Materials and methods

Production and purification of RmtC

The *rmtC* gene was amplified with the P1 primer (5′-GGA ATT CCA TAT GAA AAC CAA CGA TAA TT-3′: NdeI restriction site added), the P2 primer (5′-GCT CTA GAT TAC AAT CTC GAT ACG ATA-3′: XbaI restriction site added), and the pET-His-rmtC vector (Wachino *et al.*, 2006) as a DNA template. The amplified fragments were digested with endonucleases, cloned into pCold-II vector (Takara), and introduced into *Escherichia coli* BL21(DE3)-pLysS. Cells were grown until $A_{600\,nm}$ 0.5 at 37 °C in Luria–Bertani medium. After the addition of isopropyl-β-D-1-thiogalactopyranoside (0.5 mM), cells were grown at 15 °C for 24 h, and disrupted with a French press. Protein purification using nickel-nitrilotriacetic acid was performed according to the manufacturer's instructions (GE Healthcare). The eluted recombinant protein was loaded on size-exclusion chromatography column Superdex^{TM} 200 10/300GL (GE Healthcare), and eluted with 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 1 mM dithiothreitol. Finally, the purified protein (His_6-RmtC) was concentrated using an Amicon Ultra-15 Centricon (Millipore). The purity of the protein was proven by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro methylation of 16S rRNA

Ribosomal subunits were extracted from *E. coli* JM109 using ultracentrifugation with the sucrose density gradient. Methylation assay was performed as described elsewhere (Wachino *et al.*, 2007). In brief, purified His_6-RmtC, *S*-adenosyl-L-[methyl-^{3}H]methionine (GE Healthcare), and the substrate (30S ribosomal subunit, 50S ribosomal subunit, or naked 16S rRNA) were mixed and incubated at 35 °C. Aliquots were taken at 0, 5, 15, and 30 min, and purified using an RNeasy mini kit (Qiagen), according to the instructions provided by the manufacturer. The radioactivity of the samples was determined with a scintillation counter.

RNase protection assay

[^{3}H]-methyl-labeled 16S rRNA produced by RmtC was hybridized with oligonucleotides spanning the A-site of *E. coli* 16S rRNA. The oligonucleotides used were the same as those in our previous study (Wachino *et al.*, 2007). RNaseA (Wako) was added and incubated at 37 °C. The reaction was quenched by the addition of ice-cold trichloroacetic acid (TCA). The samples were passed through cellulose nitrate filters and washed with ice-cold trichloroacetic acid (TCA). The filter was dissolved in scintillation fluid, and the radioactivity of the samples was measured using a scintillation counter.

Primer extension analysis

The 16S rRNA was extracted from *E. coli* JM109 (pBC-KB1) that expresses RmtC. The recombinant plasmid, pBC-KB1, was constructed in our previous study (Wachino *et al.*, 2006). The 16S rRNA was then treated with borohydride and aniline as described previously (Liou *et al.*, 2006). The primer extension was performed using the primer (5′-biotin CCA ACC GCA GGT TCC CCT ACG G-3′) complementary to nucleotide 1530–1509 positions. The cDNA transcripts were analyzed using PAGE.

Detection of 7-methylguanosine (m^7G) by HPLC

The 16S rRNA of three *E. coli* strains, BW25113, BW25113Δ*gidB*, and BW25113Δ*gidB*(pBC-KB1) expressing RmtC, were extracted and treated with nuclease P1 (Wako) and alkaline phosphatase (Takara). The resulting product was analyzed using HPLC with an HRC-ODS column [4.6 mm (inner diameter) by 250 mm; Shimadzu].

Introduction of *rmtC* into Gram-positive microorganisms

The *rmtC* gene and its promoter region were amplified with the P3 primer (5′-CGC GGA TCC AGT GTA TGA AAA ATG TCT GG-3′: BamHI restriction site added) and the P4 primer (5′-CCC AAG CTT GGT GTG TTA GAA TTT GCC T-3′: HindIII restriction site added), and then cloned into the pHY300PLK shuttle vector (Takara). The recombinant plasmid, pHY300*rmtC*, was introduced into *B. subtilis* strain ISW1214 and *Staphylococcus aureus* strain RN4220 by electroporation. The *rmtC* gene was also amplified with the P5 primer (5′-TTT TTC GGC CGG CAT GAA AAC CAA CGA TAA TT-3′: Eco52I restriction site added) and the P6 primer (5′-ATT TTT CGC GAC AAT CTC GAT ACG ATA AA-3′: NruI restriction site added), cloned into *E. coli–S. aureus* shuttle expression vector pMGS100 (Fujimoto & Ike, 2001), and expressed in *S. aureus* RN4220. MICs of kanamycin, gentamicin, and neomycin for the transformants were measured according to the protocol recommended by the Clinical and Laboratory Standards Institute (2009).

Results and discussion

Assay of the methylation activity of RmtC

In the previous study (Wachino *et al.*, 2006), we purified C-terminal histidine-tagged RmtC with the combination of a pET29a vector and an *E. coli* BL21(DE3)pLysS. However, only approximately 1 mg of protein was purified from a 1 L culture. Therefore, we generated another expression

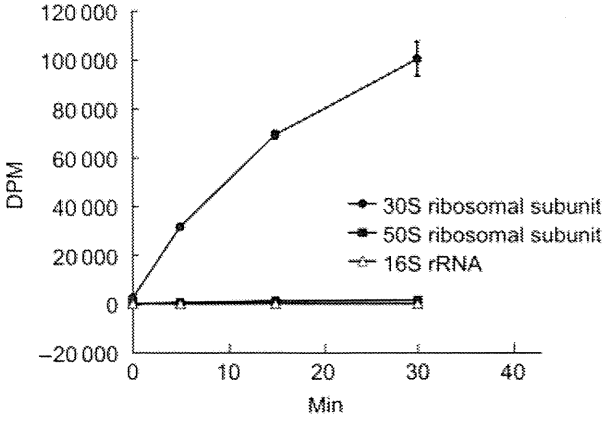

Fig. 1. Methyl acceptor activity of the 30S ribosomal subunit, the 50S ribosomal subunit, and naked 16S rRNA using [^{3}H]-*S*-adenosyl-L-methionine and purified His_6-RmtC. Values are the averages of three measurements. Error bars indicate SDs.

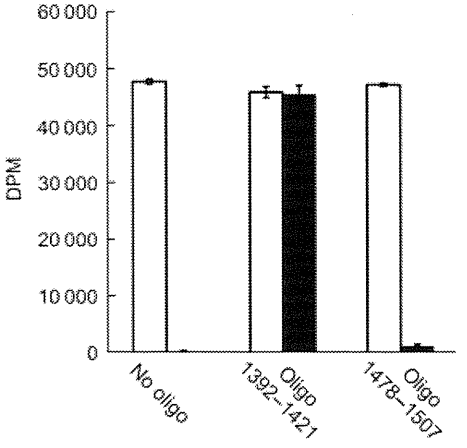

Fig. 2. RNase protection assay with [^{3}H]-methyl-labeled 16S rRNA and DNA oligonucleotides complementary to the regions from positions 1392 to 1421 or positions 1478 to 1507. Values are the averages of three measurements. Error bars indicate SDs. Open bars, undigested; solid bars, digested with RNaseA.

construct consisting of a pCold-II vector and an *E. coli* BL21(DE3)pLysS to improve the protein purification productivity. Optimized conditions yielded 8 mg of purified protein per 1 L culture, and its purity seemed very high on SDS-PAGE (data not shown). The native molecular weight (*M*) of the His_6-RmtC protein was determined to be approximately 34 000 by gel filtration chromatography.

Purified His_6-RmtC protein specifically had an MTase activity against an assembled 30S ribosomal subunit consisting of 16S rRNA and several ribosomal proteins, but no methylation activity was detected against protein-free 16S rRNA in the presence of the methyl group donor *S*-adenosyl-L-methionine (Fig. 1). This substrate specificity of RmtC to the 30S ribosomal subunit, not to naked 16S rRNA, has been commonly observed among 16S rRNA MTases such as ArmA (G1405), RmtB (G1405), Sgm (G1405), RsmF[YebU] (C1407), and NpmA (A1408), which methylate the nucleotide within the ribosomal A-site (Andersen & Douthwaite, 2006; Liou *et al.*, 2006; Wachino *et al.*, 2007; Savic *et al.*, 2008; Cubrilo *et al.*, 2009; Schmitt *et al.*, 2009). The specific activity of these 16S rRNA MTases for the 30S ribosomal subunit would indicate that ribosomal proteins play a crucial role in the precise substrate recognition.

