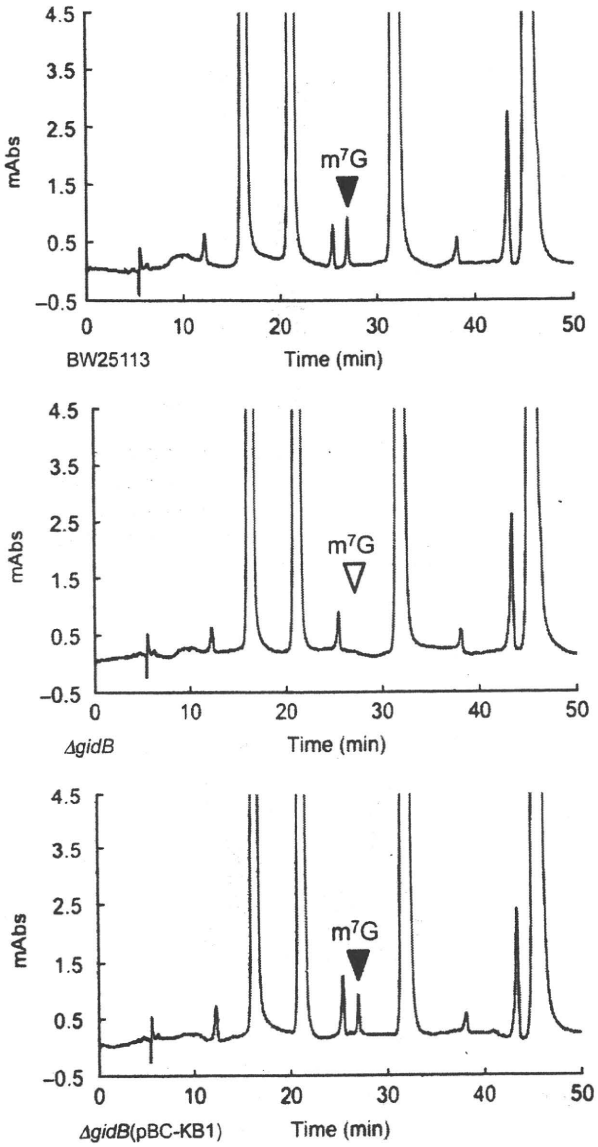

Fig. 4. HPLC analysis of methylated guanosine residue. BW25113, wild-type *Escherichia coli* 16S rRNA; Δ*gidB*, *E. coli* 16S rRNA lacking G527 methylation; Δ*gidB* (pBC-KB1), 16S rRNA of RmtC-producing *E. coli* lacking G527 methylation. mAb, milliabsorbance units.

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.

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References

Andersen NM & Douthwaite S (2006) YebU is a m5C methyltransferase specific for 16S rRNA nucleotide 1407. *J Mol Biol* **359**: 777–786.

Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY & Jiang HX (2007) Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *J Antimicrob Chemoth* **59**: 880–885.

Clinical and Laboratory Standards Institute (2009) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard, 8th ed. Document M07-A8*. CLSI, Wayne, PA.

Cubrilo S, Babic F, Douthwaite S & Vlahovicek GM (2009) The aminoglycoside resistance methyltransferase Sgm impedes RsmF methylation at an adjacent rRNA nucleotide in the ribosomal A site. *RNA* **15**: 1492–1497.

Davis MA, Baker KN, Orfe LH, Shah DH, Besser TE & Call DR (2010) Discovery of a gene conferring multiple-aminoglycoside resistance in *Escherichia coli*. *Antimicrob Agents Ch* **54**: 2666–2669.

Doi Y, Yokoyama K, Yamane K *et al.* (2004) Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. *Antimicrob Agents Ch* **48**: 491–496.

Doi Y, de Oliveira Garcia D, Adams J & Paterson DL (2007) Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. *Antimicrob Agents Ch* **51**: 852–856.

Fujimoto S & Ike Y (2001) pAM401-based shuttle vectors that enable overexpression of promoterless genes and one-step purification of tag fusion proteins directly from *Enterococcus faecalis*. *Appl Environ Microb* **67**: 1262–1267.

Galimand M, Courvalin P & Lambert T (2003) Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. *Antimicrob Agents Ch* **47**: 2565–2571.

Liou GF, Yoshizawa S, Courvalin P & Galimand M (2006) Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. *J Mol Biol* **359**: 358–364.

Okamoto S, Tamaru A, Nakajima C *et al.* (2007) Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol Microbiol* **63**: 1096–1106.

Perichon B, Courvalin P & Galimand M (2007) Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. *Antimicrob Agents Ch* **51**: 2464–2469.

Savic M, Tomic TI, Macmaster R, Vasiljevic B & Conn GL (2008) Critical residues for cofactor binding and catalytic activity in the aminoglycoside resistance methyltransferase Sgm. *J Bacteriol* **190**: 5855–5861.

Savic M, Lovric J, Tomic TI, Vasiljevic B & Conn GL (2009) Determination of the target nucleosides for members of two families of 16S rRNA methyltransferases that confer resistance to partially overlapping groups of aminoglycoside antibiotics. *Nucleic Acids Res* **37**: 5420–5431.

Schmitt E, Galimand M, Panvert M, Courvalin P & Mechulam Y (2009) Structural bases for 16S rRNA methylation catalyzed by ArmA and RmtB methyltransferases. *J Mol Biol* **388**: 570–582.

Wachino J, Yamane K, Shibayama K *et al.* (2006) Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. *Antimicrob Agents Ch* **50**: 178–184.

Wachino J, Shibayama K, Kurokawa H *et al.* (2007) Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated *Escherichia coli* strain resistant to structurally diverse aminoglycosides. *Antimicrob Agents Ch* **51**: 4401–4409.

Yamane K, Wachino J, Suzuki S *et al.* (2007) 16S rRNA methylase-producing, gram-negative pathogens, Japan. *Emerg Infect Dis* **13**: 642–646.

Yokoyama K, Doi Y, Yamane K *et al.* (2003) Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet* **362**: 1888–1893.

