly lower ($P < 0.01$) than that in *qepA*-nonharboring *E. coli* KAM32(pSTV28) or *qepA*-disrupted *E. coli* KAM32 (pSTVqepA). The accumulation of norfloxacin in *E. coli* KAM32(pSTVqepA) increased significantly after the addition of CCCP (Fig. 5), suggesting the proton potential-dependent efflux of norfloxacin by QepA.

DISCUSSION

We first characterized a new quinolone efflux pump protein (QepA) responsible for FQ resistance. QepA showed a con-

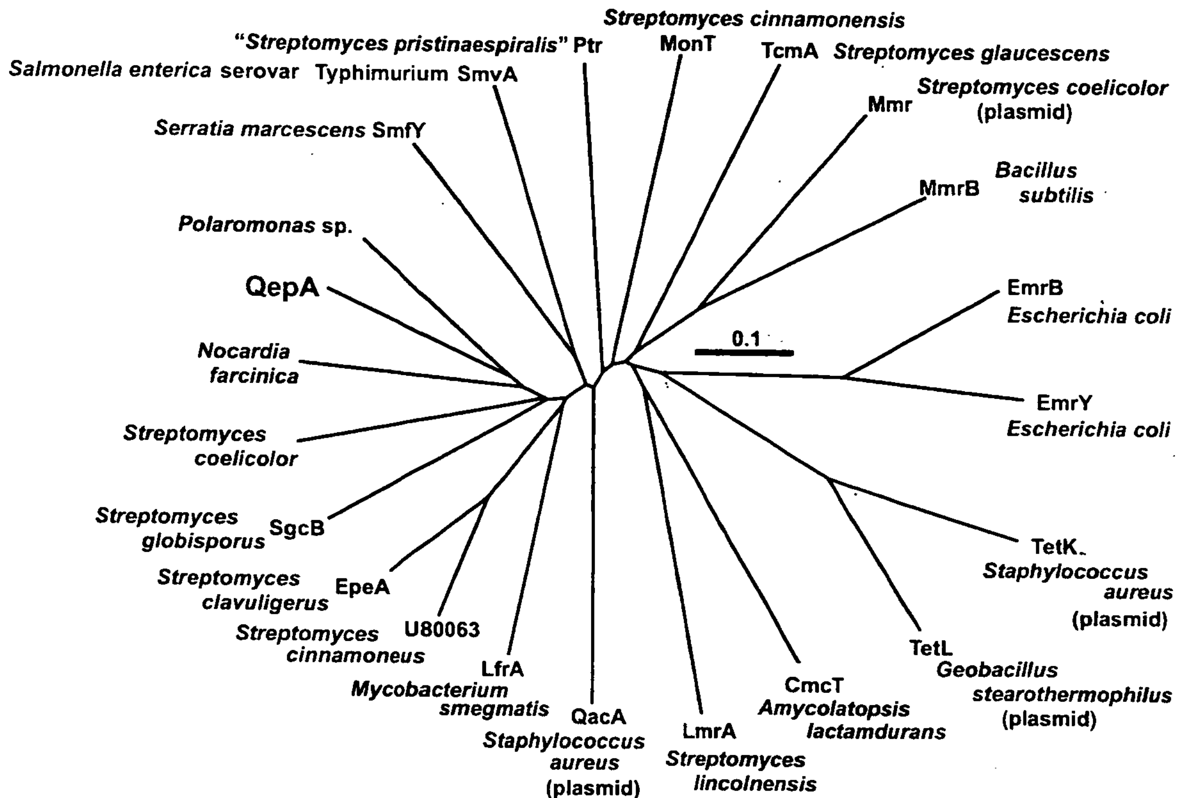

FIG. 4. Phylogenetic tree of 14-TMS efflux pumps belonging to MFS transporters. Each amino acid sequence was subjected to analysis with reference to the following sources: LfrA, a *Mycobacterium smegmatis* proton antiporter efflux pump (E/G/D accession number U40487); Ptr, a "*Streptomyces pristinaespiralis*" pristinamycin compound and rifampin resistance protein (E/G/D accession number X84072); TcmA, a *Streptomyces glaucescens* tetracenomycin C resistance and export protein (E/G/D accession number M80674); EmrB, an *E. coli* multidrug efflux system protein (E/G/D accession number AAC75733); EmrY, an *E. coli* multidrug resistance protein (E/G/D accession number D78168); TetK, a *Staphylococcus aureus* tetracycline efflux protein (E/G/D accession number M16217); TetL, a *Geobacillus stearothermophilus* tetracycline efflux protein (NCBI Protein Data Bank accession number P07561); Mmr, a *Streptomyces coelicolor* methylenomycin A resistance protein (E/G/D accession number M18263); MmrB, a *Bacillus subtilis* methylenomycin A resistance protein (E/G/D accession number X66121); CmcT, an "*Amycolatopsis lactamdurans*" cephamycin export protein (NCBI Protein Data Bank accession number Q04733); LmrA, a *Streptomyces lincolnensis* lincomycin resistance protein (E/G/D accession number X59926); QacA, an *S. aureus* antiseptic resistance protein (E/G/D accession number X56628); SmfY, a *Serratia marcescens* multidrug efflux pump (E/G/D accession number AB251607); SmvA, a *Salmonella enterica* serovar Typhimurium methyl-viologen resistance protein (NCBI Protein Data Bank accession number P37594); U80063, a *Streptomyces cinnamoneus* proton-dependent transport protein (E/G/D accession number U80063); EpeA, a *Streptomyces clavuligerus* DHA2 subfamily multidrug transporter (E/G/D accession number AJ302083); *Nocardia farcinica*, *N. farcinica* strain IFM 10152, probable arabinose efflux permease AraJ (NCBI Protein Data Bank accession number BAD57397); *Polaromonas* sp., *Polaromonas* sp. strain JS666, a probable MFS efflux pump (NCBI Protein Data Bank accession number ABE43318); QepA, an *E. coli* plasmid-mediated FQ efflux pump (E/G/D accession number AB263754); *Streptomyces coelicolor*, a probable membrane transporter of *S. coelicolor* (NCBI Protein Data Bank accession number CAC37879); SgcB, a *Streptomyces globisporus* transmembrane efflux protein (E/G/D accession number AAL06672); and MonT, a *Streptomyces cinnamonensis* putative monensin transporter (NCBI Protein Data Bank accession number AAO65793). Blue, actimomycetes; brown, gram-positive cocci; green, gram-negative rods; purple, *Mycobacterium smegmatis*. The horizontal bar (0.1) indicates a 10% change in the amino acid residues calculated by the CLUSTAL W program.

siderable similarity to the MFS-type efflux pumps belonging to the 14-TMS family of environmental microorganisms, including actinomycetes (Fig. 2). Norfloxacin accumulation in the *qepA*-expressing *E. coli* strain was significantly lower than that in the *qepA*-nonexpressing strains. Moreover, the addition of CCCP readily augmented the accumulation of norfloxacin even in the *qepA*-expressing strain (Fig. 5). These findings clearly revealed that the QepA protein is involved in the excretion of norfloxacin from the cytoplasm to the exterior of bacterial cells. As with FQs, the MICs of erythromycin, acriflavine, and ethidium bromide for the *qepA*-expressing clinical isolate and transconjugant were higher than those for the *qepA*-nonexpressing strains. However, the chemical substances tested, except FQs, showed at most twofold elevations in the MICs for a strain carrying only the *qepA* gene (Table 2). Therefore, it was strongly suggested that the QepA protein is involved mainly with the excretion of FQs as a proton antiporter efflux pump system.

Interestingly, the *qepA* gene had a high G+C content (72%), and the deduced amino acid sequence of QepA showed considerable homology to the probable efflux pumps belonging to the MFS-type membrane transporters found in *Polaromonas* spp. and members of the order *Actinomycetales* (Fig. 4). Moreover, considerable similarities between the codon usage patterns of *qepA* and genes for the MFS-type efflux pumps of *Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus* were observed, and both *qepA* and *rmtB* genes were mediated by a probable transposable element flanked by