Determination of methylation site by RmtC

To determine the precise nucleotide position modified by RmtC, we used three approaches: RNase protection assay, primer extension, and HPLC. [^{3}H]-methyl-labeled 16S rRNA modified by RmtC *in vitro* was hybridized with oligonucleotides complementary to the rRNA region, and treated with RNaseA, and the radioactivity was measured to confirm the region to which the [^{3}H]-methyl group was added. The hybridization of oligonucleotides spanning from G1392 position to the G1421 position was able to maintain

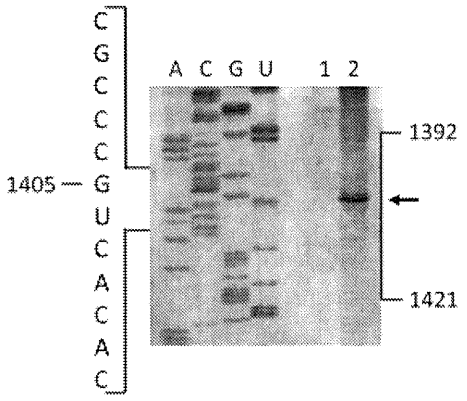

Fig. 3. Primer extension analysis of methylated 16S rRNA by RmtC. Lanes: 1, without aniline/borohydride treatment; 2, aniline/borohydride treatment. Dideoxy sequencing lanes (C, A, U, and G) were generated with the amplified PCR products of *Escherichia coli* 16S rRNA gene as the template. Primer extension termination at position U1406 is indicated by an arrow.

the radioactivity after RNaseA treatment (Fig. 2), suggesting that the nucleotide residue methylated by RmtC was located between G1392 and G1421 of 16S rRNA.

A primer extension analysis was performed as the second assay for the determination of the methylated position. The 16S rRNA prepared from the 30S ribosomal subunit of *E. coli* JM109 expressing RmtC was treated with $NaBH_4$/aniline before the primer extension reaction, and the site of methylation was determined by acrylamide gel electrophoresis. By primer extension analysis, one reverse transcription stop was observed at position U1406 as a result of β-elimination at the 3′-end of the N7-position of the nucleotide G1405 (Fig. 3). The termination of transcription at U1406 was not observed without the treatment with $NaBH_4$/aniline (Fig. 3). Thus,

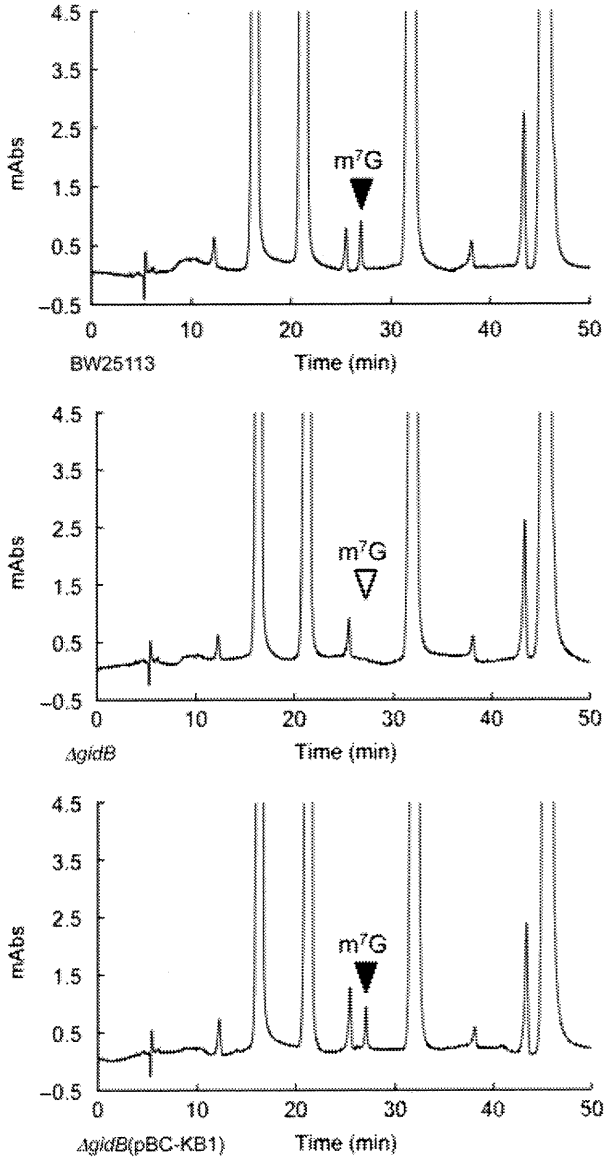

Fig. 4. HPLC analysis of methylated guanosine residue. BW25113, wild-type *Escherichia coli* 16S rRNA; Δ*gidB*, *E. coli* 16S rRNA lacking G527 methylation; Δ*gidB* (pBC-KB1), 16S rRNA of RmtC-producing *E. coli* lacking G527 methylation. mAb, milliabsorbance units.

these results clearly demonstrated that RmtC induced methylation at the N7-position of nucleotide G1405.

To confirm the above finding, we used an HPLC to examine the N7-methylation on the guanosine of 16S rRNA. As mentioned in the previous report (Okamoto *et al.*, 2007), the 16S rRNA of wild-type *E. coli* includes one m^7G at position 527 modified by GidB, which is widely conserved among both Gram-positive and Gram-negative bacteria. Therefore, we introduced the recombinant plasmid, pBC-KB1 carrying *rmtC*, into the Δ*gidB E. coli* mutant that lacks the innate m^7G in 16S rRNA, and observed the reversion of

Table 1. Susceptibility testing

	Minimal inhibitory concentration (μg mL^{-1})			
	B. subtilis ISW1214		*S. aureus* RN4220	
Aminoglycosides	pHY300*rmtC*	pHY300PLK	pMGS*rmtC*	pMGS100
Kanamycin	> 256	0.25	> 256	0.5
Gentamicin	128	≤ 0.06	> 256	0.5
Neomycin	≤ 0.06	≤ 0.06	0.5	0.5

the peak corresponding to the m^7G formed by RmtC. When the 16S rRNA of wild-type *E. coli* strain BW25113 was digested with nuclease P1 and alkaline phosphatase, a peak corresponding to m^7G was detected (Fig. 4). On the other hand, no peak corresponding to m^7G was observed when 16S rRNA of the Δ*gidB E. coli* mutant was treated (Fig. 4). The digestion of 16S rRNA extracted from Δ*gidB E. coli* mutant expressing RmtC revealed the reversion of the m^7G peak as expected (Fig. 4). These findings clearly indicated that RmtC indeed introduced the N7-methylation at the guanosine.

Liou *et al.* (2006) earlier revealed that methylation at the N7-position of nucleotide G1405 by ArmA interfered with the binding of gentamicin to the target 16S rRNA. The m^7G methylation at 1405 position by RmtC and ArmA probably induces a steric clash and electrostatic repulsion between G1405 and ring III of 4,6-disubstituted 2-DOS. This might well directly block the binding of aminoglycosides to the target A-site of 16S rRNA, and this would confer resistance in bacteria to various aminoglycosides belonging to the 4,6-disubstituted 2-DOS.

Introduction of *rmtC* into Gram-positive microorganisms

All the plasmid-mediated 16S rRNA MTases have been found exclusively in Gram-negative bacilli to date, despite the wide distribution of the chromosomally encoded 16S rRNA MTases among aminoglycoside-producing actinomycetes, including *Streptomyces* species. Therefore, we tested whether or not the RmtC could be produced and could function in Gram-positive microorganisms. A recombinant plasmid, pHY300*rmtC*, which carries the *rmtC* gene on the same fragment derived from the plasmid pBC-KB1 (Wachino *et al.*, 2006), was introduced into *B. subtilis* ISW1214 and *S. aureus* RN4220. Consequently, the introduction of *rmtC* could provide a high level of resistance to 4,6-disubstituted 2-DOS only in *B. subtilis* (Table 1), but not in *S. aureus* (data not shown). It was thought that the original promoter regions of *rmtC* are not suitable for the expression in *S. aureus*; hence, *rmtC* was cloned in an *E. coli–S. aureus* shuttle expression vector, pMGS100, and the recombinant plasmid, pMGS*rmtC*, was introduced into *S. aureus*

RN4220. As a result, the transformant of *S. aureus* RN4220 harboring *rmtC* showed resistance to 4,6-disubstituted 2-DOS as found in *B. subtilis* (Table 1).

The 16S rRNA MTase genes (*sgm*, *grmA*, *kgmB*, and *krm*) derived from high G+C content environmental actinomycetes have been functionally expressed and found to confer aminoglycoside resistance in the heterologous Gram-negative bacteria, *E. coli* (Savic *et al.*, 2009). Regardless of the provenance of the 16S rRNA MTase gene responsible for aminoglycoside resistance, the enzyme seems to be functional to some extent in any bacterial species, although each bacteria species would need the optimal promoter region in each 16S rRNA MTase gene for its expression.

Acknowledgements

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Correlation between reduced susceptibility to disinfectants and multidrug resistance among clinical isolates of *Acinetobacter* species

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Background: The aim of this study was to investigate the susceptibility profiles to disinfectants and antimicrobial agents of 283 non-repetitive *Acinetobacter* clinical isolates obtained in 97 Japanese hospitals in March 2002.

Methods: Susceptibility profiles of the above isolates to four disinfectants, six antimicrobial agents and two dyes were investigated. MICs were measured by the agar dilution method recommended by the CLSI (formerly NCCLS). MBC measurements and time–kill assays were performed using a slightly modified quantitative suspension test based on the European Standard EN 1040.

Results: No evident resistance to disinfectants was seen among the 283 strains of *Acinetobacter* spp. isolated in 2002, but the MIC_{90}s of chlorhexidine gluconate, benzalkonium chloride and alkyldiaminoethylglycine hydrochloride were 50, 50 and 400 mg/L, respectively. Interestingly, the MICs of alkyldiaminoethylglycine hydrochloride and benzethonium chloride for four and three clinical isolates, respectively, reached 800 mg/L (approximately half the in-use concentration). The MBCs for the 28 disinfectant reduced susceptibility (DRS) isolates, for which the MICs of at least one of the four disinfectants tested were higher than the MIC_{90}, were comparable to those for susceptible isolates, in general; however, significant differences ($P<0.01$) were observed between disinfectant-susceptible and DRS isolates in the time–kill assays of chlorhexidine gluconate, benzalkonium chloride and benzethonium chloride. Furthermore, DRS isolates tended to demonstrate multiresistance profiles to ceftazidime, ciprofloxacin and amikacin ($P<0.05$).