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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 (~$1–9\times10^8$ 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 ~10^4 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 (~10^8 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

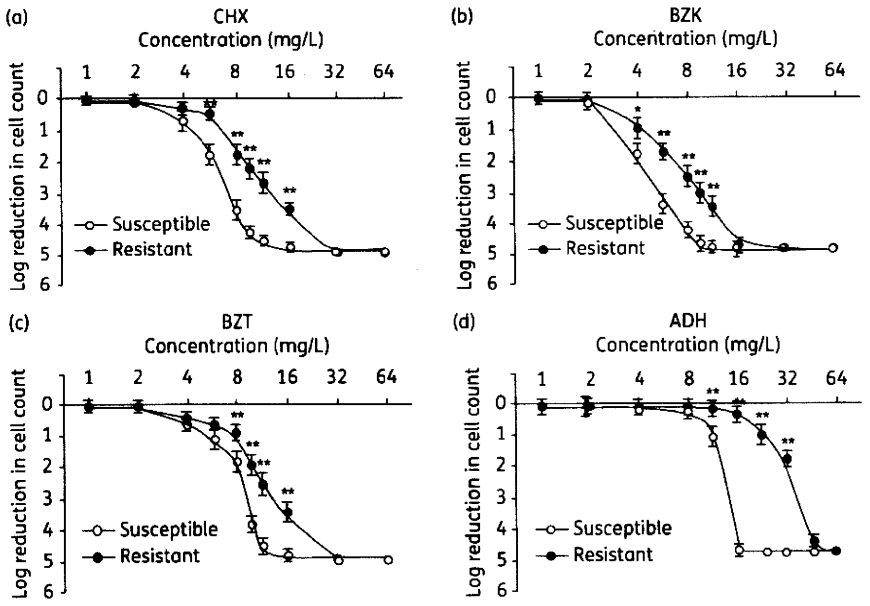

Figure 1. Comparison of MBCs. Twenty-eight isolates for which MICs of at least one disinfectant were more than the MIC_{90} measured by the agar dilution method were defined as isolates with DRS. MBCs were compared between the 28 DRS isolates and the 20 disinfectant-susceptible isolates. Results are 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 $\leq$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

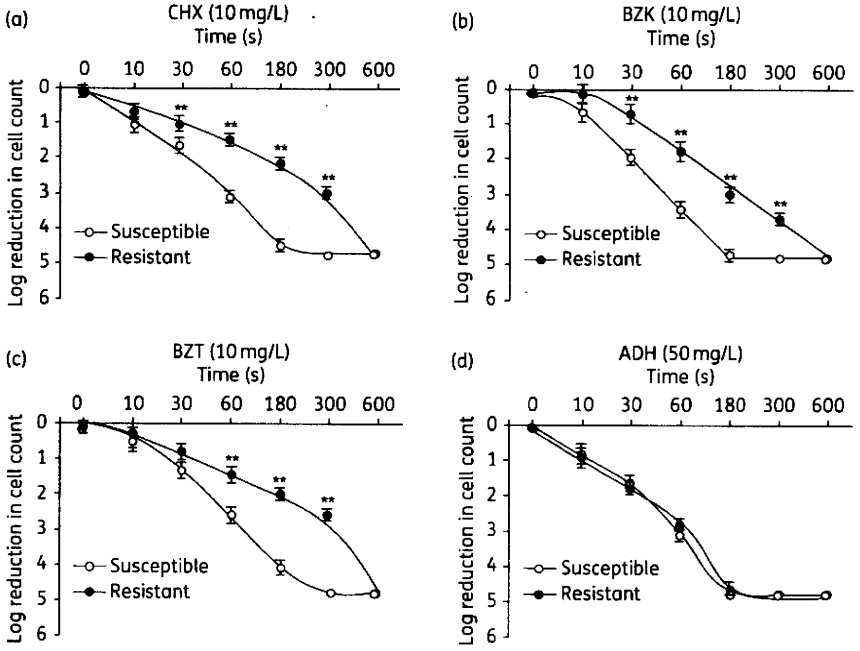

Figure 2. Results of time-kill assays. Time-kill assays were performed for the 28 DRS isolates selected in the same manner as for the MBC assay. Bacterial cells were treated with each disinfectant at 20±2 C. Each bacterial test sample was removed at 10, 30, 60, 180, 300 and 600 s, respectively. Cell 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