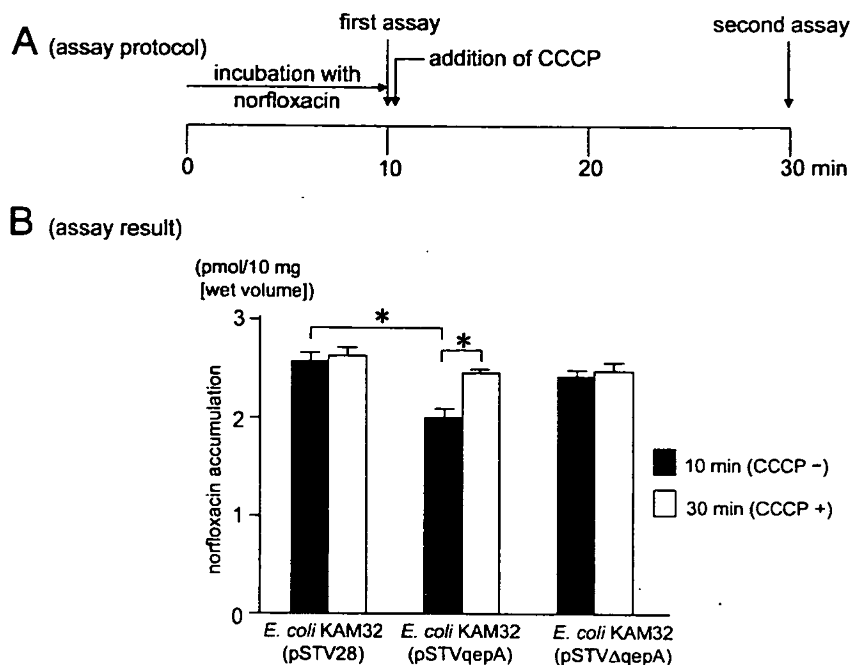

FIG. 5. Assay protocol and accumulation of norfloxacin in *E. coli* cells. The accumulations of norfloxacin in transformants that harbor a vacant plasmid (pSTV28), a *qepA*-carrying plasmid (pSTVqepA), and a disrupted *qepA*-carrying plasmid (pSTVΔqepA) are shown. *, statistical significance ($P < 0.01$) in the reduction of intracellular accumulation of norfloxacin that was seen in *E. coli* strains that produce QepA compared to QepA-nonproducing *E. coli* strains. The effect of CCCP on the accumulation of norfloxacin was also statistically significant in the QepA-producing strain. However, no statistical significance was seen in the norfloxacin levels of *E. coli* KAM32(pSTVqepA) cells between a sample taken at 10 min and a sample taken at 30 min in the absence of CCCP.

two copies of IS*26* on a transferable plasmid, pHPA. We therefore speculated that the chromosomal fragment of some actinomycetes with high G+C contents carrying both *qepA* and *rmtB* genes might be introduced into an IncFII plasmid by transposition and that pHPA could have been subsequently transferred to *E. coli*.

Antibiotic-producing actinomycetes have their own intrinsic genes for antibiotic efflux transporters for excretion of their metabolic products. FQs, however, are fully synthetic chemical agents, so no innate FQ transporter is expected to exist in the microbial world. It is possible, however, that some bacterial transporters that excrete antibiotics or bioactive agents possessing structural similarities to FQs might become transporters that can excrete FQs. Thus, we speculate that the QepA protein may well have originated from environmental microbes that produce bioactive metabolites, including antibiotics with structural similarity to FQs. A similar finding has been reported for OqxAB, a resistance nodulation division-type efflux pump that gives resistance to an artificial growth promoter, olaquindox (10).

Since resistance to broad-spectrum β-lactams has already developed among gram-negative bacteria, the emergence of multidrug-resistant gram-negative bacteria that harbor plasmids bearing *qepA* and/or *qnr*, as well as *rmtB* and genes for CTX-M-type extended-spectrum β-lactams and/or metallo-β-lactamases, could become a serious clinical concern. Although the MICs of FQs for QepA-producing strains are not high at present and the potential impact of *qepA*-harboring strains on FQ therapy has not been fully understood, the additive effect of QepA production on FQ resistance caused by mutations in genes for DNA gyrase and/or topoisomerase IV might promote further spread of FQ-resistant strains in clinical settings.

Quite recently, the *qepA* gene was also found in an RmtB-producing *E. coli* strain isolated in a Belgian hospital (23), suggesting a probable worldwide dissemination of *qepA* accompanied by *rmtB* that has already been identified in various gram-negative bacteria isolated in many countries of Asia (3, 5, 32) and Europe (2). QepA production confers resistance to enrofloxacin, a veterinary FQ, and *rmtB*-producing *E. coli* strains have frequently been isolated from pigs (3), for which aminoglycosides have tended to be used as a growth promoter in some countries or geographical areas. Thus, coproduction of QepA and RmtB may well give an advantage to bacteria to survive in livestock breeding environments as well as in human clinical settings. Hence, active surveillance of *qepA*-harboring gram-negative bacteria in animals might reveal a greater prevalence of such kinds of multidrug-resistant microbes.

In conclusion, we have newly identified a plasmid-mediated novel efflux pump, QepA, that is responsible for the elevation of levels of resistance to several clinically important FQs, such as ciprofloxacin, norfloxacin, and veterinary enrofloxacin.

ACKNOWLEDGMENTS

We thank Tomofusa Tsuchiya, a professor of the faculty of Pharmaceutical Sciences, Okayama University, for donating *E. coli* KAM32.

This work was funded by grants from the Ministry of Health, Labor and Welfare, Japan (H15-Shinkou-9, H15-Shinkou-10), and in part by a grant from the Kurozumi Medical Foundation.

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Journal of Antimicrobial Chemotherapy (2008) **61**, 568–576
doi:10.1093/jac/dkm498
Advance Access publication 12 January 2008

JAC

Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material

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Received 30 July 2007; returned 15 October 2007; revised 27 November 2007; accepted 28 November 2007

***Objectives*: In clinical *Acinetobacter* species, the reduction effects of organic material on bactericidal activities of four major disinfectants were investigated: chlorhexidine gluconate (CHX), benzethonium chloride (BZT), benzalkonium chloride (BZK) and alkyl diaminoethyl glycine hydrochloride (ADH).**

***Methods*: The bactericidal activities of the four disinfectants against 283 strains of *Acinetobacter* species recovered from 97 Japanese hospitals in March 2002 were investigated by four different tests: MIC measurements, MBC measurements, time-killing assays and adaptation assays. Moreover, disinfectant efficacy was examined in the presence of BSA in two tests: MBC measurements and time-killing assays.**

***Results*: No clinical isolates were able to withstand the in-use concentrations of the four disinfectants, although the MIC_{90} of ADH reached 100 mg/L. Strains for which MICs of at least two disinfectants were higher than MIC_{90} measured by the broth microdilution method were defined as isolates with 'disinfectant reduced susceptibility (DRS)'. In the presence of 3.0% BSA, the MBCs of BZK, BZT and ADH for DRS isolates rose to 512 and 1024 mg/L, which were about half their in-use concentrations. Moreover, the times for bacterial complete killing were remarkably prolonged in DRS isolates even after a 10 min of exposure to 1000 mg/L of ADH, a half of its in-use concentration. The MICs of CHX for DRS isolates rose to 640 mg/L after repetitive passages in subinhibitory concentrations of CHX.**

***Conclusions*: Given that the bactericidal effects of the four major disinfectants were considerably reduced in the presence of organic material (BSA) and DRS isolates tended to adapt to CHX, continuous surveys of the profiles of susceptibility to disinfectants among clinically isolated *Acinetobacter* species are very necessary from the standpoint of nosocomial infection control.**

Keywords: susceptibility profiles, bovine serum albumin, adaptation

Introduction

Acinetobacter species have recently been recognized as one of the major hospital-acquired pathogens that cause opportunistic infections such as pneumonia, urinary tract infections, septicaemia and surgical site infections, particularly in immunocompromised patients[1,2] accommodated in intensive care units where they commonly undergo invasive medical procedures and tend to receive various broad-spectrum antimicrobial agents.[3,4] Moreover, *Acinetobacter* species have rapidly developed multidrug resistance capabilities over the past 10 years, and the increasing difficulties encountered in the treatment of infections caused by this opportunistic pathogen have become a serious clinical concern.[5–7] The ability of this microbe to survive long-term in hospital environments even on dry surfaces has also been considered to play a crucial role in hospital-acquired infection.[8,9]

Biocides, including quaternary ammonium compounds (QACs) and bisbiguanides, have been assiduously used in hospitals and healthcare facilities, and have significantly contributed to maintaining sanitary conditions and preventing hospital-acquired infections.[10] However, concerns have also been raised that widespread use of disinfectants could serve to select disinfectant-insusceptible

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microbes among hospital-acquired pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.[11,12] Evidence for the development of reduced susceptibility due to exposure to disinfectants has been reported. For example, a steady increase in MICs of chlorhexidine for *P. aeruginosa* has resulted from the exposure to residual disinfectants at subinhibitory concentrations.[13] It is possible that *Acinetobacter* species may also have developed insusceptibility to disinfectants because of their innate ability to survive long-term on body surfaces and in hospital environments in which various disinfectants have been consumed.

Another cause for concern is that organic materials are known to reduce the effectiveness of disinfectants and antiseptics. Because these agents have been extensively used in medical and healthcare facilities for the disinfection of mucous membranes and wounds, and for the sterilization of medical instruments and equipment surfaces, which often tend to be contaminated with organic materials, the influence of such materials should not be ignored from the viewpoint of practical use of disinfectants. Therefore, it is very important to understand the susceptibility status of *Acinetobacter* species against disinfectants, as well as the influences of organic materials upon reduction in their efficacies. Since little is known about the present susceptibility status of *Acinetobacter* species to disinfectants, the aim of this study was to assess the susceptibility profiles of clinically isolated *Acinetobacter* species, isolated in 2002. For the isolates with 'disinfectant reduced susceptibility (DRS)' selected from clinical isolates, the bactericidal activities of QACs, bisbiguanides and ampholytic detergents used widely in medical facilities were evaluated both with and without the organic materials. In addition, the adaptive resistance to four disinfectants was also investigated to predict the potential development of resistance to disinfectants in *Acinetobacter* species.