Conclusions: Since several *Acinetobacter* clinical isolates have developed augmented resistance to multiple antimicrobials and disinfectants, it is worth checking the susceptibility to disinfectants if multidrug-resistant *Acinetobacter* spp. are recurrently isolated clinically.

Keywords: time-dependent survey, nosocomial pathogen, antimicrobial agent, infection control

Introduction

Acinetobacter spp., especially *Acinetobacter baumannii*, have emerged as a major cause of nosocomial infections, particularly in intensive care units (ICUs), where immunocompromised patients are routinely prescribed various antimicrobial agents.[1–4] Furthermore, *Acinetobacter* spp. have an innate ability to readily accept foreign DNA, including genetic determinants for antimicrobial resistance, so as to adapt to and survive in environments that are hazardous to bacterial growth.[5,6] Therefore, they have a propensity for developing resistance to multiple classes of antimicrobial agents, including broad-spectrum β-lactams, fluoroquinolones and aminoglycosides.[1,3] Recently, even in military medical facilities, a series of infections, such as osteomyelitis and cutaneous infections, caused by multidrug-resistant *A. baumannii* was reported.[7] The intrinsic abilities of this microbe to rapidly develop antimicrobial multiresistance and to survive long-term on dry surfaces have also been considered to play a crucial role in hospital-acquired infections.[8,9]

Biocides, including antiseptics and disinfectants, have been used extensively in hospitals and other healthcare settings for the sterilization of various medical devices and surfaces of nosocomial environments. In particular, disinfectants play an essential role in infection control and the prevention of nosocomial transmission of infectious microorganisms.[10] The benefits of the introduction of comprehensive disinfection on the reduction of healthcare-associated infections have been described,[11] although reduced susceptibility to biocides has been described

for various nosocomial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.[12,13] Unlike these pathogens, few studies have investigated the susceptibility to disinfectants in *Acinetobacter* spp.[14,15] More recently, we reported that repeated exposure to subinhibitory concentrations of chlorhexidine gluconate gradually elevated its MIC for *Acinetobacter* spp., especially for those isolates demonstrating reduced susceptibility to disinfectants.[16]

Together with the development of multiple antimicrobial resistance in nosocomial pathogens, correlations between the levels of multiple antimicrobial resistance and MICs of biocides have been investigated.[11,17] However, the available information about the linkage of resistance profiles to disinfectants and antimicrobial agents has so far been limited to a few bacterial species. Accordingly, presently existing data seem insufficient for the empirical application of disinfectants. *Acinetobacter* spp. have rapidly acquired resistance to multiple antimicrobial agents over the past 10 years, so greater understanding of the susceptibility to disinfectants among *Acinetobacter* spp. and information on the correlation between their susceptibility profiles to disinfectants and antimicrobials would contribute to the control of this microbe in both hospital and long-term healthcare settings.

Materials and methods

Bacterial strains

In March 2002, 283 non-repetitive clinical isolates identified as *Acinetobacter* spp. were collected from 97 hospitals located in different geographical areas of Japan. Since these isolates were speculated to be a probable causative microbe of infection in each patient, they were subjected to identification and antimicrobial susceptibility tests. They were biochemically identified as 273 *A. calcoaceticus-baumannii* complex, 7 *Acinetobacter lwoffii* and 3 *Acinetobacter junii* using the API20NE system (BioMérieux Japan, Tokyo, Japan) and a complementary test for their growth at 37, 41 and 44°C.

Disinfectants, antimicrobial agents and susceptibility tests

Disinfectants and antimicrobial agents were obtained from the following sources: chlorhexidine gluconate, benzethonium chloride, gentamicin, amikacin, acriflavine, tetracycline and ethidium bromide, Wako Pure Chemical Industries, Osaka, Japan; benzalkonium chloride, Kanto Chemical, Tokyo, Japan; alkyldiaminoethylglycine hydrochloride [TEGO 51TM, 10% (w/v) solution], Alfresa Pharma, Osaka, Japan; ceftazidime, GlaxoSmithKline, Tokyo, Japan; imipenem, Banyu Pharmaceutical, Tokyo, Japan; and ciprofloxacin, Bayer Pharmaceutical, Leverkusen, Germany. MICs of disinfectants and antimicrobial agents were determined by the agar dilution method, according to the protocol recommended by the CLSI (formerly NCCLS) in document M100-S14.[18] Appropriate dilutions of antimicrobial or disinfectant solutions were added to Mueller-Hinton agar (Becton Dickinson, Sparks, MD, USA) that had been allowed to equilibrate in a water bath to 50–55°C. The agar and antimicrobial or disinfectant solution were mixed thoroughly, and the mixture was poured into Petri dishes on a level surface to result in an agar depth of 3–4 mm. Each bacterial culture was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard ($\sim 1-9\times10^8$ cfu/mL for most species) and was then diluted 1:10 in sterile Mueller-Hinton broth (Becton Dickinson, Sparks, MD, USA). A 5 μL aliquot of each diluted bacterial suspension containing $\sim10^4$ cfu was spotted onto the agar surface using an inocula-replicating device [microplanter model MIT-P (Sakuma, Tokyo, Japan)] within 15 min of preparation and the plates were incubated at 35°C for 20 h. The MIC was recorded as the lowest concentration of the antimicrobial agent or disinfectant that completely inhibited growth, except for a single colony or a faint haze caused by the inoculum. *A. calcoaceticus* ATCC 33304 and 33305 were purchased from the ATCC, and served as the control strains in the antimicrobial susceptibility tests.

Assay of bactericidal activity: quantitative suspension test

The bactericidal effects of disinfectants on *Acinetobacter* spp. were measured using a slightly modified quantitative suspension test based on the protocol of European Standard EN 1040.[19] Although a 5 min contact time is required in EN 1040, we changed the contact time to a more challenging 3 min to evaluate subtle differences between disinfectant-susceptible isolates and the disinfectant reduced susceptibility (DRS) isolates, as observed in the Results section. Each isolate was cultivated in Luria-Bertani (LB) broth (Becton Dickinson) until its optical density (OD) reached 0.90 at 660 nm. After washing once with PBS (pH 7.4), the bacterial test suspensions were adjusted to an OD of 0.08 at 660 nm ($\sim10^8$ cfu/mL). A 100 μL test suspension was added to 900 μL of disinfectant solutions and this reaction mixture was left for 3 min at 20±2°C. One hundred microlitres of the reaction mixture was then added to 900 μL of neutralizer solution [10% Tween 80, 3% lecithin, 0.1% histidine, 0.5% sodium thiosulphate and PBS (pH 7.4)], kept at 20±2°C for 3 min and then serially diluted in PBS. After dilution, 50 μL of the mixture was spread immediately onto LB agar plates and incubated for 18 h at 35°C. The number of colonies growing on each plate was counted and cell survival rates were calculated with those obtained by a test using a bacterial suspension treated with PBS instead of disinfectant as the control. The experiments were repeated three times on different days. In addition, the possible toxicity of the neutralizer towards the test organisms and the inactivation of the bactericidal activity of each disinfectant by the neutralizer were also assessed, as described previously.[16]

Measurement of MBCs and time–kill assays

MBC measurements and time-kill assays were performed to evaluate the bactericidal effects of four disinfectants using the quantitative suspension test described above. The MBC was determined using the disinfectant solutions at different concentrations made by serial 2-fold dilutions of each disinfectant. The MBC was defined as the lowest disinfectant concentration that resulted in a 5 $\log_{10}$ reduction in the number of live bacterial cells for each disinfectant. Time-kill assays were performed at the lowest MIC of each disinfectant obtained by the agar dilution method, and exposure times to each disinfectant were 0, 10, 30, 60, 180, 300 and 600 s, respectively. Although dilution conditions employed for the MBC measurements and time-kill assays were usually selected in consideration of clinical practice, concentrations lower than the in-use concentrations of disinfectants were used in this study to evaluate the subtle differences between isolates with disinfectant-susceptible and reduced-susceptibility phenotypes. The experiments were repeated three times on different days.

Evaluation of bactericidal activity and statistical analyses

Data were analysed using the statistical program SPSS for Windows version 11.0J (SPSS Inc., Chicago, IL, USA). The correlation between MIC values of each disinfectant and the correlation between MICs of disinfectants and antimicrobial agents were determined using Spearman rank correlation. The Mann-Whitney *U*-test was performed to compare the bactericidal activities measured by both MBC and time-kill assays, as

described above, and to compare MIC distributions between isolates with both disinfectant susceptibility and DRS. A value of $P<0.05$ was considered to be statistically significant.