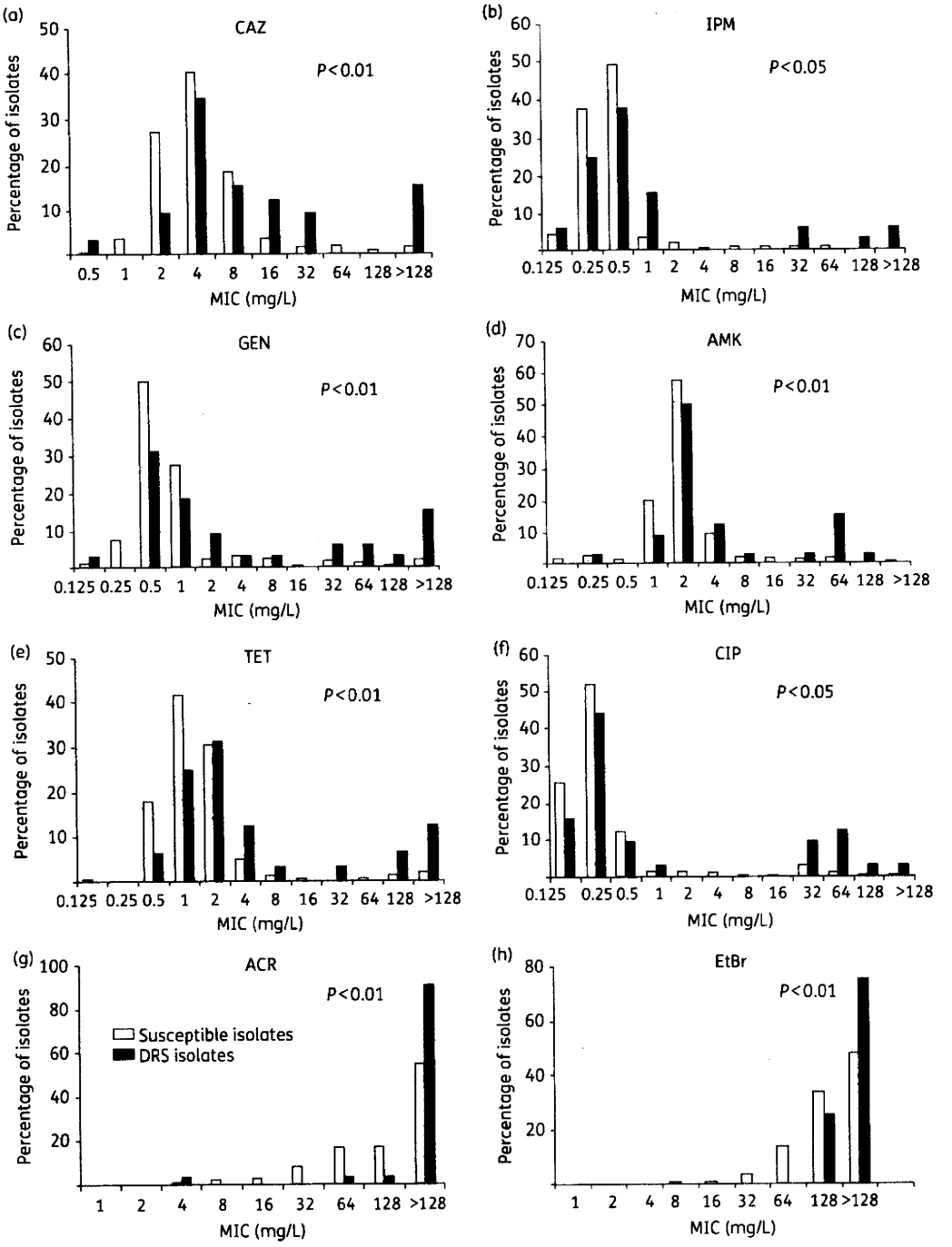

Figure 3. Comparison of MIC distributions between disinfectant-susceptible and DRS isolates. MICs of six antibiotics and two dyes for 283 clinical isolates of *Acinetobacter* spp. were compared between disinfectant-susceptible and DRS isolates. For the 28 clinical DRS isolates, MIC distributions apparently 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/).

References

1 Bergogne-Bérézin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical and epidemiological features. *Clin Microbiol Rev* 1996; **9**: 148–65.

2 Munoz-Price LS, Robert AW. *Acinetobacter* infection. *N Engl J Med* 2008; **358**: 1271–81.

3 Paterson DL. The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clin Infect Dis* 2006; **43** Suppl 2: S43–8.

4 Pournaras S, Markogiannakis A, Ikonomidis A *et al.* Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit. *J Antimicrob Chemother* 2006; **57**: 557–61.

5 de Vries J, Wackernagel W. Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proc Natl Acad Sci USA* 2002; **99**: 2094–9.

6 Nielsen KM, van Weerelt MDM, Berg TN *et al.* Natural transformation and availability of transforming DNA to *Acinetobacter calcoaceticus* in soil microcosms. *Appl Environ Microbiol* 1997; **63**: 1945–52.

7 Davis KA, Moran KA, McAllister CK *et al.* Multidrug-resistant *Acinetobacter* extremity infections in soldiers. *Emerg Infect Dis* 2005; **11**: 1218–24.

8 Webster C, Towner KJ, Humphreys H. Survival of *Acinetobacter* on three clinically related inanimate surfaces. *Infect Control Hosp Epidemiol* 2000; **21**: 246.

9 Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 2006; **6**: 130.

10 Rutala WA. APIC guidelines for selection and use of disinfectants. *Am J Infect Control* 1996; **24**: 313–42.

11 Makris AT, Morgan L, Gaber DJ *et al.* Effect of a comprehensive infection control program on the incidence of infections in long-term care facilities. *Am J Infect Control* 2000; **28**: 3–7.

12 Russell AD. Bacterial resistance to disinfectants: present knowledge and future problems. *J Hosp Infect* 1999; **43** Suppl: S57–68.

13 Block C, Furman M. Association between intensity of chlorhexidine use and micro-organisms of reduced susceptibility in a hospital environment. *J Hosp Infect* 2002; **51**: 201–6.

14 Martro E, Hernández A, Ariza J *et al.* Assessment of *Acinetobacter baumannii* susceptibility to antiseptics and disinfectants. *J Hosp Infect* 2003; **55**: 39–46.

15 Wisplinghoff H, Schmitt R, Wöhrmann A *et al.* Resistance to disinfectants in epidemiologically defined clinical isolates of *Acinetobacter baumannii*. *J Hosp Infect* 2007; **66**: 174–81.

16 Kawamura-Sato K, Wachino J, Kondo T *et al.* Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material. *J Antimicrob Chemother* 2008; **61**: 568–76.

17 Kõljalg S, Naaber P, Mikelssar M. Antibiotic resistance as an indicator of bacterial chlorhexidine susceptibility. *J Hosp Infect* 2002; **51**: 106–13.

18 National Committee for Clinical Laboratory Standards. *Performance Standards for Antimicrobial Susceptibility Testing: Fourteenth Informational Supplement M100-S14*. NCCLS, Wayne, PA, USA, 2004.

19 European Committee for Standardization. *European Standard EN 1040: Chemical Disinfectants and Antiseptics. Basic Bactericidal Activity. Test Method and Requirements (Phase 1)*. Brussels, Belgium: CEN, 1997.

20 Dijkshoorn L, Nemec A, Seifert H. An increasing threat in the hospital: multidrug- resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* 2007; **5**: 939–51.

21 Nemec A, Dijkshoorm L, van der Reijden TJK. Long-term predominance of two pan-European clones among multi-resistant *Acinetobacter baumannii* strains in the Czech Republic. *J Med Microbiol* 2004; **53**: 147–53.

22 Nemec A, Krizová L, Maixnerová M *et al.* Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant strains of European clone II. *J Antimicrob Chemother* 2008; **62**: 484–9.

23 Nagano N, Nagano Y, Cordevant C *et al.* Nosocomial transmission of CTX-M-3 β-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. *J Clin Microbiol* 2004; **42**: 3978–84.

24 Doi Y, Wachino J, Yamane K *et al.* Spread of novel aminoglycoside resistance gene *aac(6′)-Iad* among *Acinetobacter* clinical isolates in Japan. *Antimicrob Agents Chemother* 2004; **48**: 2075–80.

25 Paul M, Weinberger M, Siegman-Igra Y *et al. Acinetobacter baumannii*: emergence and spread in Israel hospitals 1997–2002. *J Hosp Infect* 2005; **60**: 256–60.

26 Nagai K, Murate T, Ohta S *et al.* Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a *Pseudomonas fluorescence* strain. *Microbiol Immunol* 2003; **47**: 709–15.

27 Russell AD, Tattawasart U, Maillard J-Y *et al.* Possible link between bacterial resistance and use of antibiotics and biocides. *Antimicrob Agents Chemother* 1998; **42**: 2151.

28 Kampf G, Jarosch R, Rüden H. Limited effectiveness of chlorhexidine based hand disinfectants against methicillin-resistant *Staphylococcus aureus* (MRSA). *J Hosp Infect* 1998; **38**: 297–303.

29 Fraise AP. Susceptibility of antibiotic-resistant cocci to biocides. *J Appl Microbiol Symp Suppl* 2002; **92**: 158S–62S.