Materials and methods

Bacterial strains and culture media

In March 2002, 283 non-repetitive clinical isolates identified as *Acinetobacter* species were collected from 97 hospitals located in different geographical areas of Japan. Since these isolates were speculated to be major causative microbes of infection in each patient, they were subjected to identification and antibiotic-susceptibility tests. They were identified as 273 *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex, 7 *Acinetobacter lwoffii* and 3 *Acinetobacter junii* by their biochemical identification using the API20NE system (bioMérieux Japan, Ltd, Tokyo, Japan) and a complementary test for the ability to grow at 37, 41 and 44°C. After re-identification, each isolate was grown on Luria–Bertani (LB) agar plates or in LB broth (Becton–Dickinson Diagnostic System, Sparks, MD, USA) for further studies.

Disinfectants and susceptibility testing

The disinfectants were obtained from the following sources: chlorhexidine gluconate (CHX), benzethonium chloride (BZT), Wako Pure Chemical Industries, Ltd, Osaka, Japan; benzalkonium chloride (BZK), Kanto Chemical Co., Inc., Tokyo, Japan; alkyl diaminoethyl glycine hydrochloride [ADH; TEGO 51TM, 10% (w/v) solution]. The MICs of the disinfectants were determined by the broth microdilution method using Mueller–Hinton broth (Becton–Dickinson Diagnostic System) according to the protocol recommended by the CLSI (formerly NCCLS) in document M100-S14.[14]

Assay of bactericidal activity: quantitative suspension test

The bactericidal effects of disinfectants on *Acinetobacter* species were measured using a slightly modified quantitative suspension test referring to the European Standard EN 1040.[15] The neutralizer solution used in the suspension test contained the following: 10% Tween 80 (v/v), 3% lecithin (w/v), 0.1% histidine (w/v), 0.5% sodium thiosulphate (w/v) and phosphate-buffered saline (PBS, pH 7.4), all obtained from Wako Pure Chemical Industries.

Each isolate was cultivated in LB broth until its optical density (OD) of 0.90 at 660 nm was reached and washed once with PBS (pH 7.4). The bacterial test suspensions were then adjusted to an optical density of 0.08 at 660 nm ($\sim 10^8$ cfu/mL), and bacterial test suspensions were prepared for each strain. A 100 µL of test suspension was added to tubes containing 900 µL of disinfectant solutions at different concentrations and left for 3 min at 20 ± 2°C. Since the test results obtained at room temperature demonstrated poor reproducibility, all reactions were performed as much as possible at 20 ± 2°C in an incubator using bacterial suspensions, disinfectant solutions and neutralizer solutions preincubated at 20 ± 2°C. Aliquots of the reaction mixture (100 µL) of containing bacterial cells and disinfectant were then added to 900 µL of neutralizer solution at 20 ± 2°C for 3 min and serially diluted in PBS (pH 7.4). After dilution, 50 µL of the mixture was spread immediately onto LB agar plates and incubated for 18 h at 35°C. The numbers of colonies surviving on each plate were counted, and cell survival rates were calculated with those obtained by a test using a bacterial suspension treated with PBS (pH 7.4) instead of disinfectant, as the control. BSA (Sigma) was used to imitate organic soiling, thus ensuring that the 'dirty' test simulated practical 'in-use' conditions. The quantitative suspension tests with BSA were performed according to recommendation EN 1276.[16] Each test was repeated to simulate 'dirty' conditions by mixing the test suspension with 0.3% or 3.0% (w/v) BSA solution before adding the 100 µL of test suspension to the 900 µL of disinfectant solution. The experiments were repeated three times on different days. In addition, the neutralizer was checked for its possible toxicity for the test organisms. Aliquots of a diluted bacterial suspension (100 µL) containing $1-3 \times 10^4$ cfu/mL was added to 900 µL of neutralizer and left at 20 ± 2°C for 3 min. Two samples of 100 µL of mixture described above were then spread onto LB agar plates and incubated as above. The values obtained on each plate were 100–300 cfu/100 µL. Finally, the inactivation of the bactericidal activity of each disinfectant by neutralizer was also validated. Nine hundred microlitres of the solutions containing different concentrations of each disinfectant was added to 100 µL of the PBS (pH 7.4) and left at 20 ± 2°C for 3 min. Then, 100 µL of the mixture was transferred into 800 µL of neutralizer and left at 20 ± 2°C for 3 min. Aliquot of a diluted bacterial suspension (100 µL) containing $1-3 \times 10^4$ cfu/mL was then added and left at 20 ± 2°C for 3 min. Two samples of 100 µL mixture containing disinfectant, neutralizer and bacteria, were then spread onto LB agar plates and incubated as described above. The numbers of colonies grown on each plate ranged from 100 to 300 cfu/100 µL.

MBC

MBC was determined using the quantitative suspension test described above. The disinfectant solutions at different concentrations were made by serial 2-fold dilutions of each disinfectant.

MBC was defined as the lowest concentration of disinfectants that completely suppressed bacterial growth in each disinfectant.

Measurement of bactericidal activity: time-killing assay

Time-killing assays were performed to evaluate the bactericidal effects of disinfectants using a modified quantitative suspension test referring to the European Standards EN1040 and EN1276. Bacterial cell suspension (100 μL) was added to 900 μL of disinfectant solution and 50 μL aliquots of the mixtures were sampled at 0.5, 1, 2.5, 5, 10, 20, 30, 60, 90 and 120 min, respectively. Each sample was immediately added to 450 μL of neutralizer solution at 20 ± 2 C and left for 3 min, then serially diluted in PBS (pH 7.4). Fifty microlitres of each diluent was spread onto LB agar plate and incubated for 18 h at 35°C. The numbers of colonies that grew on each plate were counted, and cell survival rates were calculated by similar methods using a bacterial suspension as the control treated with PBS (pH 7.4) instead of the mixture containing disinfectant and neutralizer solution. The test was repeated to simulate 'dirty' conditions using the test suspension containing a 0.3% or 3.0% (w/v) BSA by the same method employed in the MBC measurement. The experiments were repeated three times on different days.

Adaptation to disinfectants

Twelve strains, including five disinfectant-susceptible clinical isolates and seven DRS isolates, strains 1, 2, 4, 5, 6, 7 and 10 shown in Table 2, were subjected to the adaptation test. Five susceptible clinical isolates were randomly selected from the group of strains for which the MICs of all four disinfectants were less than MIC_{50} as shown in Table 1. Aliquots of the overnight culture (100 μL) were added to nutrient broth containing each agent at 1/2 of the MIC for each strain. The 1/2 MIC culture was incubated at 37 C with shaking for 72 h, and bacterial growth was assessed visually. When a culture density of higher than $\sim 1 \times 10^8$ cfu/mL was observed, 100 μL of the 1/2 MIC culture was spread onto nutrient agar plates containing the same concentration of disinfectant, and the agar plates were incubated overnight at 37 C. The colonies grown on each agar plate were selected and cultured for further testing using nutrient broth containing 1/2 MIC disinfectant. An aliquot of cell culture was stored at −80 C as passage 1 (P1), and MICs and MBCs for P1 were re-determined. Any isolates that had shown an increase in MICs and MBCs were then inoculated into the broth media containing twice the original concentration of disinfectant, and the others not shown augmented MICs and MBCs were inoculated into media containing the disinfectant with the previous concentration. This procedure was repeated four times every 5 days (including 3 days for culture and 2 days for assay) after the selection of P1. If culture density was lower than 1×10^8 cfu/mL, the passage was not advanced to the next step, because no $>5 \log_{10}$ reduction could be detected in this condition. The stabilities of the MICs and MBCs for P5 isolates demonstrating highest disinfectant-reduced susceptible profile were checked four times every 5 days by repetitive culture with disinfectant-free broth. Moreover, the bactericidal activities of each disinfectant for these adapted isolates were evaluated by the quantitative suspension tests. The experiments were repeated three times on different days.

Statistical analysis

Data were analysed using the statistical program SPSS for Windows version 11.0J (SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U*-test was performed to compare bactericidal activities measured by both MBC and time-killing assays. Dunnett's and Bonferroni's multiple comparison tests were performed to determine statistically significant differences between passage groups of the adaptation test. A *P* value of ≤0.05 was considered statistically significant.

Results

Susceptibility to disinfectants and selection of DRS isolates

Distributions of MICs of CHX, BZK, BZT and ADH for 283 clinical isolates are shown in Table 1. *Acinetobacter* species tended to be susceptible to CHX, BZK and BZT, and MIC_{90}s obtained by the broth microdilution method in the absence of BSA were ≤25 mg/L. However, MICs of ADH were relatively higher than those of the other three disinfectants, and the MIC_{90} of ADH obtained was 100 mg/L (Table 1).

The isolates with DRS possessing relatively high MICs of CHX, BZK, BZT and/or ADH were selected. In the present study, the DRS isolates were defined as those for which MICs of at least two among the four disinfectants were higher than MIC_{90} when measured by the broth microdilution method. As a result, 14 isolates (8 *A. baumannii*, 4 *A. calcoaceticus* and 2

Table 1. Distributions of MICs of various disinfectants by the broth microdilution method

	Number of strains at each MIC (mg/L) of disinfectant										
Disinfectant	2.5	5	10	25	50	100	200	400	800	MIC_{50}	MIC_{90}
CHX	0	126[a]	80	62	<u>14</u>[b]	<u>1</u>	0	0	0	10	25
BZK	0	168[a]	104	<u>9</u>	<u>2</u>	0	0	0	0	5	10
BZT	0	2	196[a]	68	<u>15</u>	<u>2</u>	0	0	0	10	25
ADH	0	0	1	21	176[a]	85	0	0	0	50	100

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride.