Results

Susceptibility to disinfectants

Distributions of MICs of chlorhexidine gluconate, benzalkonium chloride, benzethonium chloride and alkyldiaminoethylglycine hydrochloride for 283 clinical isolates are shown in Table 1. *Acinetobacter* spp. tended to be susceptible to both chlorhexidine gluconate and benzalkonium chloride, and the MIC_{90}s of these agents were 50 mg/L. However, the MICs of alkyldiaminoethylglycine hydrochloride were relatively higher than those of the other three disinfectants and the MIC_{90} of alkyldiaminoethylglycine hydrochloride was 400 mg/L. For several isolates, benzethonium chloride and alkyldiaminoethylglycine hydrochloride showed a high MIC (800 mg/L), which was approximately half of the in-use concentration [0.2% (w/v)=2000 mg/L]. Table 1 shows a summary of the in-use concentration stipulated in the manuals for hand disinfection and the sterilization of various medical devices.

Correlation between MIC values of disinfectants

The possible correlation between the MIC values was examined. MICs of benzalkonium chloride for each isolate were correlated with those of benzethonium chloride ($r=0.631$, $P<0.01$), because these two agents belong to the same class on the basis of chemical structure and mode of action. However, significant correlations were found between the MIC values of the other disinfectants for which the chemical structures and modes of action are dissimilar, e.g. benzalkonium chloride and chlorhexidine gluconate ($r=0.632$, $P<0.01$), benzethonium chloride and chlorhexidine gluconate ($r=0.425$, $P<0.01$), chlorhexidine gluconate and alkyldiaminoethylglycine hydrochloride ($r=0.643$, $P<0.01$), benzalkonium chloride and alkyldiaminoethylglycine hydrochloride ($r=0.657$, $P<0.01$) and benzethonium chloride and alkyldiaminoethylglycine hydrochloride ($r=0.520$, $P<0.01$).

Selection of isolates with reduced susceptibility to disinfectants

In the present study, the DRS isolates were defined as those for which the MICs of at least one among the four disinfectants were higher than the MIC_{90} when measured by the agar dilution method. As a result, 28 (9.9%) (19 *A. baumannii*, 5 *A. calcoaceticus*, 1 *A. lwoffii* and 3 *A. junii*) of 283 isolates were provisionally defined as DRS isolates in the present study (Table 2). Most of the DRS isolates tended to demonstrate reduced susceptibility to two or more disinfectants.

Bactericidal activity

To evaluate the difference between isolates with disinfectant-susceptible and reduced-susceptibility phenotypes, 20 isolates for which MICs of all four disinfectants were lower than the MIC_{50} were defined as 'disinfectant-susceptible isolates' and randomly selected as the control strains for the MBC measurements and time–kill assays. Figure 1 shows that MBC values (mg/L) of chlorhexidine gluconate, benzalkonium chloride, benzethonium chloride and alkyldiaminoethylglycine hydrochloride for the disinfectant-susceptible isolates and the DRS isolates were 16 and 32, 12 and 32, 16 and 32, and 16 and 64, respectively, and 2- or 4-fold differences were observed for the MBCs of each disinfectant between disinfectant-susceptible isolates and DRS isolates. Moreover, a statistical significance ($P<0.05$) was found between the disinfectant-susceptible isolates and DRS isolates in the reduction of bacterial cells after exposure to chlorhexidine gluconate (8 and 16 mg/L), benzalkonium chloride (4 and 8 mg/L), benzethonium chloride (8 and 16 mg/L) and alkyldiaminoethylglycine hydrochloride (16 and 32 mg/L) (Figure 1).

As shown in Figure 2, bacterial cells of the disinfectant-susceptible isolates showed significant (5 $\log_{10}$) reductions after 180 s of exposure to all four disinfectants. On the other hand, the live bacterial cells of the DRS isolates gradually decreased after exposure to chlorhexidine gluconate, benzalkonium chloride and benzethonium chloride in comparison with the disinfectant-susceptible isolates, and the exposure times

Table 1. Distributions of MICs (mg/L) of various disinfectants by the agar dilution method

Disinfectant	In-use concentration (mg/L)[a]	Number of strains with the MIC (mg/L) indicated									MIC_{50}[c]	MIC_{90}[c]
		2.5	5	10	25	50	100	200	400	800		
CHX	5000	0	0	**175**[b]	28	67	4	8	1	0	10	50
BZK	2000	0	0	**41**	**153**	76	6	7	0	0	25	50
BZT	2000	0	0	**10**	**12**	**122**	113	7	16	3	50	100
ADH	2000	0	0	0	0	**7**	**160**	72	40	4	100	400

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyldiaminoethylglycine hydrochloride.
[a]In-use concentrations stipulated in the manuals are shown. These concentrations are generally used for hand hygiene and the disinfection of non-critical items, including medical devices, in hospital and other healthcare settings.
[b]Bold numbers indicate groups of clinical isolates demonstrating the susceptible phenotype to each disinfectant. The 20 strains used for MBC measurements and time–kill assays were randomly selected from these groups.
[c]Standard MIC_{50} and MIC_{90} measurements (quantal measurement of 50% and 90% of the population) are given. Twenty-eight isolates for which the MICs of at least CHX, BZK, BZT and ADH were above the MIC_{90} were defined as isolates with DRS and selected as candidates for MBC measurements and time–kill assays.

Table 2. Susceptibility profiles of 28 isolates with DRS properties

	MIC (mg/L)										
	disinfectant[a]				antimicrobial agent[b]						
Strain no.	CHX	BZK	BZT	ADH	CAZ	CIP	AMK	IPM	GEN	TET	Species
1	**200**	50	**400**	400	2	0.13	2	0.25	2	1	*A. baumannii*
2	50	50	**200**	400	8	0.25	2	0.25	**128**	1	*A. baumannii*
3	10	50	**400**	200	4	0.25	4	0.5	1	2	*A. baumannii*
4	50	50	**200**	100	4	0.25	2	0.5	0.5	2	*A. baumannii*
5	**200**	**200**	**800**	200	0.5	0.13	1	0.13	0.5	1	*A. junii*
6	10	25	**200**	200	4	0.25	2	0.25	1	1	*A. baumannii*
7	**100**	50	**200**	**800**	8	**64**	2	0.5	**64**	**512**	*A. baumannii*
8	25	**100**	**400**	400	**32**	0.25	**64**	1	4	2	*A. baumannii*
9	50	50	100	**800**	8	**128**	4	0.5	**256**	**512**	*A. baumannii*
10	**200**	**200**	**400**	400	4	**64**	2	0.5	0.5	2	*A. baumannii*
11	25	**100**	**400**	400	**32**	0.5	**64**	1	2	2	*A. baumannii*
12	50	50	100	**800**	4	0.25	2	0.25	1	1	*A. baumannii*
13	**100**	**200**	**400**	200	16	1	**32**	1	**512**	4	*A. baumannii*
14	**200**	**200**	**800**	400	4	0.25	2	0.25	1	2	*A. calcoaceticus*
15	**100**	50	**400**	200	**256**	0.13	**64**	**32**	**128**	0.5	*A. baumannii*
16	**200**	50	**400**	400	8	0.25	2	0.5	2	2	*A. baumannii*
17	10	25	**200**	200	16	0.5	2	0.5	1	4	*A. baumannii*
18	**100**	50	**400**	100	**512**	**64**	**128**	**128**	**512**	4	*A. junii*
19	50	50	**200**	400	2	0.13	1	0.25	0.5	0.5	*A. calcoaceticus*
20	50	**100**	**400**	**800**	4	64	4	0.5	**64**	**512**	*A. baumannii*
21	**200**	**200**	**800**	400	**512**	0.25	4	**32**	8	1	*A. calcoaceticus*
22	10	50	**200**	200	16	**32**	2	1	0.5	8	*A. baumannii*
23	10	**100**	**400**	100	4	0.13	0.25	0.13	0.13	4	*A. lwoffii*
24	10	**200**	**400**	100	2	0.5	1	1	1	**32**	*A. junii*
25	50	**200**	**400**	200	8	0.25	2	0.25	0.5	1	*A. baumannii*
26	**400**	50	**400**	200	16	0.25	1	0.5	0.5	**128**	*A. baumannii*
27	**200**	**100**	**400**	200	**512**	**32**	**64**	**256**	**32**	2	*A. calcoaceticus*
28	**200**	**100**	**400**	200	**512**	**32**	**64**	**256**	**32**	2	*A. calcoaceticus*
ATCC 33304	10	25	50	100	2	0.13	0.25	0.13	0.25	0.5	
ATCC 33305	10	25	50	100	1	0.13	0.13	0.13	0.25	0.5	

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyldiaminoethylglycine hydrochloride; CAZ, ceftazidime; CIP, ciprofloxacin; AMK, amikacin; IPM, imipenem; GEN, gentamicin; TET, tetracycline.
[a]Bold numbers indicate MICs higher than the MIC_{90}.
[b]Bold numbers indicate MICs judged as resistant according to CLSI recommendation M100-S14.

needed for their complete killing were prolonged to ~600 s in the DRS isolates. Significant differences ($P<0.01$) were also observed between disinfectant-susceptible isolates and DRS isolates in the time-kill assays of chlorhexidine gluconate (10 mg/L, at 30–300 s), benzalkonium chloride (10 mg/L, at 30–300 s) and benzethonium chloride (10 mg/L, at 60–300 s). The bactericidal activity of alkyldiaminoethylglycine hydrochloride on the DRS isolates was similar to that on the disinfectant-susceptible isolates and their bacterial cells showed a 5 $\log_{10}$ reduction after 180 s of exposure to disinfectants (Figure 2d).