30 Dance DAB, Pearson AD, Seal DV *et al.* A hospital outbreak caused by chlorhexidine and antibiotic-resistant *Proteus mirabilis*. *J Hosp Infect* 1987; **10**: 10–6.

31 Russell AD. Do biocides select for antibiotic resistance? *J Pharm Pharmacol* 2000; **52**: 227–33.

32 Turton JF, Gabriel SN, Valderrey C *et al.* Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007; **13**: 807–15.

33 Adams-Haduch JM, Paterson DL, Sidjabat HE *et al.* Genetic basis of multidrug resistance in *Acinetobacter baumannii* clinical isolates at a tertiary medical center in Pennsylvania. *Antimicrob Agents Chemother* 2008; **52**: 3837–43.

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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

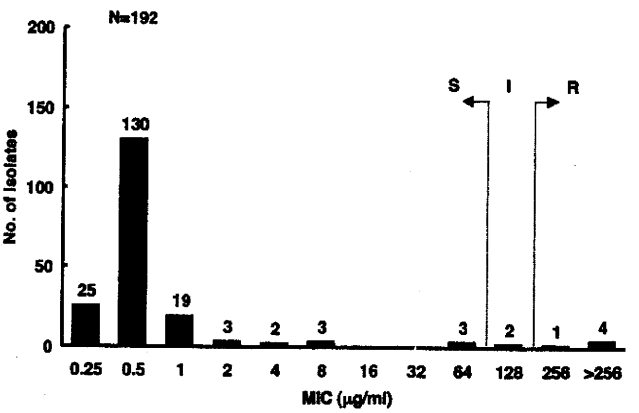

FIG. 1. Distribution of fosfomycin MICs for the 192 CTX-M-producing *E. coli* isolates.

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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.

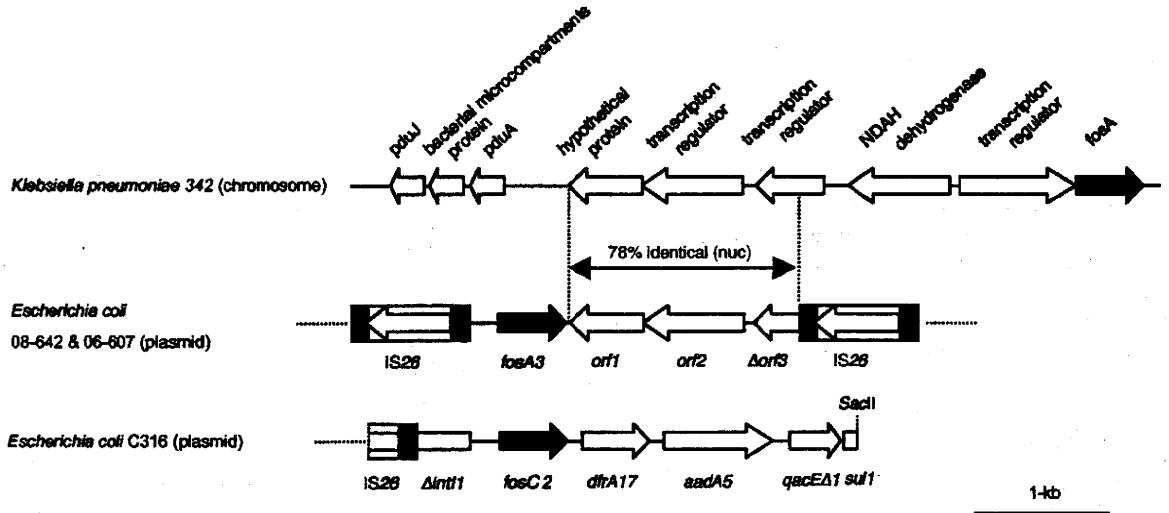

FIG. 2. Genetic environment of transferable fosfomycin resistance determinants and their neighboring regions in *E. coli* strains 08-624, 06-607, and C316.

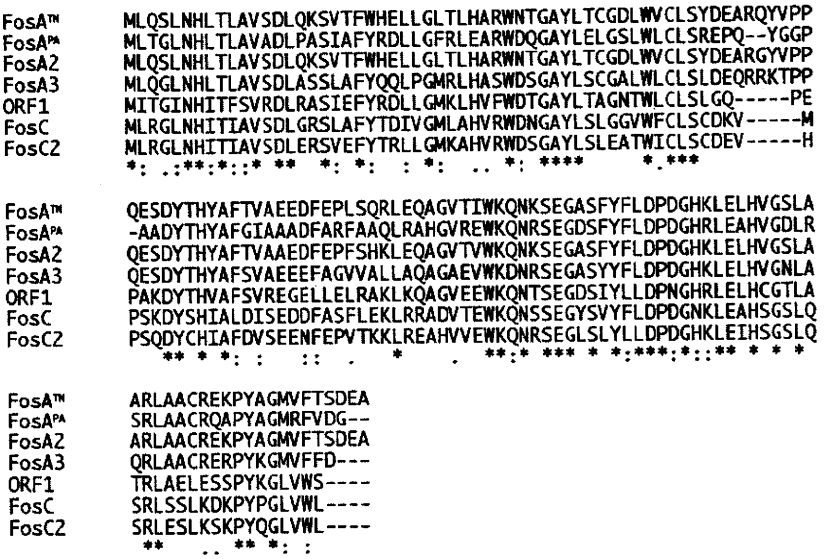

FIG. 3. Predicted amino acid sequences of fosfomycin resistance determinants. *, amino acid residues conserved among the seven fosfomycin resistance determinants; colons and dots, amino acid substitutions that result in homologous amino acid residues. Proteins (GenBank accession no.): $FosA^{TN}$ (AAA98399); $FosA^{PA}$ (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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REFERENCES

1. **Beharry, Z., and T. Palzkill.** 2005. Functional analysis of active site residues of the fosfomycin resistance enzyme FosA from *Pseudomonas aeruginosa*. J. Biol. Chem. **280:**17786–17791.
2. **Bernat, B. A., L. T. Laughlin, and R. N. Armstrong.** 1999. Elucidation of a monovalent cation dependence and characterization of the divalent cation binding site of the fosfomycin resistance protein (FosA). Biochemistry **38:** 7462–7469.
3. **Bernat, B. A., L. T. Laughlin, and R. N. Armstrong.** 1997. Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. Biochemistry **36:**3050–3055.
4. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 8th ed. Document M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Endimiani, A., G. Patel, K. M. Hujer, M. Swaminathan, F. Perez, L. B. Rice, M. R. Jacobs, and R. A. Bonomo.** 2009. In vitro activity of fosfomycin against bla_{KPC}-containing *Klebsiella pneumoniae* isolates, including those nonsusceptible to tigecycline and/or colistin. Antimicrob. Agents Chemother. **54:**526–529.
6. **Falagas, M. E., A. C. Kastoris, A. M. Kapaskelis, and D. E. Karageorgopoulos.** 2010. Fosfomycin for the treatment of multidrug-resistant, including

extended-spectrum β-lactamase producing, *Enterobacteriaceae* infections: a systematic review. Lancet Infect. Dis. **10**:43–50.