[a]Numbers indicate a group of clinical isolates demonstrating the phenotype susceptible to each disinfectant, and five strains used for adaptation tests were randomly selected in each group.

[b]Standard MIC_{50} and MIC_{90} measurements (quantal measurement of 50% and 90% of the population) are given. Underlined numbers indicate number of isolates for which MICs of each disinfectant were higher than MIC_{90}. MIC_{90} of ADH for all isolates was 100 mg/L. Fourteen isolates for which MICs of at least CHX, BZK and BZT were above MIC_{90} were defined as 'isolates with reduced disinfectant susceptibility' and selected as candidates for MBC and time-killing assays.

Table 2. Susceptibility profiles of 14 isolates with 'disinfectant-reduced susceptible' properties

Strain number	MIC (mg/L) of disinfectant[a]				Species
	CHX	BZK	BZT	ADH	
1[b]	50	10	50	100	*A. baumannii*
2[b]	25	25	50	50	*A. junii*
3	25	25	50	100	*A. baumannii*
4[b]	50	25	50	50	*A. baumannii*
5[b]	25	25	100	100	*A. baumannii*
6[b]	25	25	100	100	*A. baumannii*
7[b]	50	50	50	100	*A. calcoaceticus*
8	50	10	50	100	*A. baumannii*
9	50	10	50	100	*A. baumannii*
10[b]	100	25	50	100	*A. calcoaceticus*
11	10	50	50	50	*A. junii*
12	25	25	50	100	*A. baumannii*
13[c]	50	10	50	100	*A. calcoaceticus*
14[c]	50	10	50	100	*A. calcoaceticus*

[a]MICs of disinfectants were determined using the broth microdilution method.
[b]Strains 1, 2, 4, 5, 6, 7 and 10 were selected for adaptation tests.
[c]Strains 13 and 14 were isolated from different patients in the same hospital.

A. junii) out of 283 were provisionally defined as DRS isolates in the present study (Table 2).

MBCs of four disinfectants

To evaluate the phenotypes of DRS isolates selected, the MBC and bactericidal activity of the four disinfectants were measured under both the so-called 'clean' and 'dirty' conditions. Figure 1 shows cell survival rates of DRS isolates after exposure to CHX, BZK, BZT and ADH, with concentrations ranging from 1 to 1024 mg/L. The MBC values of the four disinfectants in the presence of BSA were higher than those in the absence of BSA, and MBC values of BZK, BZT and ADH obtained by the addition of 3.0% BSA showed high values (512 and 1024 mg/L), which were about half of the in-use concentration of these agents (Figure 1).

Time-killing assay

Time-killing assays were also performed to evaluate the bactericidal effects of the four disinfectants on DRS isolates from the viewpoint of exposure duration. As can be seen in Figure 2, at low concentrations (MIC_{50} of each disinfectant obtained by the broth microdilution method), the bacterial cell count in the absence of BSA reached a 5 log_{10} reduction after 10 min. On the other hand, the presence of 0.3% BSA simulating the 'dirty' condition elevated the cell survival rate, and, even after 120 min of exposure, reduction of bacterial cells was less than 1 log_{10} under the test conditions employed for BZK, BZT and ADH.

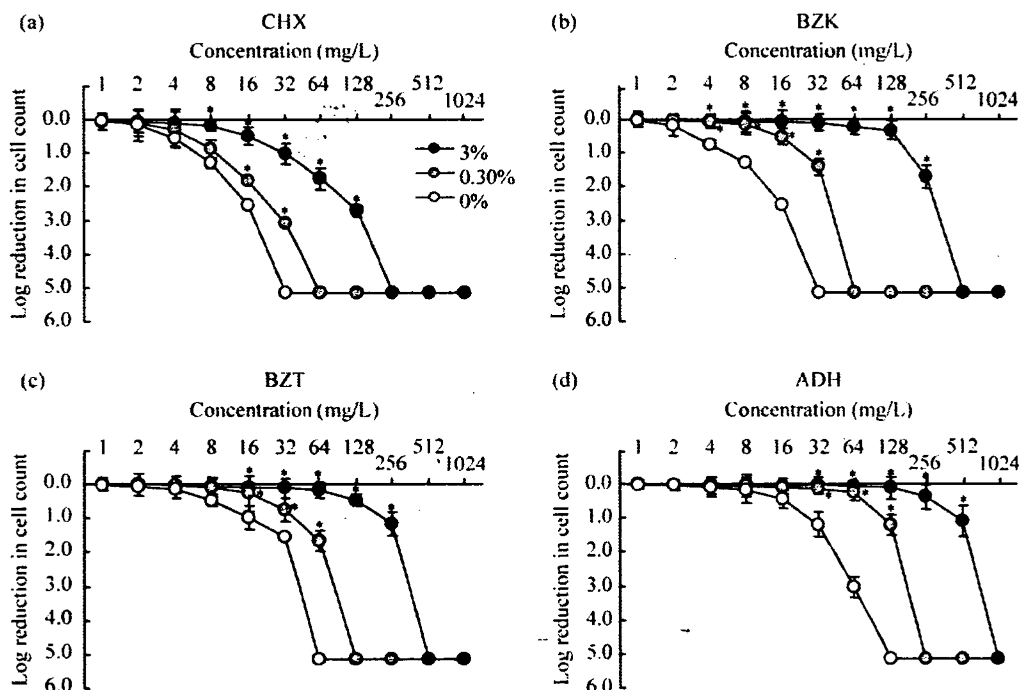

Figure 1. Comparison of MBC. Fourteen isolates for which MICs of at least two disinfectants were more than MIC_{90} measured by the broth microdilution method were defined as isolates with DRS. MBCs of the four disinfectants tested on the DRS isolates are shown. Results are expressed as log_{10} reduction in cell counts compared with those of the control sample treated with PBS. The viable cell count before the exposure to disinfectant was $2.21 \pm 0.323 \times 10^7$ cfu/mL. White circles, 'clean' condition (0% BSA); grey circles, 'dirty' condition (0.3% BSA); black circles, 'dirty' condition (3% BSA). Error bars represent standard deviations of results from three experiments, and the asterisks indicate a significant difference ($^*P < 0.05$, as determined by Mann–Whitney *U*-test). (a) Chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH).

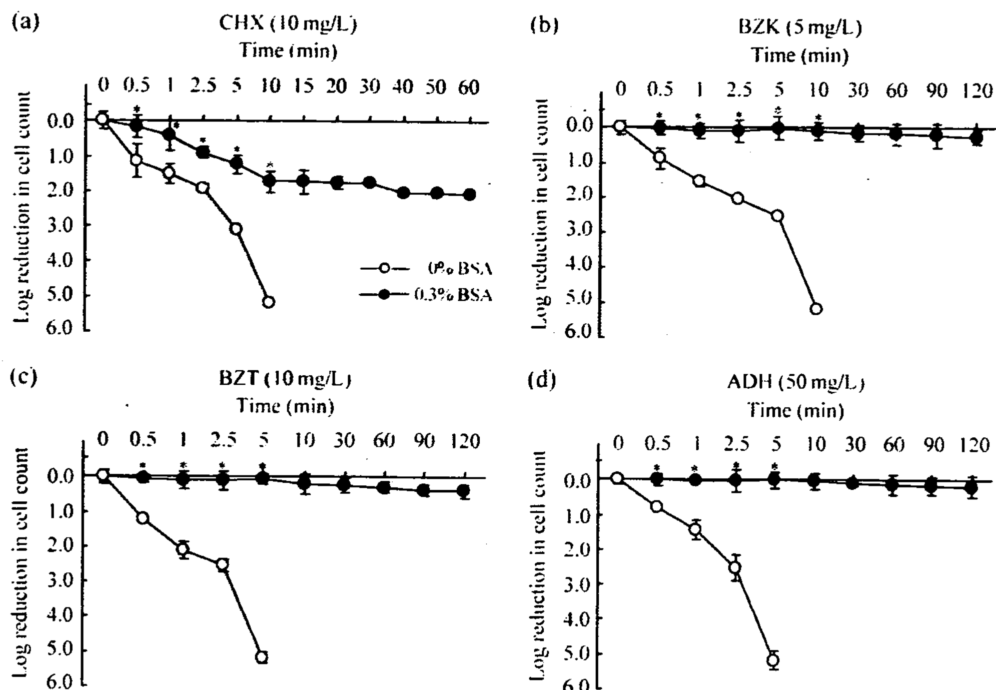

Figure 2. Results of time-killing assays. Time–kill assays were performed for the 14 DRS isolates selected in the same manner as in the MBC assay. The concentration of each disinfectant is as follows: (a) chlorhexidine gluconate (CHX), 10 mg/L; (b) benzalkonium chloride (BZK), 5 mg/L; (c) benzethonium chloride (BZT), 10 mg/L; (d) alkyl diaminoethyl glycine hydrochloride (ADH), 50 mg/L. Bacterial cells were treated with each disinfectant at 20 ± 2°C. Each bacterial test sample was removed at 0.5, 1, 2.5, 5, 10, 20, 30, 60, 90 and 120 min, respectively. Cell viabilities were determined by plating serially diluted cell suspensions on LB plates. Results are expressed as $\log_{10}$ reduction in cell counts compared with those of control sample treated with PBS. The viable cell count before the exposure to disinfectant was $2.60 \pm 0.530 \times 10^7$ cfu/mL. White circles, 'clean' condition (0% BSA); black circles, 'dirty' condition (0.3% BSA). Error bars represent standard deviations of results from three experiments, and the asterisks indicate a significant difference ($*P < 0.05$, as determined by Mann–Whitney *U*-test).