Susceptibility to antimicrobial agents and dyes

For all 283 clinical isolates, the MICs of six antimicrobial agents and two dyes were determined. For the 28 clinical DRS isolates, MIC distributions apparently shifted towards resistance for all antimicrobials and dyes tested in comparison with those for disinfectant-susceptible isolates, suggesting acquisition of a higher level of resistance to these agents in the DRS isolates than in the disinfectant-susceptible isolates ($P<0.05$) (Figure 3). Moreover, susceptibilities of 283 isolates of *Acinetobacter* spp. to amikacin, ceftazidime, ciprofloxacin and imipenem were categorized into 'susceptible' (amikacin, ≤16 mg/L; ceftazidime, ≤8 mg/L; ciprofloxacin, ≤1 mg/L; and imipenem, ≤4 mg/L), 'intermediate' (amikacin, 32 mg/L; ceftazidime, 16 mg/L; ciprofloxacin, 2 mg/L; and imipenem, 8 mg/L) and 'resistant' (amikacin, ≥64 mg/L; ceftazidime, ≥32 mg/L; ciprofloxacin, ≥4 mg/L; and imipenem, ≥16 mg/L) in accordance with CLSI recommendations, and correlation coefficients between MICs of disinfectants and four antimicrobial agents

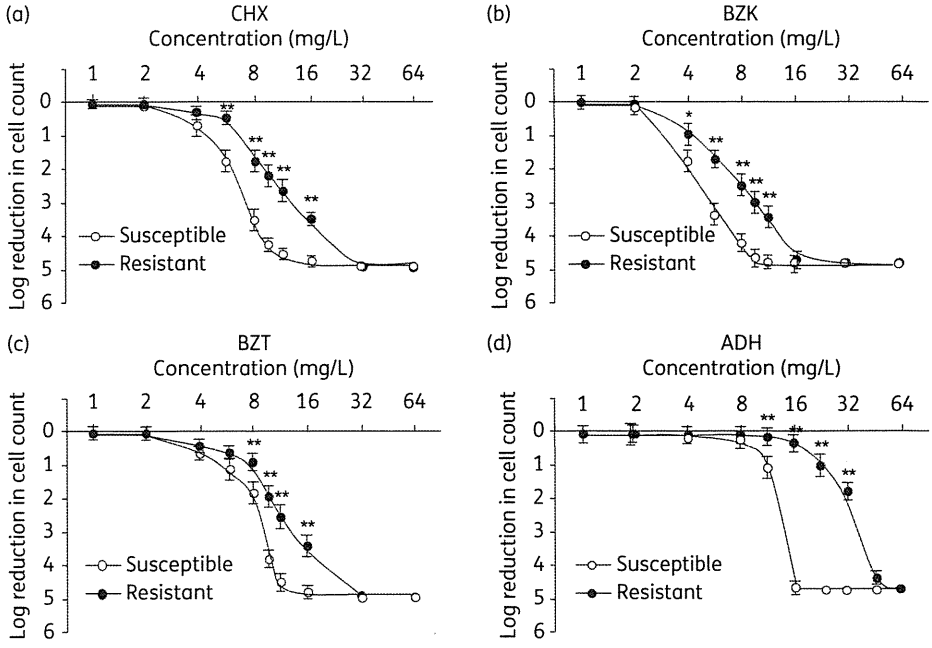

Figure 1. Comparison of MBCs. Twenty-eight isolates for which MICs of at least one disinfectant were more than the MIC_{90} measured by the agar dilution method were defined as isolates with DRS. MBCs were compared between the 28 DRS isolates and the 20 disinfectant-susceptible isolates. Results are expressed as the $\log_{10}$ reduction in cell counts compared with that of the control sample treated with PBS. Error bars represent standard deviations of results from three experiments. (a) Chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); and (d) alkyldiaminoethylglycine hydrochloride (ADH).

were determined. MICs of the four disinfectants were well correlated with those of three antimicrobial agents; ceftazidime, amikacin and ciprofloxacin ($P<0.05$) [Table 3 and Figure S1 (available at *JAC* Online (http://jac.oxfordjournals.org/)].

Discussion

Bacterial strains belonging to *Acinetobacter* spp. are very common environmental microbes growing in soil, compost and drainage, but they have become one of the critical nosocomial pathogens due to the recent increase in their isolation from clinical samples.[1,2] Extensive genotypic investigations on *Acinetobacter* spp. have elucidated that, within *A. baumannii*, clusters of genetically very similar lineages have been disseminated worldwide. Of these, the European (EU) clones I, II and III have been widely spread across Europe as well as several countries outside of Europe, and the majority of the multidrug-resistant strains belong to EU clones I and II.[20–22] The ability of specific clones such as EU clone II to acquire multidrug resistance would give them an advantage in antimicrobial-rich hospital environments and may have substantially facilitated their spread as nosocomial pathogens. Thus, the increasing frequency of infections caused by multidrug-resistant *A. baumannii* would accelerate the use of disinfectants as effective measures to prevent their inhabitation and transmission in healthcare settings. Many studies have focused on the antimicrobial resistance issues in this species;[23–25] however, there have been few studies on the possible acquisition or development of reduced susceptibilities to disinfectants. Hence, the present study, focused on the correlations of susceptibilities of *Acinetobacter* spp. to antimicrobials and disinfectants isolated in 2002, is meaningful given the potential shift in the susceptibility profiles of *Acinetobacter* spp. to both antimicrobials and disinfectants.

In general, the term 'at-use concentration' has been used as the basis to define disinfectant-tolerant strains, though disinfectants are used for a variety of purposes. Thus, the 'at-use concentrations' vary considerably depending on the purposes of disinfection. In the present study, we used 'in-use concentrations', considering the hand hygiene of healthcare workers, and the disinfection of various non-critical medical devices used in clinical and healthcare settings. At present, the emergence of bactericidal-resistant clinical isolates, as was observed for *Pseudomonas* spp.[26] that can withstand in-use concentrations of disinfectants, was not found for the *Acinetobacter* spp. isolated in 2002. More precisely, 283 non-repetitive clinically isolated *Acinetobacter* spp. were subjected to susceptibility tests for four disinfectants and the MIC_{90}s of the disinfectants were found to be ≤400 mg/L, lower than their in-use concentrations. These results are consistent with previous reports by Martró *et al.*[14] and Wisplinghoff *et al.*,[15] who found no apparent development of resistance to disinfectants among clinically isolated *Acinetobacter* spp. Indeed, no evident correlation was found, e.g. between the resistance profiles to antimicrobial agents and biocides for the nine *Acinetobacter* spp. that caused a sustained ICU outbreak in Spain.[14] However, the correlation between the resistance levels to antimicrobial agents and disinfectants observed in the present study differed from previous observations.[14,15] In the present study, the exposure times required

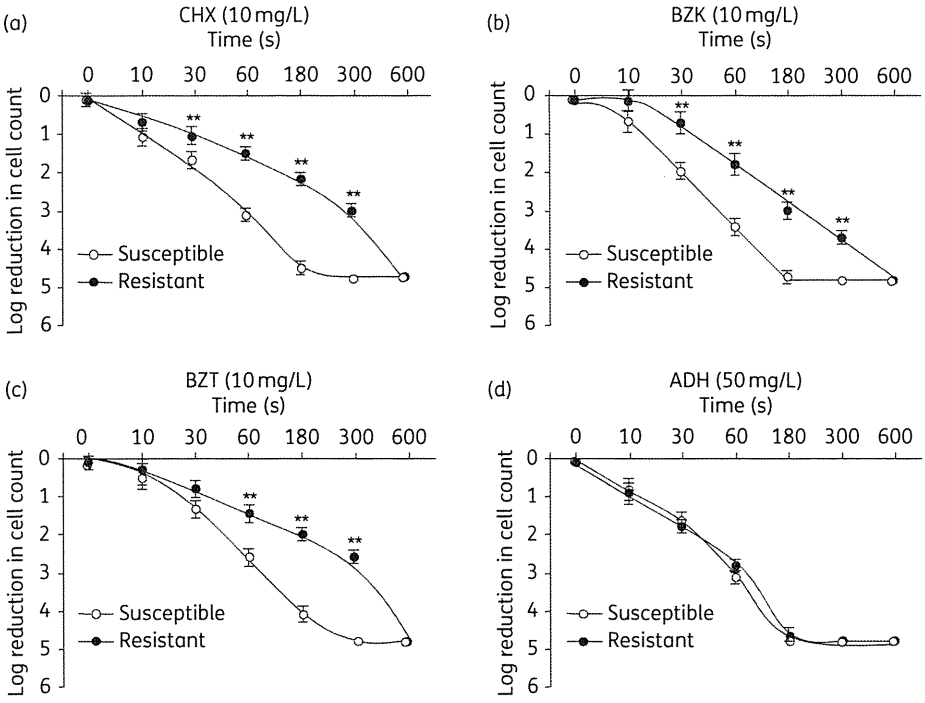

Figure 2. Results of time–kill assays. Time–kill assays were performed for the 28 DRS isolates selected in the same manner as for the MBC assay. Bacterial cells were treated with each disinfectant at 20 ± 2°C. Each bacterial test sample was removed at 10, 30, 60, 180, 300 and 600 s, respectively. Cell viabilities were determined by plating serially diluted cell suspensions on LB plates. Results are expressed as the $\log_{10}$ reduction in cell counts compared with that of the control sample treated with PBS. Error bars represent standard deviations of results from three experiments. (a) Chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); and (d) alkyldiaminoethylglycine hydrochloride (ADH).

for complete killing were significantly more prolonged in DRS isolates than in disinfectant-susceptible isolates, and the DRS isolates also demonstrated considerably higher resistance levels to ceftazidime, imipenem, ciprofloxacin and/or aminoglycosides. These findings may well suggest divergence in the mode of acquisition of reduced susceptibility to disinfectants and the development of multiple antimicrobial resistance in *Acinetobacter* spp. isolated on separate continents or in different regions worldwide.