7. **Fouts, D. E., H. L. Tyler, R. T. DeBoy, S. Daugherty, Q. Ren, J. H. Badger, A. S. Durkin, H. Huot, S. Shrivastava, S. Kothari, R. J. Dodson, Y. Mohamoud, H. Khouri, L. F. Roesch, K. A. Krogfelt, C. Struve, E. W. Triplett, and B. A. Methe.** 2008. Complete genome sequence of the N2-fixing broad host range endophyte Klebsiella pneumoniae 342 and virulence predictions verified in mice. PLoS Genet. **4**:e1000141.
8. **Giamarellou, H., and G. Poulakou.** 2009. Multidrug-resistant Gram-negative infections: what are the treatment options? Drugs **69**:1879–1901.
9. **Mendoza, C., J. M. Garcia, J. Llaneza, F. J. Mendez, C. Hardisson, and J. M. Ortiz.** 1980. Plasmid-determined resistance to fosfomycin in *Serratia marcescens*. Antimicrob. Agents Chemother. **18**:215–219.
10. **Nilsson, A. I., O. G. Berg, O. Aspevall, G. Kahlmeter, and D. I. Andersson.** 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **47**:2850–2858.
11. **Rife, C. L., R. E. Pharris, M. E. Newcomer, and R. N. Armstrong.** 2002. Crystal structure of a genomically encoded fosfomycin resistance protein (FosA) at 1.19 Å resolution by MAD phasing off the L-III edge of Tl(+). J. Am. Chem. Soc. **124**:11001–11003.
12. **Takahata, S., T. Ida, T. Hiraishi, S. Sakakibara, K. Maebashi, S. Terada, T. Muratani, T. Matsumoto, C. Nakahama, and K. Tomono.** 2010. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*. Int. J. Antimicrob. Agents **35**:333–337.
13. **Yamane, K., J. Wachino, S. Suzuki, K. Kimura, N. Shibata, H. Kato, K. Shibayama, T. Konda, and Y. Arakawa.** 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **51**:3354–3360.

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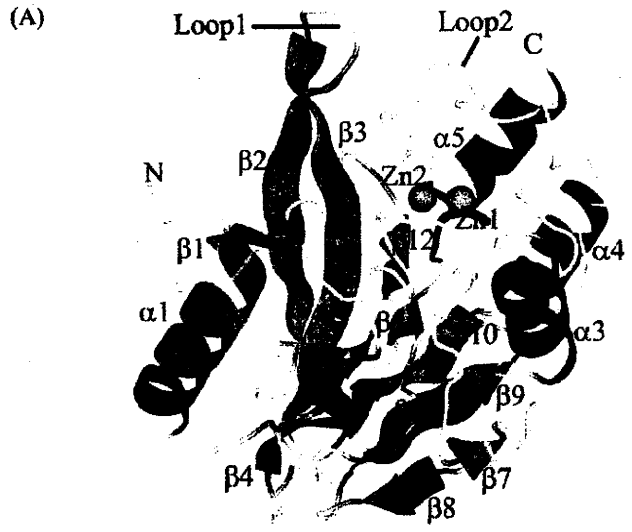

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Structure of metallo-β-lactamase IND-7 from a *Chryseobacterium indologenes* clinical isolate at 1.65-Å resolution

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The X-ray crystal structure of metallo-β-lactamase from *Chryseobacterium indologenes* IND-7 was determined at a resolution of 1.65 Å. The overall structure adopted a four-layered αβ/βα sandwich structure with a dinuclear zinc(II) active site, in which the zinc(II) ions were denoted as Zn1 and Zn2. The overall structure of IND-7 is analogous to those of subclass B1 metallo-β-lactamases, as determined by X-ray crystallography. A significant structural difference, however, was observed in the dinuclear zinc(II) active site: the coordination geometry around Zn1 changed from tetrahedral, found in other subclass B1 metallo-β-lactamases, to distorted trigonal bipyramidal, whereas that of Zn2 changed from trigonal bipyramidal to tetrahedral. Arg121(101), which is located in the vicinity of the dinuclear zinc(II) active site, may affect the binding affinity of Zn2 due to an electronic repulsion between the zinc(II) ion(s) and a positively charged guanidyl group of Arg121(101). Moreover, the hydrogen-bonding interaction of Arg121 with Ser71(53), which is conserved in IND-1, IND-3 and IND-5–IND-7, appeared to have important consequences for the binding affinity of Zn2 in conjunction with the above electrostatic effect.

Keywords: β-lactams/metallo-β-lactamase/crystal structure/X-ray crystallography/zinc(II).

Abbreviations: BBL, class B β-lactamase; BcII, metallo-β-lactamase from *Bacillus cereus*; BlaB, metallo-β-lactamase from *Cryseobacterium meningosepticum*; CcrA, metallo-β-lactamase from *Bacteroides fragilis*; GIM-1, metallo-β-lactamase from *Pseudomonas aeruginosa*; IMP-1, metallo-β-lactamase from *Serratia marcescens*; IND-1, IND-2, IND-2a, IND-3, IND-4, IND-5, IND-6 and IND-7; metallo-β-lactamases from *Chryseobacterium indologenes*; PDB, Protein Data Bank; SIM-1, metallo-β-lactamase from *Acinetobacter baumannii*; SPM-1, metallo-β-lactamase from *Pseudomonas aeruginosa*; VIM-2, metallo-β-lactamase from *Pseudomonas aeruginosa*.

Metallo-β-lactamases are zinc(II)-dependent enzymes that catalyze the hydrolysis of the amide bond in most β-lactams, including carbapenems, and are associated with one of the prevalent mechanisms of bacterial resistance against β-lactams (*1–3*). Metallo-β-lactamases (referred to as class B β-lactamases) are grouped into the molecular subclasses B1 – B3 based on their primary amino acid sequence (*4–6*). Subclasses B1 and B2 metallo-β-lactamases have the amino acid sequences, H*X*H*X*D and N*X*H*X*D, respectively. Subclass B3 metallo-β-lactamases have the amino acid sequence H(Q)*X*H*X*DH. Subclasses B1 and B3 metallo-β-lactamases are able to bind up to two zinc(II) ions (*7–10*) and can hydrolyze a wide range of β-lactams including penicillins, cefalosporins and carbapenems (*11*). On the other hand, subclass B2 metallo-β-lactamases are mononuclear zinc(II) enzymes, which exhibit specificity toward carbapenems (*11–17*).