Indeed, higher concentrations of disinfectants were reasonably more effective, especially under 'clean' conditions (Table 3). However, the bacterial cell numbers failed to show a 5 $\log_{10}$ reduction after a 1 min of exposure to all four disinfectants at 1/5 of the in-use concentrations in the presence of 3.0% BSA. As for ADH, bacterial cells survived even after a 10 min of exposure to 1000 mg/L, a half of the in-use concentration, with 3.0% BSA (Table 3).

Adaptation to disinfectants

Five susceptible isolates and 7 DRS isolates selected from 283 isolates were subjected to an adaptation test. The adaptation profiles of these isolates are shown in Figure 3. For five susceptible strains, the MICs of BZK, BZT and ADH were not significantly elevated after repetitive passages through the broth media containing each disinfectant. These five susceptible isolates were most susceptible to CHX among the four disinfectants, but these isolates could adapt to only 2-fold higher concentrations of CHX after five passages. The MICs of BZK, BZT and ADH for DRS isolates were only slightly elevated (<2-fold) during five passages. However, MICs of CHX were significantly elevated ~10-fold (up to 640 mg/L) after the repeated passages. Similar findings were observed in the elevation of MBC values throughout the experimental process of passage as were seen in MIC (data not shown). The stability of disinfectant-reduced susceptible profile among strains demonstrating the highest adaptation ability was checked by culturing for 20 days in disinfectant-free broth. In some cases, the MIC or MBC of CHX for the adapted strains decreased approximately to 50%; however, no case declined to the level of parent strains (data not shown). Time-killing assays by suspension test were also performed to evaluate the bactericidal effect of CHX on both DRS and adapted isolates obtained through each passage process (Table 4). No considerable changes in the bactericidal activity of BZK, BZT and ADH for adapted isolates obtained after passage in CHX were observed (data not shown). On the other hand, an apparent elevation of the resistance level to CHX in the adapted isolates was observed by suspension test. As can be seen in Table 4, the bacterial cell counts of both DRS and adapted isolates showed a 5 $\log_{10}$ reduction after a 1 min of exposure to 5000 mg/L CHX, that is, its in-use concentration. However, in P4 and P5 strains adapted for disinfectants, a 5 $\log_{10}$ reduction of bacterial cells failed after a 1 min of exposure to 1000 mg/L CHX, 1/5 of its in-use concentration (Table 4).

Discussion

In healthcare settings, it has become more difficult to treat infections caused by *Acinetobacter* species because of their acquisition of consistent resistance to major groups of antimicrobial agents.[5–7] Difficulties in the infection control practices as well as in chemotherapy of infectious diseases are due to the intrinsic capacities of *Acinetobacter* species for long-term survival in

Table 3. Bactericidal effects of four disinfectants on 'disinfectant reduced-susceptible' isolates

Condition and exposure time	Reduction in live bacterial cell numbers[a]							
	CHX (mg/L)				BZK (mg/L)			
	5000[b]	2500	1000	500	1000[b]	500	200	100
0% BSA, 1 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23
0.3% BSA, 1 min	>5.23	>5.23	>5.23	4.86 ± 0.08	>5.23	>5.23	>5.23	4.55 ± 0.09
3.0% BSA, 1 min	>5.23	>5.23	4.86 ± 0.09	3.89 ± 0.10	>5.23	>5.23	2.86 ± 0.16[c]	2.50 ± 0.10
3.0% BSA, 10 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	4.16 ± 0.09	2.53 ± 0.10
	BZT (mg/L)				ADH (mg/L)			
	2000[b]	1000	400	200	2000[b]	1000	400	200
0% BSA, 1 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23
0.3% BSA, 1 min	>5.23	>5.23	>5.23	4.35 ± 0.09	>5.23	>5.23	3.82 ± 0.10	3.22 ± 0.14
3.0% BSA, 1 min	>5.23	>5.23	2.77 ± 0.16[c]	2.53 ± 0.12	>5.23	2.82 ± 0.14[c]	2.15 ± 0.14	2.06 ± 0.18
3.0% BSA, 10 min	>5.23	>5.23	3.85 ± 0.12	2.72 ± 0.10	>5.23	4.89 ± 0.14	2.27 ± 0.21	2.32 ± 0.13

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride.
The bacterial cell concentration exposed to each disinfectant was $3.40 \pm 0.81 \times 10^7$ cfu/mL.
[a]Shown as $\log_{10}$ reduction in bacterial cell number in both 'clean' (0% BSA) and 'dirty' (0.3% and 3% BSA) conditions: mean ± SD.
[b]Numbers indicate 'in-use' concentration (mg/L) of each disinfectant.
[c]Disinfectants failed to provide a 5 $\log_{10}$ reduction in live bacterial cell counts in the presence of BSA.

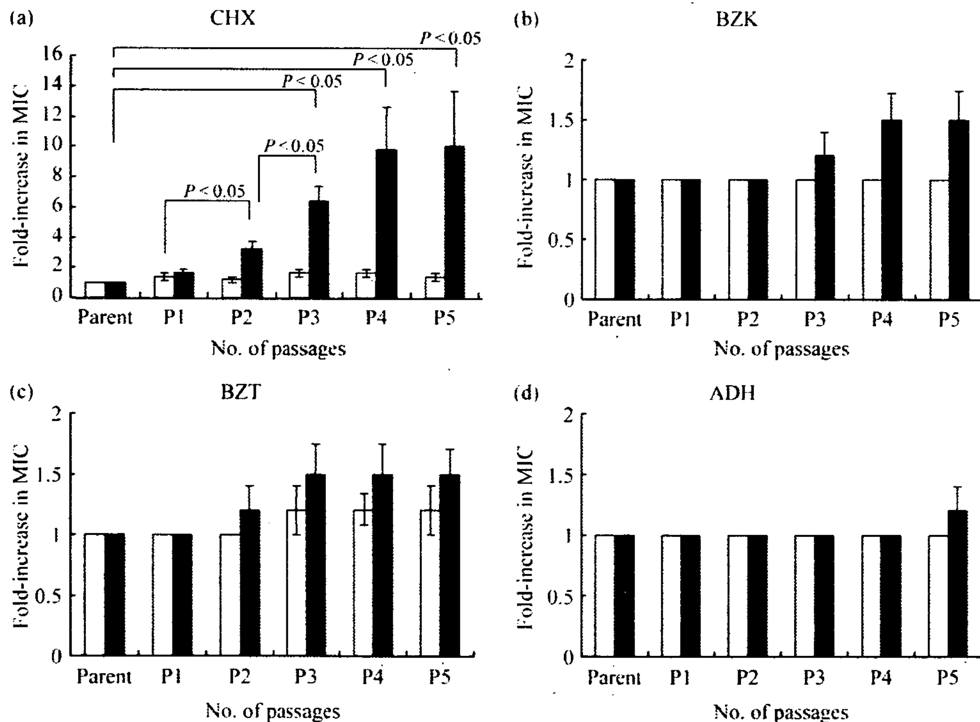

Figure 3. Adaptation to four disinfectants after persistent passages. Adaptations to disinfectants were generated by repeated subculture in nutrient broths containing each disinfectant (a) chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH). Five disinfectant-susceptible clinical isolates selected randomly and seven DRS isolates (strains 1, 2, 4, 5, 6, 7 and 10 shown in Table 2) were subjected to this test. The 1/2 MIC cultures were incubated at 37 C with shaking for 72 h, and growth was assessed visually for cultures grown in media containing disinfectants. MICs were checked, and any isolates that showed an increase in MICs were then inoculated into media containing twice the original concentration. This procedure was repeated a total of five times every 5 days (including 3 days for culture and 2 days for assay). Stabilities of adaptive resistances of most resistant bacterial cells (P5) were determined by further passages through disinfectant-free broths and checked four times every 5 days. The experiments were performed three times on different days. White bars, five disinfectant-susceptible isolates selected; black bars, seven DRS isolates. Error bars represent standard deviations, and a significant difference is indicated by $P < 0.05$ (as determined by Dunnett's and Bonferroni's multiple-comparison tests).