Thus far, the positive linkage between bacterial resistance and the use of biocides has been suggested. Russell *et al.*[27] revealed that chlorhexidine gluconate resistance in *Pseudomonas stutzeri* correlated with resistance to polymyxin B, gentamicin, erythromycin and ampicillin. Similar observations were found for other nosocomial pathogens, such as MRSA and *P. aeruginosa*.[11,17,28,29] Our results showed no apparent correlations between specific disinfectants and antimicrobial agents, but our observations imply a trend towards overall cross-resistance between multiple antimicrobials and disinfectants among clinically isolated *Acinetobacter* spp. A hospital outbreak caused by a strain of *Proteus mirabilis* demonstrating resistance to several antimicrobial agents, including gentamicin as well as chlorhexidine gluconate, was reported.[30] Thus, the increased isolation of *Acinetobacter* spp. that had acquired multiple resistance to antimicrobials would be a good indicator for early recognition of the emergence of *Acinetobacter* DRS isolates in both acute and long-term healthcare settings.

As combined resistance mechanisms to antimicrobials and disinfectants, augmented efflux pump functions and changes in permeability of the bacterial outer membrane have been reported.[11,31] Although the antimicrobial resistance mechanisms of DRS isolates observed in the present study have not been well characterized, the recent rapid development of multiple antimicrobial resistance suggests the presence of potential common molecular mechanisms for augmented resistance levels to both disinfectants and antimicrobials. It seems difficult to conclude whether disinfectant exposure or antibiotic use is responsible for this phenomenon, but our data suggest the presence of a close genetic or mechanistic link between the antimicrobial resistance and DRS found among the clinical isolates.

In-use concentrations of disinfectants are much higher than their MICs and low-level resistance or DRS profiles have not been recognized as a possible clinical hazard to date. However, the clinical significance of emerging DRS *Acinetobacter* isolates deserves evaluation hereafter, given the close correlation with multiple antimicrobial resistance properties. Recent works report that carbapenem-resistant *A. baumannii* epidemic isolates belong to EU clonal complexes I or II and harbour $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-51-like}}$ or $bla_{\text{OXA-58-like}}$ genes that have spread worldwide.[32,33] Since the endemic *A. baumanii* strains isolated in Japanese clinical settings do not necessarily belong to the epidemic clonal complexes, susceptibility profiles to disinfectants among the global epidemic *A. baumannii* isolates belonging to the clonal complexes would be worth investigating, together

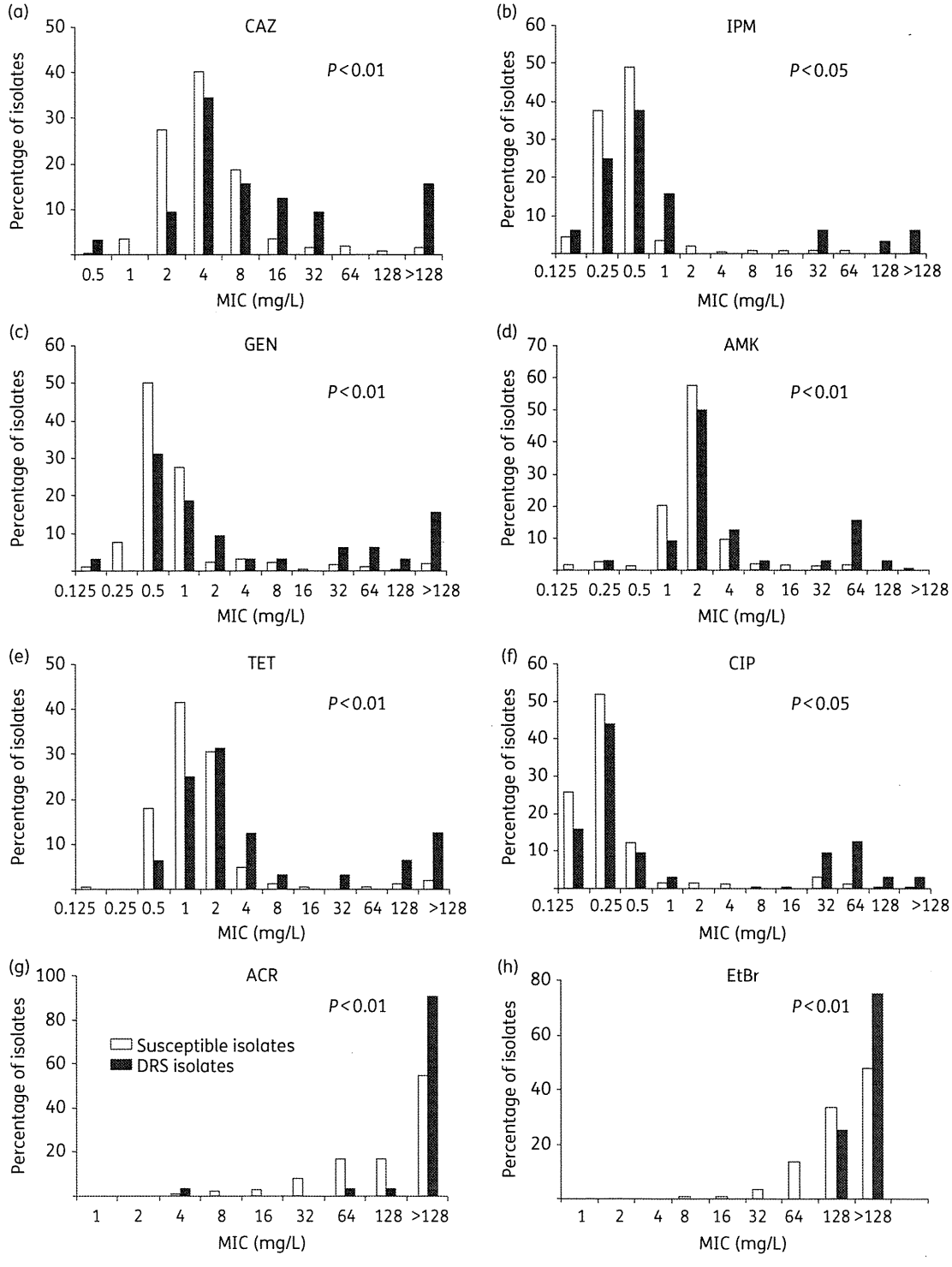

Figure 3. Comparison of MIC distributions between disinfectant-susceptible and DRS isolates. MICs of six antibiotics and two dyes for 283 clinical isolates of *Acinetobacter* spp. were compared between disinfectant-susceptible and DRS isolates. For the 28 clinical DRS isolates, MIC distributions apparently shifted to the right for all antimicrobials and dyes tested in comparison with those for disinfectant-susceptible isolates, suggesting acquisition of a higher level of resistance to these agents in the DRS isolates than in the disinfectant-susceptible isolates. White bars, MIC distributions for 255 disinfectant-susceptible isolates; black bars, MIC distributions for 28 DRS isolates. A significant difference between disinfectant-susceptible isolates and DRS isolates was indicated ($P<0.05$; as determined by Mann–Whitney *U*-test). CAZ, ceftazidime; IPM, imipenem; GEN, gentamicin; AMK, amikacin; TET, tetracycline; CIP, ciprofloxacin; ACR, acriflavin; EtBr, ethidium bromide.

Table 3. Correlation between MICs of disinfectants and antimicrobial agents

Antimicrobial agent	Category[a]	Number of strains	Median MIC (mg/L)	Spearman's correlation coefficient (*P* value)			
				CHX	BZK	BZT	ADH
CAZ	S	247	4	0.336 ($P<0.01$)	0.300 ($P<0.01$)	0.193 ($P<0.01$)	0.292 ($P<0.01$)
	I	13	16				
	R	23	64				
IPM	S	268	0.5	0.095 ($P=0.114$)	0.130 ($P<0.05$)	0.035 ($P=0.559$)	0.008 ($P=0.900$)
	I	2	8				
	R	13	32				
CIP	S	252	0.25	0.224 ($P<0.01$)	0.319 ($P<0.01$)	0.128 ($P<0.05$)	0.287 ($P<0.01$)
	I	4	2				
	R	27	32				
AMK	S	268	2	0.189 ($P<0.01$)	0.193 ($P<0.01$)	0.142 ($P<0.05$)	0.223 ($P<0.01$)
	I	4	32				
	R	11	64				

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyldiaminoethylglycine hydrochloride; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; AMK, amikacin.
[a]Susceptibilities of 283 isolates of *Acinetobacter* spp. to CAZ, IPM, CIP and AMK were categorized into susceptible (S), intermediate (I) and resistant (R) in accordance with CLSI criteria.

with their antimicrobial susceptibilities. Measures to prevent nosocomial infections caused by *A. baumannii* should include appropriate usage of disinfectants. Indeed, the DRS isolates tend to be more tolerant of a group of biocides, but enforcement of correct disinfection regimens would still be essential when multidrug-resistant *Acinetobacter* spp. are recurrently or continuously isolated in clinical settings despite enhanced performance of appropriate standard and contact precautions.