Among them, subclass B1 metallo-β-lactamases are gaining popularity worldwide, and their genes are encoded either on the bacterial chromosome or on mobile genetic elements such as plasmids and transposons (*6*, *18–20*). For instance, chromosomally encoded metallo-β-lactamases include BcII from *Bacillus cereus* (*21*), BlaB from *Chryseobacterium meningosepticum* (*22*), CcrA from *Bacteroides fragilis* (*23*) and IND-1 from *Chryseobacterium indologenes* (*24*), whereas IMP-1 from *Serratia marcescens* (*25*), VIM-2 from *Pseudomonas aeruginosa* (*18*), SPM-1 from *P. aeruginosa* (*26*), SIM-1 from *Acinetobacter*

baumannii, and GIM-1 from *P. aeruginosa* (*27*) are encoded by mobile genes on a plasmid. So far, the three-dimensional structures of the subclass B1 metallo-β-lactamases have been solved for BcII (*7*; *28–30*), BlaB (*31*), CcrA (*32–34*), IMP-1 (*35*, *36*), VIM-2 (*37*, *38*) and SPM-1 (*10*).

In 1999, the chromosome-encoded *C. indologenes* metallo-β-lactamase IND-1 was first isolated (*24*), and six IND variants, designated IND-2 to IND-6 and IND-2a, have been detected to date (*39–41*). *Chryseobacterium indologenes* are aetiological agents of infection associated with the use of indwelling devices (*42*). The amino acid sequences of the above-mentioned six IND variants share 72–92% identity with that of IND-1 (Fig. 1). Of these enzymes, kinetic parameters are available for IND-1, IND-2, IND-5 and IND-6, though in the case of IND-1, only a comparison of the K_m values is available (*24*, *39–41*).

In 2005, Arakawa *et al.* isolated a chromosomal metallo-β-lactamase produced by a *C. indologenes* clinical isolate from the urinary tract infection of a patient in Japan (MRY040066 strain). Based on a comparison of the amino acid sequence of metallo-β-lactamase from *C. indologenes* MRY040066 (denoted as IND-7) with that of IND-1, IND-7 identified most with IND-1 (99%) where an isoleucine at position 90(72) in IND-1 was replaced by a valine in IND-7. (In this paper, the amino acid residues are designated by a BBL numbering (*4*), and the amino acid sequence number from the N-terminus for mature protein—the latter is in parenthesis.)

The aim of this study is to fully characterize the three-dimensional structure of IND-7 from *C. indologenes*, because the X-ray crystal structures of IND variants are not available. We report here the crystal structure of metallo-β-lactamase IND-7 at a resolution of 1.65 Å.

Materials and Methods

Cloning

The bla_{IND-7} gene was amplified with the primers IND-F (5′-CAT ATG AAA AAA AGC ATC CGT TTT-3′), which have an NdeI linker (underlined), and IND-R (5′-GGA TCC CTA TTT TTT ATT CAG AAG TT-3′), which have a BamHI linker (underlined), using an Expand High-Fidelity PCR system (Roche). The template DNA was extracted from the clinically isolated *C. indologenes* strain MRY040066. The amplicon was ligated with a pGEM-T vector (Promega), and was partially excised by digestion with NdeI and BamHI. The excised fragment was subcloned into the expression vector pET29a(+) (Novagen) to yield plasmid pET29a(+)/bla_{IND-7}.

Expression and purification

A 25-ml overnight preculture of *Escherichia coli* BL21(DE3), which was transformed by plasmid pET29a(+)/bla_{IND-7}, was used to inoculate 10 l of LB broth supplemented with 50 μg/ml kanamycin, and the resulting culture was grown at 37°C with shaking until the culture reached an OD_{600} of about 1.0. The culture was developed in 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and shaken at 37 C for 2 h. The culture was centrifuged at 5,000*g* for 10 min at 4°C. The pellets (about 40 g, wet weight) were washed by resuspension in 40 ml of 20 mM Tris–HCl pH 7.4, with repeat centrifugation. The pellets were resuspended in 40 ml of the same buffer, disrupted by sonication for 5 min and centrifuged at 100,000*g* for 75 min at 4°C. After removal of insoluble components by filtration (MILLEX-GP 0.45 μm, Millipore), the filtrate was loaded into an SP Sepharose Fast Flow column (φ 2.6 × 10 cm, GE Healthcare) pre-equilibrated with 20 mM Tris–HCl pH 7.4, and the proteins were eluted with a linear gradient of 0–0.5 M NaCl. Fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by their ability to turn over nitrocefin as a substrate. Fractions containing the desired activity were pooled and concentrated to a volume of 10 ml by Ultracel YM-10 (Millipore). The concentrated protein was loaded into a Sepharose G-75 column (φ 1.6 × 10 cm, GE Healthcare) and was eluted with 20 mM Tris–HCl pH 7.4 containing 0.3 M NaCl. Fractions containing the IND-7 protein were pooled and concentrated to a volume of 6 ml. The protein was then reloaded into a Sephacryl HR-100 column (φ 1.6 × 10 cm, GE Healthcare) and was eluted with 20 mM Tris–HCl pH 7.4 containing 0.3 M NaCl. The protein was further concentrated to 14 mg/ml using both Ultracel YM-10 (Millipore) and Amicon Ultra-15 (Millipore). The purity of the IND-7 protein was estimated to be greater than 95% by SDS-PAGE at a protein concentration of 1 mg/ml. Prior to the X-ray diffraction experiments, a buffer solution of the IND-7 protein was converted from 20 mM Tris–HCl containing 0.3 M NaCl to 20 mM HEPES–NaOH pH 7.5. For crystallization, the purified IND-7 protein was concentrated to about 14 mg/ml using both Amicon YM-10 (Millipore) and Amicon Ultra (Millipore).

Crystallization

Screening for IND-7 crystallization conditions was performed at 20 C by the hanging-drop method using Crystal Screen and Crystal Screen II kits (Hampton Research): drops mixed 2 μl of a protein solution with 2 μl of a reservoir solution. The best crystals suitable for X-ray diffraction study were obtained from condition No. 17 using the Crystal Screen kit [30%(w/v) PEG4000, 0.1 M Tris–HCl pH 8.5 and 0.2 M Lithium sulphate monohydrate], which grew as plates within 3 months at 20°C.