Table 4. Bactericidal activity of CHX against adapted isolates with reduced susceptibility to CHX

Passage[a]	Bacterial cells exposed to disinfectant[b]	exposure time (30 s)		exposure time (60 s)		exposure time (180 s)	
		viable cell concentration[d]	log_{10} reduction	viable cell concentration[d]	log_{10} reduction	viable cell concentration[d]	log_{10} reduction
		CHX (5000 mg/L)[c]					
Parent	$5.36 \pm 1.20 \times 10^7$	NC	>5.43	NC	>5.43	NC	>5.43
P1	$4.43 \pm 1.73 \times 10^7$	NC	>5.34	NC	>5.34	NC	>5.34
P2	$8.00 \pm 1.00 \times 10^7$	NC	>5.60	NC	>5.60	NC	>5.60
P3	$8.85 \pm 2.06 \times 10^7$	NC	>5.65	NC	>5.65	NC	>5.65
P4	$6.34 \pm 1.73 \times 10^7$	NC	>5.50	NC	>5.50	NC	>5.50
P5	$3.37 \pm 0.70 \times 10^7$	NC	>5.23	NC	>5.23	NC	>5.23
		CHX (1000 mg/L)[c]					
Parent	$5.36 \pm 1.20 \times 10^7$	NC	>5.43	NC	>5.43	NC	>5.43
P1	$4.43 \pm 1.73 \times 10^7$	$2.54 \pm 1.05 \times 10^2$	5.24	NC	>5.34	NC	>5.34
P2	$8.00 \pm 1.00 \times 10^7$	$5.54 \pm 1.25 \times 10^2$	5.16	NC	>5.60	NC	>5.60
P3	$8.85 \pm 2.06 \times 10^7$	$3.10 \pm 1.43 \times 10^3$	4.46	NC	>5.65	NC	>5.65
P4	$6.34 \pm 1.73 \times 10^7$	$1.88 \pm 0.88 \times 10^3$	4.53	$9.80 \pm 0.78 \times 10^1$[e]	5.50	NC	>5.50
P5	$3.37 \pm 0.70 \times 10^7$	$3.64 \pm 0.89 \times 10^3$	3.97	$3.60 \pm 0.68 \times 10^2$	4.97	NC	>5.23
		CHX (500 mg/L)[c]					
Parent	$5.36 \pm 1.20 \times 10^7$	$2.26 \pm 0.99 \times 10^3$	4.38	NC	>5.43	NC	>5.43
P1	$4.43 \pm 1.73 \times 10^7$	$2.50 \pm 1.21 \times 10^3$	4.25	NC	>5.34	NC	>5.34
P2	$8.00 \pm 1.00 \times 10^7$	$4.68 \pm 1.31 \times 10^3$	4.23	NC	>5.60	NC	>5.60
P3	$8.85 \pm 2.06 \times 10^7$	$5.46 \pm 1.56 \times 10^3$	4.21	$2.80 \pm 0.92 \times 10^2$	5.28	NC	>5.65
P4	$6.34 \pm 1.73 \times 10^7$	$4.75 \pm 0.65 \times 10^3$	4.12	$4.37 \pm 1.31 \times 10^2$	5.31	NC	>5.50
P5	$3.37 \pm 0.70 \times 10^7$	$2.56 \pm 0.41 \times 10^4$	3.12	$3.60 \pm 0.68 \times 10^3$	3.99	$2.71 \pm 1.65 \times 10^2$	5.09

CHX, chlorhexidine gluconate.

For seven DRS isolates, the experiments were repeated three times on different days. First, the viable cell counts of each isolate were calculated by the average of three test results performed on different days, and then average and distribution of the viable cell counts of the seven DRS isolates were again calculated.

NC indicates negative culture. Viable cell counts were estimated to be $< 2 \times 10^2$ cfu/mL in all three tests for seven DRS isolates selected.

[a]Strains were obtained throughout adaptation process passaged in increasing concentrations of CHX as shown in Figure 3.

[b]Average viable cell concentration (cfu/mL ± SE) measured in PBS instead of disinfectant solution.

[c]Concentration of CHX to which bacterial cells were exposed before inoculation onto agar plate for counting surviving bacterial colonies.

[d]Average viable cell concentration (cfu/mL ± SE) after the exposure to disinfectant.

[e]Since no colonies grew on several plates, viable cell count became below the detection limit after the statistical analysis.

hospital environments and transmission among patients. Therefore, performance of contact-precautions including hand-hygiene and disinfection is crucial to interrupt patient-to-patient transmission of this microbe. Indeed numerous studies have focused on the issues of *Acinetobacter* species resistant to antimicrobial agents,[17,18] but only a few have so far explored the susceptibility profiles to antiseptics and disinfectants in this microbe. Thus, investigations on the susceptibility status of clinically isolated *Acinetobacter* species' susceptibility to antiseptics and disinfectants assessed by four different test methods, MIC measurements, MBC measurements by quantitative suspension tests, time-killing assays and adaptation tests, would provide instructive new insights into coping with *Acinetobacter* species in various healthcare settings.

In the present study, the MIC_{90}s of the four disinfectants tested for 283 *Acinetobacter* species isolates were $\leq$100 mg/L, which is lower than the actual in-use concentration of each disinfectant. Moreover, the 14 DRS isolates selected were also confirmed to be susceptible to disinfectants by multiple tests, including MBC measurement, and time-killing assay, in the absence of organic materials. The results are consistent with those of Martró *et al.*[19] and Wisplinghoff *et al.*[20] who found no apparent development of resistance to disinfectants among clinically isolated *Acinetobacter* species. They assessed the susceptibility of *A. baumannii* to respective disinfectants and antiseptics by suspension test without adding organic materials. However, since *Acinetobacter* species inhabit hospital environments often contaminated with a variety of organic materials and colonize various body sites of patients, one must never fail to take into account the reduction effects of organic materials on antiseptics and disinfectants in practical use. Therefore, we further extended their studies to evaluate the properties of DRS isolates against antiseptics and disinfectants by MBC measurements and time-killing assays in the presence of BSA. The 10 min of exposures of DRS isolates to CHX at much lower than its in-use concentration in the presence of 3.0% BSA provided a 5 $\log_{10}$ reduction in bacterial cell numbers, whereas 10 min of exposure of these isolates to 200 mg/L BZK or 400 mg/L BZT failed to produce a 5 $\log_{10}$ reduction of bacterial cells in the presence of 3.0% of BSA. In addition, the 10 min of exposure to 1000 mg/L ADH, half of its in-use concentration, failed to eliminate the live bacterial cells in the presence of 3.0% BSA. This finding is crucial, because ADH is one of the most frequently used disinfectants for medical instruments and hospital environments.

Acinetobacter species usually cause hospital-acquired infections, including urinary- and respiratory-tract infections, and particularly ventilator-associated pneumonia, especially in debilitated individuals.[1,2,21] Indeed, no apparent resistance properties of these DRS isolates against disinfectants were observed from the viewpoints of MIC and MBC measurements in the absence of organic materials, but the results obtained by the suspension test in the presence of BSA suggested that these DRS isolates may well survive in conditions of contamination by organic materials such as blood and exudation. Thus, care should be taken in monitoring the susceptibility profile of *Acinetobacter* species against disinfectants, especially when this microbe is frequently or continuously isolated from clinical samples.

To our knowledge, no adaptive resistance to disinfectants in strains belonging to *Acinetobacter* species has been reported to date. Our results demonstrate that repeated exposure to subinhibitory concentrations of CHX gradually elevated its MIC (at most 10-fold, up to 640 mg/L) for the DRS isolates. Furthermore, P4 and P5 strains obtained after several passages in 1/2 MICs of CHX survived after a 1 min of exposure to 1000 mg/L CHX, 20% of its in-use concentration (0.5% = 5000 mg/L), in suspension test. CHX has been demonstrated to have a persistent or residual effect after applications to skin[22] and mucous membranes.[23]

Irizarry *et al.*[24] suggested that environmental residues of CHX and cetylpyridinium chloride might confer some selective advantage on MRSA, an organism that has also been known as relatively resistant to CHX on dry surfaces. A recent study by Thomas *et al.*[13] gives some experimental support to the idea that repetitive exposure to subinhibitory concentrations of CHX results in a stable increase in MICs of CHX for *P. aeruginosa*. *Acinetobacter* species have also become very common in hospital environments as well as MRSA and *P. aeruginosa* known as the major hospital-acquired pathogens. Our results suggest that the DRS isolates, which have been exposed to some extent to subinhibitory amounts of disinfectants remaining on environmental surfaces or even on the skin, would also develop the properties of adaptive resistance to CHX. It would therefore be necessary to carefully screen and select appropriate disinfectants based on a sufficient understanding of each for use in medical facilities.

In conclusion, no resistance to CHX, BZX, BZT and ADH was detected among clinically isolated *Acinetobacter* species by MIC measurements. However, the bactericidal effects of BZK, BZT and ADH, especially on the DRS isolates, were remarkably reduced in the presence of an organic material (3% BSA). Furthermore, the DRS isolates tended to adapt a higher concentration of CHX after repetitive passages in 1/2 MIC concentrations of CHX. To prevent hospital-acquired infections caused by this kind of microbe, the profile of susceptibility to disinfectants, as well as to antimicrobial agents, must be carefully monitored and checked among *Acinetobacter* species isolated from both clinical specimens and environments. Disinfectants are indispensable to perform appropriate infection control. Hence, this study highlights the need to ensure that these agents are being used appropriately in practice at the correct concentrations and for adequate contact times.

Acknowledgements

We are grateful to all the medical institutions that submitted bacterial isolates to the National Reference Laboratory.

Funding

This work was supported by an H18-Shinkou-11 grant from the Ministry of Health, Labour and Welfare, Japan and in part an H17-Gakushin grant from Nagoya University Graduate School.

Transparency declarations

None to declare.