In conclusion, no apparent acquisition of resistance to disinfectants was observed in this time-dependent survey using the 283 strains of *Acinetobacter* spp. clinically isolated in Japan in 2002. About 10% of the isolates (28 strains) were found to demonstrate reduced susceptibility to disinfectants and these DRS isolates also tended to show resistances to various antimicrobial agents. Compared with the disinfectant-susceptible isolates using *in vitro* stepwise exposure including MBC measurements and time-kill assays, the DRS isolates tend to survive much longer in sub-MIC concentrations of several disinfectants. Thus, susceptibility to disinfectants must be carefully checked on a case-by-case basis if several multidrug-resistant *A. baumannii* are recurrently isolated from clinical specimens despite proper precautionary measures.

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Prevalence of Fosfomycin Resistance among CTX-M-Producing *Escherichia coli* Clinical Isolates in Japan and Identification of Novel Plasmid-Mediated Fosfomycin-Modifying Enzymes▽

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We evaluated the *in vitro* activity of fosfomycin against a total of 192 CTX-M β-lactamase-producing *Escherichia coli* strains isolated in 70 Japanese clinical settings. Most of the isolates (96.4%) were found to be susceptible to fosfomycin. On the other hand, some of the resistant isolates were confirmed to harbor the novel transferable fosfomycin resistance determinants named FosA3 and FosC2, which efficaciously inactivate fosfomycin through glutathione *S*-transferase activity.

Clinical efficacy of an old antibiotic, fosfomycin, is being reassessed owing to its *in vitro* high potent activity against multidrug-resistant Gram-negative bacilli belonging to the family *Enterobacteriaceae* (5, 6, 8). In the present study, we investigated the prevalence of fosfomycin resistance among CTX-M extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* clinical isolates in Japan and clarified the molecular mechanisms underlying the fosfomycin resistance, with special focus on exogenous resistance determinants, like the $FosA^{TN}$ protein (9).

A total of 192 CTX-M ESBL-producing *E. coli* isolates, which were collected from 70 medical facilities throughout Japan between 2002 and 2007, were retrospectively subjected to fosfomycin susceptibility testing with the agar dilution method according to the CLSI guideline (4). The result is shown in Fig. 1. Most of the strains (96.4%) investigated were susceptible to fosfomycin (MIC, ≤64 μg/ml), while seven isolates (3.6%) showed nonsusceptibility to fosfomycin (MIC, ≥128 μg/ml). It seems likely that CTX-M-producing *E. coli* isolates that have acquired fosfomycin resistance are infrequent in Japan, and these data suggest the probable clinical efficacy of fosfomycin for the treatment of infectious diseases, like urinary tract infections (UTIs), caused by CTX-M-producing *E. coli* to some extent.

We evaluated the fosfomycin resistance mechanism of the 10 isolates and found reduced susceptibility to fosfomycin (MIC, ≥64 μg/ml) (Fig. 1 and Table 1). The transmissibility of the fosfomycin resistance determinant in the 10 isolates was investigated, and it was found that the nature of fosfomycin resistance of three strains, 08-642, 06-607, and C316, was successfully transferred to a recipient *E. coli* strain. The cefotaxime resistance phenotype was cotransferred to a recipient strain with the fosfomycin resistance (Table 1).

The DNA fragments containing fosfomycin resistance determinants were cloned from the conjugative plasmids of *E. coli* 08-642, 06-607, and C316 strains and partially sequenced (Table 1). The fosfomycin resistance determinants and their genetic neighboring regions are shown in Fig. 2. The KpnI ca. 8-kb fragment cloned from the transferable plasmid of *E. coli* 08-642 and the SacII ca. 10-kb fragment from that of *E. coli* 06-607 included the same nucleotide region flanked by IS*26* (Fig. 2). The deduced amino acid sequences of one open reading frame (named *fosA3*) showed 70% identities to those of $FosA^{TN}$, the Mn(II)- and K^+-dependent glutathione (GSH) *S*-transferase from Tn*2921* of *Serratia marcescens* (2, 3) and 59% identities to that of $FosA^{PA}$ from *Pseudomonas aeruginosa* (Fig. 3) (1, 11). The *fosA3* gene is likely to be responsible for fosfomycin resistance in strains 08-642 and 06-607.

The 1.8-kb region containing *orf1* to Δ*orf3* at the 3′ end of *fosA3* had 78% nucleotide identity with a part of the chromosome sequence of *Klebsiella pneumoniae* strain 342 (Fig. 2) (7). Moreover, this 1.8-kb homology region on the chromosome of *K. pneumoniae* strain 342 was close to the *fosA* gene. FosA of *K. pneumoniae* strain 342 has 80% amino acid identity to the FosA3 found in the present study. Although the precise physiological function of chromosomally encoded FosA proteins of *K. pneumoniae* remains to be determined, it is speculated that

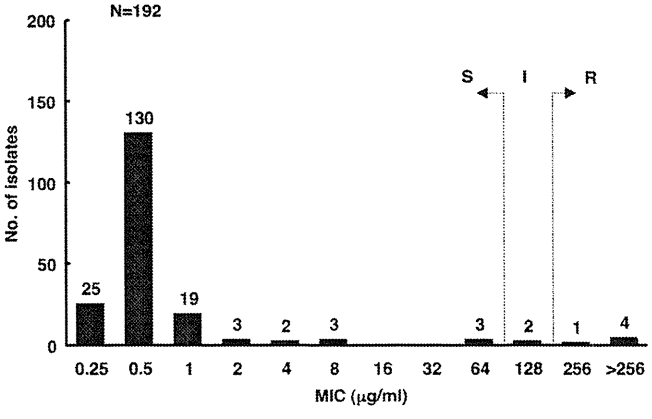

FIG. 1. Distribution of fosfomycin MICs for the 192 CTX-M-producing *E. coli* isolates.

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TABLE 1. Characteristics of *E. coli* strains used in the study

E. coli isolate	Characteristic(s) of *murA*, *uhpA*, *uhpT*, and/or *glpT* gene	MIC (μg/ml) of:	
		Fosfomycin	Cefotaxime
Clinical isolates			
08-555	*glpT* with $_{981}$[227-bp deletion]$_{1209}$	>256	>128
08-642		>256	64
06-607		>256	16
05-244	*glpT* with $_{328}$[14-bp duplication]$_{343}$, *uhpT* with $_{1173}$[96-bp deletion]$_{1270}$	128	>128
05-690	*uhpA* stop at amino acid 144	64	64
03-271	Failure in PCR amplification of *uhpA* and *uhpT*	64	32
03-285		128	>128
03-286	*glpT* with $_{405}$[5-bp duplication]$_{411}$	64	>128
03-287	*glpT* with $_{405}$[5-bp duplication]$_{411}$	>256	128
C316		256	128
CSH-2 conjugants			
CSH-2(p08-642)	Conjugant of strain 08-642	>256	128
CSH-2(p06-607)	Conjugant of strain 08-607	>256	32
CSH-2(pHPA)	Conjugant of strain C316	>256	64
CSH-2	Resistant to rifampin and nalidixic acid	1	≤0.06
DH10B transformants			
DH10B(pK-fosA3)	Contains KpnI fragment with *fosA3* from p08-642	>256	≤0.06
DH10B(pS-fosA3)	Contains SacII fragment with *fosA3* from p06-607	>256	≤0.06
DH10B(pS-fosC2)	Contains SacII fragment with *fosC2* from pHPA	>256	≤0.06
DH10B(pBCKS+)	Resistant to streptomycin and chloramphenicol	0.5	≤0.06

these proteins are the origin of a plasmid-mediated fosfomycin-modifying enzyme like FosA3.

Additionally, one open reading frame, named *fosC2*, was found in the fragment cloned from the conjugative plasmid of *E. coli* strain C316 (Table 1) (13). The amino acid sequence of FosC2 had 72%, 56%, and 51% identity to that of FosC found in *Achromobacter xylosoxidans* (GenBank accession no. DQ112222), FosA^{TN}, and FosA^{PA}, respectively (Fig. 3). The *fosC2* gene was the first gene cassette in a class 1 integron accompanied by *dfrA17* and *aadA5* (Fig. 2).

No transfer of fosfomycin resistance determinants was observed in the seven *E. coli* strains showing reduced susceptibility to fosfomycin (MIC, ≥64 μg/ml) (Table 1). Next, already-known chromosomally derived genes *glpT*, *uhpT*, *uhpA*, and *murA*, which are involved in fosfomycin resistance, were investigated (Table 1) (10, 12). The primers used in the present study are listed in Table 2. Several outcomes supposed to be involved in fosfomycin resistance were observed in six of the strains, but no remarkable change was detected in strain 03-285 among the investigated genes. Although the extent to which the fosfomycin resistance conferred by chromosomally encoded factors described above remains controversial, these factors would partially explain the fosfomycin resistance in the clinical isolates.