Data collection and refinement

All diffraction data were collected at 100 K after cryoprotection by a brief exposure to a reservoir solution containing 30% (w/v) PEG4000 at the SPring-8 (Harima, Japan) and the Photon Factory (Tsukuba, Japan). The data set used the structural analysis that was collected at the SPring-8 on a beamline 38B1 (Harima, Japan) at a resolution of 1.65 Å and a wavelength of λ = 1.00 Å. Data for the crystal were integrated, merged, and scaled using HKL-2000 (*43*). The refined structure (PDB code: 1M2X) of BlaB from *C. meningosepticum* at a 1.5 Å resolution (*31*) was used as the search model for molecular replacement using the AMoRe (*44*) and Molrep (*45*) software programs, which are a component of the CCP4 program suite v.6.0.0 (*46*). The interactive graphics programs O (*47*) and Coot v.0.1.2 (*48*) were used to build the IND-7 structure (*47*). The refinement was performed with the CNS (*49*) and Refmac (*50*) software programs. Data collection and refinement statistics are listed in Table I. Selected bond distances and angles are shown in Table II.

PDB Accession Code

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession code 3L6N.

Nucleotide Sequence Accession Number

The nucleotide sequence encoding IND-7 characterized in this study appears in the EMBL/GenBank/DDBJ databases under accession AB529520.

Results and Discussion

Overall structure of IND-7

The structure of IND-7 from *C. indologenes* was solved by molecular replacement. The final refined structural model contained residues Gln38(21)–Lys293(238), two zinc(II) ions (Zn1 and Zn2), two sulphate ions, and 327 ordered water molecules per asymmetric unit, and was refined to $R_{working} = 17.7\%$ and $R_{free} = 20.0\%$ at 1.65 Å resolution (Table I). The root mean square differences (RMSD) from the ideal

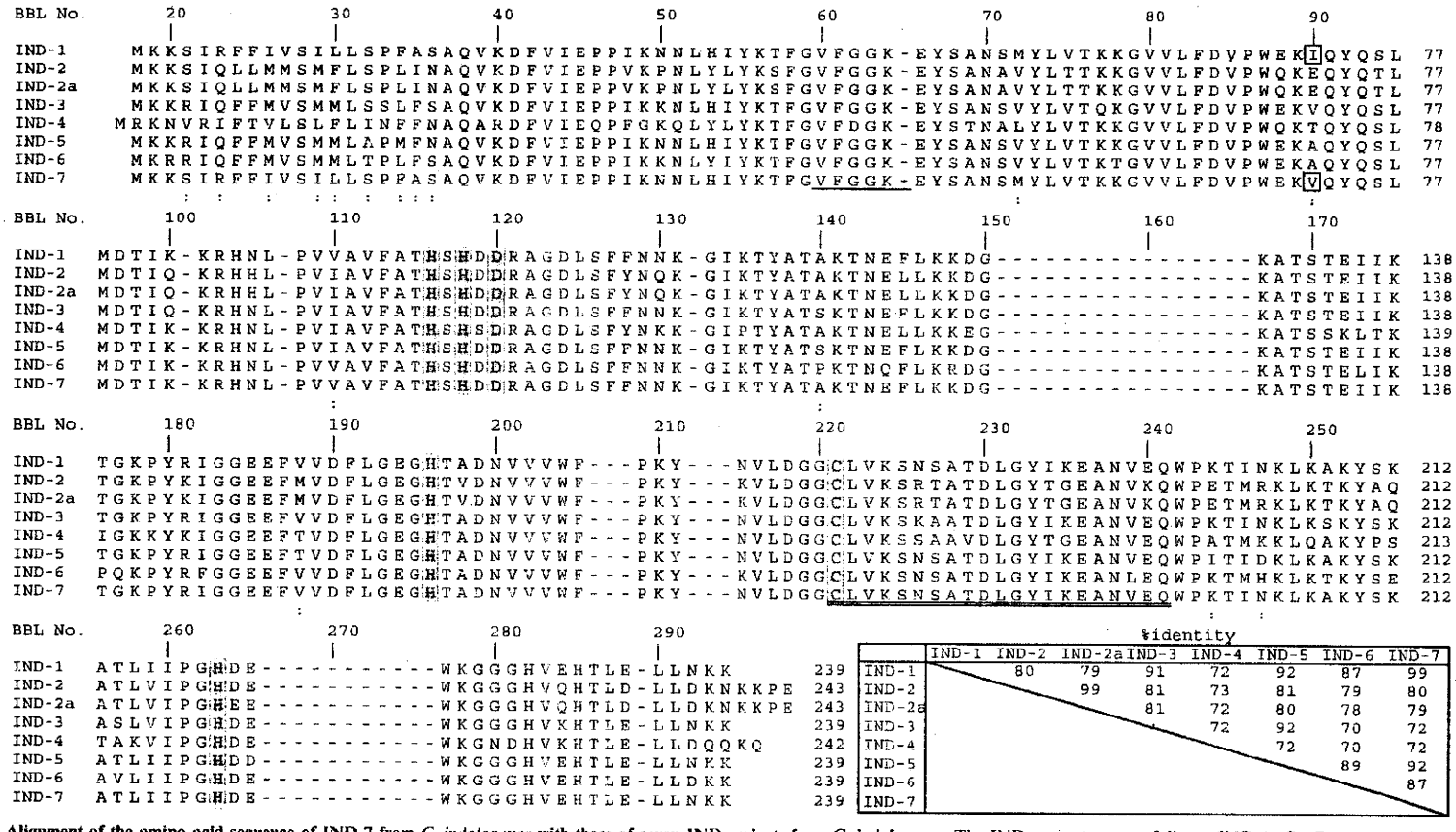

Fig. 1 Alignment of the amino acid sequence of IND-7 from *C. indologenes* with those of seven IND variants from *C. indologenes*. The IND variants are as follows: IND-1 (GenBank accession number: AF099139), IND-2 (AF219129), IND-2a (AF219130), IND-3 (AF219133), IND-4 (AF219135), IND-5 (AY504627) and IND-6 (AM087455). Sequence comparison was performed by aligning the protein amino acid sequences by use of the MULTALIN program (*66*). The class B metallo-β-lactamase numbering scheme is indicated above the sequences (*4*). Dashes indicate gaps introduced to optimize the alignment. Numbers on the right indicate numbers of amino acid residues from the N-terminus for each mature protein. Zinc(II)-coordinating residues are shaded in grey. Substitutions between IND-1 and IND-7 are circled. Substitutions between IND-5 and IND-7 are indicated by colons. The regions of loop 1 and loop 2 in IND-7 are underlined and double-underlined, respectively. The matrix at the bottom of the figure summarizes the levels of amino acid sequence identity.