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16S Ribosomal RNA Methylation: Emerging Resistance Mechanism against Aminoglycosides

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Methylation of 16S ribosomal RNA (rRNA) has recently emerged as a new mechanism of resistance against aminoglycosides among gram-negative pathogens belonging to the family *Enterobacteriaceae* and glucose-nonfermentative microbes, including *Pseudomonas aeruginosa* and *Acinetobacter* species. This event is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside-producing actinomycetes. Their presence confers a high level of resistance to all parenterally administered aminoglycosides that are currently in clinical use. The responsible genes are mostly located on transposons within transferable plasmids, which provides them with the potential to spread horizontally and may in part explain the already worldwide distribution of this novel resistance mechanism. Some of these organisms have been found to coproduce extended-spectrum β-lactamases or metallo-β-lactamases, contributing to their multidrug-resistant phenotypes. A 2-tiered approach, consisting of disk diffusion tests followed by confirmation with polymerase chain reaction, is recommended for detection of 16S rRNA methylase–mediated resistance.

Aminoglycosides continue to play an important role in the management of serious infections caused by gram-negative pathogens, often in combination with broad-spectrum β-lactams. They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis [1]. The most commonly encountered mechanism of resistance to aminoglycosides is enzymatic inactivation, which is mediated by 3 classes of enzymes: acetyltransferases, nucleotidyltransferases, and phosphotransferases [2]. They are further divided into subclasses that are based on the site of modification and the spectrum of resistance within the class of antimicrobials. Other known mechanisms of aminoglycoside resistance include defect of cellular permeability, active efflux, and, rarely, nucleotide substitution of the target molecule [1].

Received 30 November 2006; accepted 24 March 2007; electronically published 21 May 2007.

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Clinical Infectious Diseases 2007; 45:88–94
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1058-4838/2007/4501-0018$15.00
DOI: 10.1086/518605

Aminoglycosides are produced by species of actinomycetes, such as *Streptomyces* species and *Micromonospora* species. These actinomycetes are intrinsically resistant to the aminoglycosides that they produce [3]. In many cases, this resistance is caused by ribosomal protection through methylation of specific nucleotides within the A-site of 16S rRNA, which hampers binding of aminoglycosides to the 30S ribosomal subunits and serves as a means of self-protection. Until recently, this resistance mechanism was believed to be absent in clinically relevant species.

However, clinical strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* that produced 16S rRNA methylases were reported in 2003 [4, 5]. These enzymes were found to confer extraordinarily high levels of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin, and gentamicin. Since 2003, the literature on this newly recognized resistance mechanism has grown rapidly, documenting identification of new enzymes and their spread to different species in various parts of the world. In the present article, we will first review aminoglycoside resistance caused by 16S rRNA methylation in aminoglycoside-producing actinomycetes. We will then discuss the current knowledge of this emerging, plasmid-mediated resistance mechanism that is found among gram-negative path-

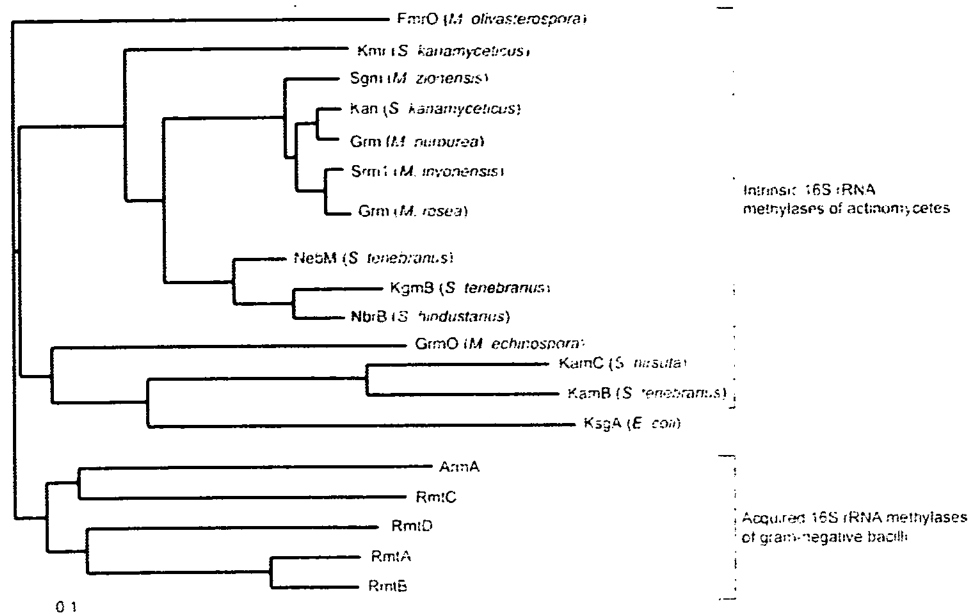

Figure 1. Dendrogram of 16S rRNA methylases of gram-negative bacteria and of representative actinomycetes. KsgA is a nonresistance 16S rRNA methylase intrinsic in *Escherichia coli*. The protein sequences were obtained from the databases of GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan and were aligned using ClustalW [11]. *M. echinospora, Micromonospora echinospora; M. inyonensis, Micromonospora inyonensis; M. olivasterospora, Micromonospora olivasterospora; M. purpurea, Micromonospora purpurea; M. rosea, Methylocystis rosea; M. zionensis, Micromonospora zionensis; S. hindustanus, Streptoalloteichus hindustanus; S. hirsuta, Saccharopolyspora hirsuta; S. kanamyceticus, Streptomyces kanamyceticus; S. tenebrarius, Streptomyces tenebrarius.*

ogens, with an emphasis on the diagnostic and therapeutic implications.

MODIFICATION OF rRNA IN BACTERIA

The ribosome is a large enzyme consisting of multiple proteins and RNA components [6]. In bacteria, it comprises 30S and 50S subunits, the former containing 16S rRNA and the latter containing 23S and 5S rRNAs. Posttranscriptional modification events of RNAs, such as methylation of nucleosides, take place following the generation of initial RNA transcripts. They are predominantly reported in tRNA, but they are also reported in rRNA. *Escherichia coli*, for example, is known to contain 10 methylated nucleosides in 16S rRNA and 14 methylated nucleotides in 23S rRNA [7]. The primary roles of rRNA methylation likely include modulation of rRNA maturation, stabilization of rRNA structures, and alteration of translation rates. For instance, mutants of *E. coli* that were deficient in the production of KsgA (RsmA) showed an increased leakiness of nonsense and frameshift mutants and alteration in decoding fidelity at both the A-site and peptidyl-tRNA site of 16S rRNA [8, 9]. In addition, some of the posttranscriptional methylation events are known to confer resistance to antimicrobials that target rRNA.

RESISTANCE IN ACTINOMYCETES MEDIATED BY 16S rRNA METHYLATION

A number of actinomycetes are known to be intrinsically resistant to aminoglycosides that they produce themselves. The mechanisms of resistance include inactivation of aminoglycosides by production of aminoglycoside-modifying enzymes and protection of 16S rRNA within the 30S ribosome subunit by production of 16S rRNA methylase. The latter mechanism results in high-level resistance to multiple aminoglycosides. It represents an efficient means to avoid inhibition of their own protein synthesis and is prevalent among aminoglycoside-producing actinomycetes (figure 1) [3].

Two sites of methylation within 16S rRNA that lead to different aminoglycoside-resistance phenotypes have been identified [10]. One group of 16S rRNA methylases, such as that produced by the istamycin producer *Streptomyces tenjimariensis*, methylates residue A1408 (figure 2). Another group of 16S rRNA methylases, exemplified by those produced by gentamicin-producer *Micromonospora purpurea*, methylates residue G1405. The former confers resistance to kanamycin and apramycin but not gentamicin, whereas the latter confers resistance to kanamycin and gentamicin but not apramycin. Both of these residues are located within the A-site–decoding region of 16S

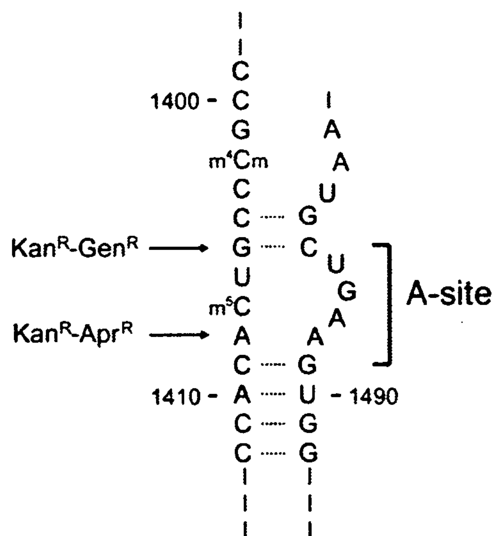

Figure 2. The positions of modifications in the aminoacyl site (A-site) decoding region in 16S rRNA (modified from Beauclerk et al. [10] with permission). ArmA is known to methylate G1405 [19]. Based on the common Kan^R-Gen^R phenotype, the other methylases in gram-negative organisms likely methylate the same residue. Methylases that modify A1408 have only been reported in actinomycetes. Apr, apramycin; Gen, gentamicin; Kan, kanamycin.

rRNA, where aminoglycosides are known to bind and interfere with accurate translation through blocking translocation of peptidyl-tRNA from the A-site to the peptidyl-tRNA site [12].