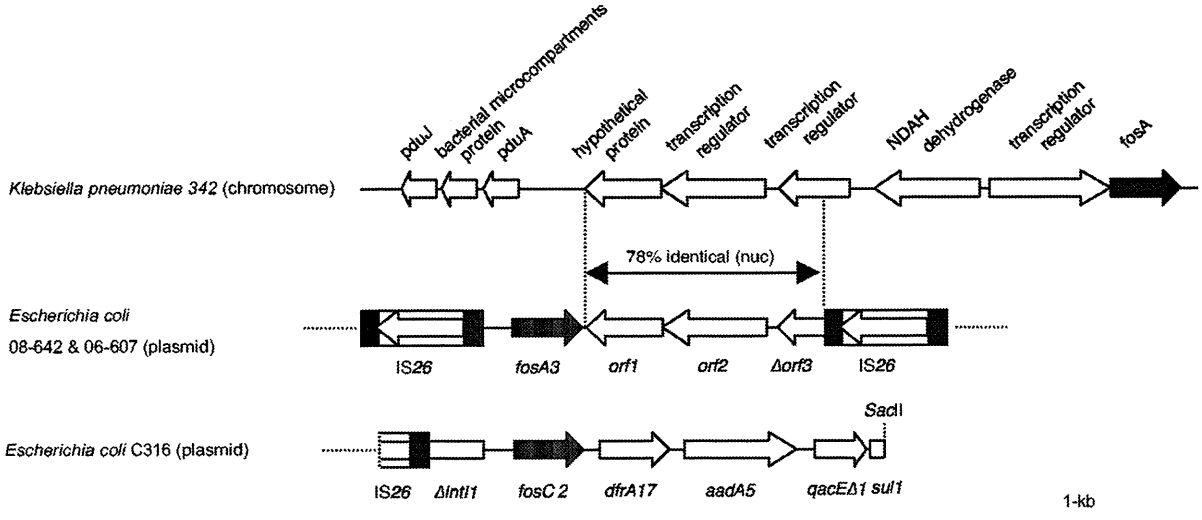

FIG. 2. Genetic environment of transferable fosfomycin resistance determinants and their neighboring regions in *E. coli* strains 08-624, 06-607, and C316.

FIG. 3. Predicted amino acid sequences of fosfomycin resistance determinants. *, amino acid residues conserved among the seven fosfomycin resistance determinants; colons and dots, amino acid substitutions that result in homologous amino acid residues. Proteins (GenBank accession no.): $FosA^{TN}$ (AAA98399); $FosA^{PA}$ (AAT49669); FosA2 (ACC85616); FosA3 (AB522970); ORF1 (AAP50248); FosC (AAZ14834); FosC2 (AB522969).

Finally, we purified C-terminal histidine-tagged FosA3 and FosC2 with the combination of a pET29a vector and *E. coli* BL21(DE3)(pLysS) and determined the enzymatic characteristics through a bioassay. Assays were performed in 50 mM HEPES buffer (pH 7.8) containing 100 mM KCl, 0.05 mM $MnCl_2$, 5 mM fosfomycin, 10 mM glutathione, and 10 μM purified protein in a final volume of 100 μl at 35°C for 30 min, and the reaction was quenched by adding methanol. Ten microliters of sample solution was added to a blank disc set on an agar plate inoculated with *E. coli* ATCC 25922, and the remaining antibacterial activity was measured as a growth inhibition zone. When a sample solution containing only fosfomycin and GSH was added, a 21-mm inhibitory zone was observed. When the same sample supplemented with FosA3 or FosC2 was added, no inhibitory zone was observed around the disc. No decrease in the growth inhibition zone was observed when the sample containing only fosfomycin and purified proteins were added. The consumption of GSH catalyzed by FosA3 and FosC2 was confirmed using Ellman's reagent. These results indicated that FosA3 and FosC2 inactivated fosfomycin by exerting GSH *S*-transferase activity, very similar to $FosA^{TN}$ and $FosA^{PA}$ (1, 3).

In conclusion, we report here the prevalence of fosfomycin resistance among CTX-M-producing *E. coli* isolates in Japan, together with the emergence of two novel plasmid-borne fosfomycin-modifying enzymes, FosA3 and FosC2. The fosfomycin resistance rate in CTX-M-producing *E. coli* is still low (3.6%) in Japan, but the fosfomycin resistance genes were already indwelling in the transferable plasmid of ESBL-producing clinical isolates. Continuous monitoring will be necessary to prevent further dissemination of fosfomycin resistance genes, together with prudent use of fosfomycin in clinical settings.

TABLE 2. Primers used in the study

Primer	Sequence
uhpT-forward	5′-ATG CTG GCT TTC TTA AAC C-3′
uhpT-reverse	5′-TTA TGC CAC TGT CAA CTG C-3′
uhpA-forward	5′-ATC ACC GTT GCC CTT ATA GA-3′
uhpA-reverse	5′-TCA CCA GCC ATC AAA CAT-3′
murA-forward	5′-CTC CAG GGC GAA GTC ACA-3′
murA-reverse	5′-GCC TTT CAC ACG CTC AAT A-3′
glpT-forward	5′-ATG TTG AGT ATT TTT AAA CC-3′
glpT-reverse	5′-TAG CCT CCG TTG CGT TTT TG-3′

Nucleotide sequence accession numbers. The nucleotide sequences for *fosA3* and *fosC2* have been deposited in GenBank under accession numbers AB522970 and AB522969, respectively.

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

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

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

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

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

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

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

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

To evaluate the sensitivity and specificity of the disk diffu-

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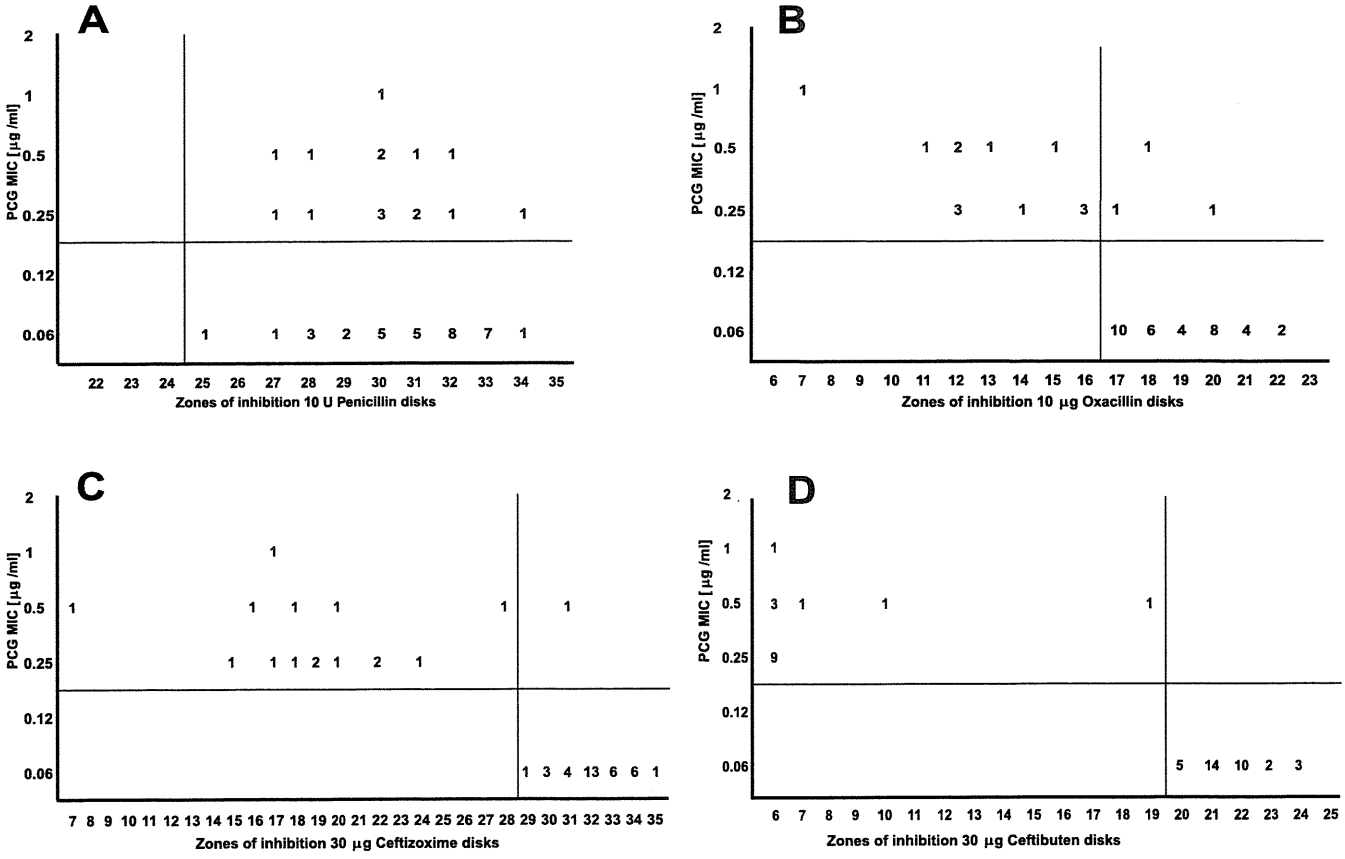

FIG. 1. Scatter diagram of MICs and the sizes of the growth-inhibitory zone. PCG MICs determined by the CLSI agar dilution method and diameters of the growth-inhibitory zone measured by CLSI-recommended standard disk diffusion method are plotted using a PCG disk (A). MICs determined by the agar dilution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

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

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

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

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

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

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

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

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

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

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

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