Table I. Data collection and refinement statistics for IND-7.

Data collection	
Resolution (Å)	50.0–1.65 (1.71–1.65)
Wavelength (Å)	1.00
Unit-cell parameters (Å, °)	$a=77.6$, $b=74.3$, $c=47.4$, $\beta=99.4$
Space group	$C2$
Redundancy	3.7 (3.5)
Completeness (%)	99.9 (99.7)
R_{merge}[a]	4.3 (0.104)
No. of unique reflections	31,928 (901)
$I/\sigma(I)>$	48.5 (16.7)
Refinement statistics	
σcut-off	None
Resolution (Å)	38.3–1.65 (1.69–1.65)
No. of reflections used	30,314
B factors (Å^2)	
Average	13.5
Protein	11.0
Water	26.0
No. of non-H atoms[b]	
Protein	1,811
Water	327
R.m.s. deviation for ideal[c]	
Bond lengths (Å)	0.008
Angles (°)	1.09
$R_{working}$[d]	0.177 (0.221)
R_{free}[e]	0.200 (0.250)

[a] $R_{merge}=\sum_{hkl}\sum_i|I_i(hkl)-<I(hkl)>|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity for reflection for hkl and $<I(hkl)>$ is the average intensity calculated for reflection hkl from replicate data. [b] Per asymmetric unit. [c] RMSD.: root mean square differences. [d] $R_{working}=\sum_{hkl}||F_o|-|F_c||/\sum_{hkl}|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. [e] $R_{free}=\sum_{hkl}||F_o|-|F_c||/\sum_{hkl}|F_o|$ for 5% of the data not used at any stage of structural refinement. Values in parentheses are for the last resolution shell.

bond distances and angles were 0.008 Å and 1.09°, respectively. A Ramachandran plot showed that 97.6% and 5.8% of the residues were in the most-favoured regions and in the additionally allowed regions, respectively, and 0.5% and 1.1% of those were in the generously allowed regions and in the disallowed regions, respectively. The exceptions were Asp50(33) and Asp84(66), which adopted ϕ and ψ angles of 79° and −51°, and 70° and 160°, respectively. Asp84 was buried in the protein and exhibited a strained main chain conformation, which is a commonly observed feature in the crystal structures of BcII, CcrA and IMP-1 metallo-β-lactamases (*28*, *32*, *35*). Therefore, it is thought that conservation of the conformation of Asp84 is important to the folding of metallo-β-lactamases.

The overall structure of IND-7 was found to be composed of five α-helixes and twelve β-strands, which fold into a four-layered αβ/βα sandwich structure (Fig. 2A). The dinuclear zinc(II) active site was located at the bottom of a shallow groove and was made up of the two zinc(II) ions (Zn1 and Zn2), the zinc(II) ion binding residues and two loops (loop 1 and loop 2). Loop 1, which is a typical feature of subclass B1 metallo-β-lactamases, was formed by residues at positions 60–66 (*51*) (BBL numbering, Figs 1 and 2A) between β2 and β3. This flexible loop is thought to be responsible for the tight binding of substrates and inhibitors in the dinuclear zinc(II) active site (*35*, *51–55*). In the IND-7 structure, loop 1 was well defined. Loop 2 was comprised residues at positions 221–241 (BBL numbering), positioned approximately opposite loop 1 cantered at about the zinc(II) binding site (Figs 1 and 2A). Thus, the overall structure of IND-7 was found to be similar to the previously solved structures of subclass B1 metallo-β-lactamases (*7*, *10*, *28–38*).

Coordination mode and occupancy of zinc(II) ions in the dinuclear zinc(II) active site

To understand the structural features of IND-7 in detail, the structures of IND-7 and structurally well-characterized CcrA from *B. fragilis*, which have 28% sequence homology (PDB code: 1ZNB; 1.85 Å resolution), were compared. As shown in Fig. 2B, superimposing the Cα atoms of IND-7 and molecule A in CcrA demonstrated a similarity in the protein folds with an overall RMSD of 1.7 Å, as mentioned above. Despite the similarity in the overall structures between these two enzymes, noticeable structural differences were found in the dinuclear zinc(II) active sites.

In the CcrA structure (Fig. 3A), Zn1 was coordinated with His116(99), His118(101), His196(162) and a water molecule or hydroxide ion (Wat1), forming a tetrahedral geometry, whereas Zn2 was coordinated with Asp120(103), Cys221(181), His263(223), one water molecule (Wat3, termed 'apical water') and Wat1, forming a trigonal bipyramidal geometry. The zinc(II) coordination geometry found in CcrA is mostly conserved in subclass B1 metallo-β-lactamases.

On the other hand, the zinc(II) coordination mode of IND-7 was different from the common feature. In the process of refinement of the IND-7 structure, we initially set the occupancies of Zn1 and Zn2 as 1.0 and refined their B-factors. As the B-factor of Zn2 (15.6 Å^2) was relatively higher than that of Zn1 (9.3 Å^2), occupancies for Zn1 and Zn2 were reset as 1.0 and 0.7, respectively, for subsequent refinement. The final B-factors equalled ~9.3 Å^2 for Zn1 and 12.3 Å^2 for Zn2. The average B-factor of the residues involved in the zinc(II) coordination, His116(96), His118(98), Asp120(100), His196(159), Cys221(178) and His263(220), was 7.1 Å^2. These results suggested that the affinity of Zn2 was lower than that of Zn1, and that Zn2 tended to escape from the dinuclear zinc(II) active site of IND-7 Moreover, a $2|F_o|-|F_c|$ electron density map around the Zn2 site (Fig. 3B) clearly showed the existence of both conformations in the side chains of Cys221(178) and His263(220), where the occupancies of Cys221A(178) and His263A(220) were refined by 0.7, and those of Cys221B(178) and His263B(220) were refined by 0.3. The former was a Zn2-coordinated form, whereas the latter was a Zn2-uncoordinated form. Interestingly, in the side chain of Asp120(100), a zinc(II) ligand, no disorder was observed, which was fixed through the interactions of Zn2 and Wat1 (*vide infra*). This finding indicates that Asp120 played an important role in helping to orient a zinc(II)-bound hydroxide ion for a nucleophilic attack on the CO group of the β-lactam ring.

The coordination geometry around Zn1 (Fig. 3B) was a distorted trigonal bipyramid with $\tau=0.88$