RESISTANCE IN GRAM-NEGATIVE PATHOGENS MEDIATED BY 16S rRNA METHYLATION

Because it became evident that many aminoglycoside-producing actinomycetes used ribosomal resistance afforded by methylation of 16S rRNA, the question was raised as to why the same resistance mechanism was not identified in clinically relevant species. It was speculated that such resistance mechanisms could exist but were possibly missed because of limited screening methods, because the resistance pattern could mimic that of organisms producing multiple aminoglycoside modifying enzymes. In 2002, a gene encoding 16S rRNA methylase, later designated ArmA, was deposited to the European Molecular Biology Laboratory and GenBank as part of a plasmid sequence from *Citrobacter freundii* in Poland (accession number AF550415). No additional findings have been published to date.

Then, in 2003, an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan was reported to produce 16S rRNA methylase [5]. The deduced amino acid sequence of this new enzyme, designated RmtA, shared modest (up to 35%) identity with 16S rRNA methylases of various actinomycetes. RmtA displayed methylation activity against 16S rRNA of 30S ribosomal subunits derived from a susceptible strain of *P. aeruginosa*. When *rmtA* was cloned and expressed in *E. coli* and *P. aeruginosa*, it was found to confer a high degree of resistance to all 4,6-disubstituted deoxystreptamines, which include gentamicin, tobramycin, and amikacin. As described above, a putative 16S rRNA methylase gene was initially found in a *C. freundii* clinical isolate from Poland. This gene was also identified in *K. pneumoniae* from France [4]. The gene product ArmA was also shown to confer high-level resistance to 4,6-disubstituted deoxystreptamines. The identity of the amino acid sequence of ArmA with those of RmtA and other 16S rRNA methylases from actinomycetes was only modest, ranging between 30% and 35%. The structural gene of RmtA was associated with a genetic element that resembled a mercury-resistance transposon Tn*5041* on a transferable plasmid [13]. The guanine cytosine content of *rmtA* was 55%, suggesting its origin from some guanine cytosine–rich microbe, including actinomycetes. The structural gene for ArmA was reported to be located on functional composite transposon Tn*1548* [14]. The guanine cytosine content of *armA* was 30%, suggesting that it was derived from some microbe with lower guanine cytosine content. These findings point to the possibility that these genes were acquired horizontally from diverse nonpathogenic environmental microbes, but their exact origins remain unknown.

Several other 16S rRNA methylases were subsequently discovered among gram-negative bacteria, and a total of 5 are known to date (figure 1). RmtB was identified in *Serratia marcescens* from Japan [15]. RmtB is closest to RmtA, sharing 82% identity at the amino acid level. The structural gene for RmtB was located adjacent to a Tn*3*–like transposon on a large transferable plasmid. RmtC was found in a *Proteus mirabilis* clinical strain from Japan that was rather distant in phylogeny from the 3 enzymes already reported [16]. The structural gene for RmtC is also located next to a transposon-mediated recombination system termed IS*Ecp1*, and the methylase gene was shown to be mobilizable from plasmid to plasmid [17]. The most recently identified 16S rRNA methylase is RmtD, which shares moderate identity (40%–42%) with RmtA and RmtB [18]. RmtD was found to be produced by a *P. aeruginosa* clinical strain from Brazil, which also produced metallo-β-lactamase SPM-1. This particular strain was, therefore, highly resistant to carbapenems as well as to aminoglycosides.

These newly identified 16S rRNA methylases in gram-negative bacilli all confer resistance to 4,6-disubstituted deoxystreptamines, including gentamicin, tobramycin, and amikacin, but not including 4,5-disubstituted deoxystreptamines, such as neomycin, 4-monosubstituted deoxystreptamines, such as paromomycin, or streptomycin, which lacks a deoxystreptamine ring. The level of resistance to tobramycin appears to be slightly lower than the level of resistance to other 4,6-disubstituted deoxystreptamines when the responsible genes are cloned and expressed in experimental strains of *E. coli*, such as XL1-Blue, DH5α, or INVaF' (MIC, 64–256 μg/mL) (table 1). However,

Table 1. Aminoglycoside resistance pattern conferred by G1405 16S rRNA methylase.

Drug	Susceptibility
4,6-disubstituted DOS	Highly resistant
Gentamicin	Highly resistant
Tobramycin	Resistant, highly resistant
Amikacin	Highly resistant
4,5-disubstituted DOS; neomycin	Susceptible
Monosubstituted DOS; apramycin	Susceptible
No DOS ring; streptomycin	Susceptible

NOTE. DOS, deoxystreptamine.

the MICs of tobramycin for the clinical strains that produce 16S rRNA methylase usually range between 512 and 1024 μg/mL or greater. This pattern of resistance resembles that of the methylase of *M. purpurea*, which is known to methylate residue G1405, as discussed above. Recently, residue G1405 of 16S rRNA in the 30S ribosomal subunit was confirmed as the site of methylation by ArmA using primer extension method (figure 2) [19]. No methylase that modifies residue A1408 has been reported in gram-negative pathogens at this time.

PREVALENCE OF 16S rRNA METHYLASE-MEDIATED RESISTANCE

Data on the prevalence of aminoglycoside resistance mediated by 16S rRNA methylation among gram-negative bacilli is still scarce. The prevalence of RmtA among clinical isolates of *P. aeruginosa* in Japan was estimated to be at least 0.4% during a national surveillance when screened by high-level multiple-aminoglycoside resistance followed by PCR confirmation [5]. Subsequently, RmtB has been detected in various species belonging to the family *Enterobacteriaceae*, including *K. pneumoniae*, *Klebsiella oxytoca*, *E. coli*, and *C. freundii* in Japan, Taiwan, South Korea, China, and Belgium [20—25]. ArmA, which was initially found in *C. freundii* and later characterized in *K. pneumoniae*, has also been identified in clinical isolates of *E. coli*, *S. marcescens*, *Enterobacter cloacae*, *Salmonella enterica*, *Shigella flexneri*, and *Acinetobacter* species from various countries in East Asia and Eastern and Western Europe (table 2) [14, 20–23]. A recent report from a university hospital in Taiwan estimated the prevalence of ArmA and RmtB to be 0.9% and 0.3%, respectively, among *K. pneumoniae* and *E. coli* when screened by resistance to amikacin and confirmed by PCR [22]. RmtA and RmtD have only been reported from *P. aeruginosa* in Japan and Brazil, respectively [5, 18, 21]. These findings indicate that 16S rRNA methylase genes are already disseminated globally among pathogenic gram-negative bacilli, although the overall prevalence appears to remain low.

Strains producing 16S rRNA methylase have been reported from livestock, as well. Plasmid-mediated *armA* and *rmtB* genes have been identified from *E. coli* in swine from Spain and China, respectively [26, 25]. A large amount of aminoglycosides, including kanamycin, gentamicin, apramycin, and streptomycin, has been consumed in veterinary medicine. This may have served as a selective pressure for enteric gram-negative organisms to acquire 16S rRNA methylase genes, possibly from nonpathogenic environmental actinomycetes that intrinsically produced aminoglycosides or similar 16S rRNA inhibitors, and then maintain and spread them to humans through the food supply chains. Monitoring for high-level aminoglycoside resistance among gram-negative pathogens mediated by this new resistance mechanism would, therefore, be important in livestock breeding environments, as well.

Table 2. Genetic association and geographic distribution of 16S rRNA methylase genes.

16S rRNA methylase gene	Guanine cytosine content, %	Molecular weight of product, kDa	IS or transposon	Associated β-lactamase genes	Bacterial species (country or countries)	Country, reference(s)
rmtA	55.4	27.4	IS*6100*, κγ element, Tn*4051*		*Pseudomonas aeruginosa* (J)	J [5, 21]
rmtB	55.6	27.4	Tn*3*	bla_{TEM-1}, $bla_{CTX-M-14}$	*Serratia marcescens* (J), *Eschericia coli* (J, T, C, Be), *Klebsiella pneumoniae* (J, T, K), *Klebsiella oxytoca* (J), *Citrobacter freundii* (K)	J [15], T [22], K [20, 23], C (DQ345788), Be [24]
rmtC	41.1	32.1	IS*Ecp1*		*Proteus mirabilis* (J)	J [16]
rmtD	59.3	27.7	IS*CR*	bla_{TEM-1}	*P. aeruginosa* (Br)	Br [18]
armA	30.4	30.2	IS*26*, Tn*1548*	$bla_{CTX-M-3}$	*S. marcescens* (J, F, K), *C. freundii* (P, K, F, Bu), *Citrobacter amalonaticus* (Be), *K. pneumoniae* (F, S,T, K, Bu, Be), *K. oxytoca* (Bu), *E. coli* (J, T, S, F, Bu, Be), *Enterobacter cloacae* (F, K, Be), *Enterobacter aerogenes* (Bu, Be), *P. mirabilis* (F), *Salmonella enterica* Enteritidis (Bu), *S. enterica* Oranienburg (P), *Shigella flexneri* (Bu), *Acinetobacter* species (J, K)	P (AY522431) [14], Bu [14], F [14], S [26], Be [24], J [21], T [22], K [20, 23]

NOTE. DQ345788 and AY522431 are European Molecular Biology Laboratory and GenBank accession numbers. Be, Belgium; Br, Brazil; Bu, Bulgaria; C, China; F, France; I, India; IS, insertion sequence; J, Japan; K, South Korea; P, Poland; S, Spain; T, Taiwan.