194 spp. in 14 of 47 prefectures.

195 Isolates obtained at the 771 medical facilities over the study period and those of
196 MDR isolates are shown in Fig. 2. The percentages for each year were similar to those
197 for the entire study period (data not shown). These results indicated that *Acinetobacter*
198 spp., including MDR isolates, mainly affected the respiratory tract.

199 *Acinetobacter* spp. isolates were identified to the spp. level at 558 medical facilities
200 in 2007, 571 in 2008, and 577 in 2009. A total of 86 834 *Acinetobacter* spp. isolates
201 were identified during the study period. As shown in the upper part of Table 3, 61 794
202 (71.2%) were *A. baumannii*, 8 983 (10.3%) were *A. lwoffii*, and 3 327 (3.8%) were *A.*
203 *calcoaceticus*. The percentages for each year were similar to those for the entire study
204 period.

205 MDR isolates were identified to the spp. level at 34 medical facilities in 2007, 33 in
206 2008, and 45 in 2009. A total of 515 MDR isolates included *A. baumannii* ($n = 423$,
207 82.1%), *A. lwoffii* ($n = 39$, 7.6%), and *A. calcoaceticus* ($n = 4$, 0.8%). As shown in the
208 lower part of Table 3, isolation rates of *A. baumannii* increased significantly during the
209 study period ($P < 0.0001$), and those of *A. lwoffii* and *A. calcoaceticus* decreased each
210 year during the study period.

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DISCUSSION

The results of the present survey showed that the percentages of MDR *P. aeruginosa* decreased significantly during the study period (April 2007 through March 2010). In addition, the number of patients with MDR isolates, as well as the number of patients per bed, decreased markedly. Our previous survey performed during the period January 2003 through 2006 showed that the percentages of MDR isolates increased significantly and the number of patients increased gradually [11]. The number of patients with MDR isolates seemed to decrease since 2007 in medical facilities in Japan, although these two surveys cannot be directly compared to each other because the hospitals surveyed were different between them. The hospitals surveyed in the present survey were institutions with over 200 beds, whereas medical facilities with 500 or more, and regional core hospitals with less than 500 beds were included in the previous study.

During the first survey, outbreaks of MDR *P. aeruginosa* had became a serious problem in medical facilities in Japan [19]. The Ministry of Health, Labour and Welfare of Japan, scientific societies on nosocomial infection controls and infectious diseases provided information about the current situation and infection control measures regarding MDR *P. aeruginosa* in medical settings in Japan. Most outbreaks were controlled by early involvement of management, including staff education, strict isolation of infected patients or carriers of MDR *P. aeruginosa*, active surveillance for drug-resistant *P. aeruginosa* and rigorous contact precautions [13]. Infection control measures especially focused on the handling of urine and urinary catheters, because the first survey suggested the importance of management of patients' urine in the prevention and control of nosocomial MDR *P. aeruginosa* infection in Japan [13].

It is essential to monitor MDR *A. baumannii* in medical facilities and to prepare

infection control measurements for patients with MDR *A. baumannii* in Japan. The present study revealed that the majority of MDR *Acinetobacter* spp. isolated from patients in Japan were *A. baumannii.* Although the number of the MDR isolates was still small, these findings agreed with those in other countries [5].

CONCLUSIONS

A large-scale investigation of multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. was performed at medical facilities in Japan. MDR *P. aeruginosa* was prevalent nationwide in Japan, but its incidence decreased significantly after 2007. MDR *Acinetobacter* spp. is an emerging problem in medical facilities in Japan.

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ABBREVIATION

JANIS: Japanese Nosocomial Infection Surveillance System; DGIZ: Diameter of the Growth Inhibition Zone; MDR: MultiDrug-Resistant; MIC: Minimum Inhibitory Concentration; TDR: Two-Drug-Resistant

COMPETING INTERESTS

All authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

TK and NMY carried out this study. All authors participated in the design of the study and they read and approved the final manuscript.

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with and without multidrug resistance in medical facilities and clinical laboratories in Japan. *J Antimicrob Chemother* 2008, **61**:612-615.

384 **Figure legends**

385 **Figure 1.** Numbers of patients with MDR *P. aeruginosa* per 1,000 beds/year during the
386 study period in each group of medical facilities categorized according to the number of
387 beds.
388 Four sets of three box plots represent the numbers of patients in all facilities, large-scale
389 facilities with ≥ 600 beds, medium-scale facilities with 300 – 599 beds, and small-scale
390 facilities with < 300 beds during the study period. The top and bottom of the boxes
391 indicate the 75th and 50th percentiles, respectively. The ends of the whiskers indicate 90th
392 and 25th percentiles.
393 §§§: $p \leqq 0.01$, §§: $p \leqq 0.05$ for Freedman's test; ***: $p \leqq 0.01$, **: $p \leqq 0.05$ for
394 Wilcoxon's test.
395
396 **Figure 2.** Tissue sources of total *Pseudomonas aeruginosa* isolates, TDR isolates, and
397 MDR isolates obtained during the study period at medical facilities.
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399 **Figure 3.** Tissue sources of total *Acinetobacter* spp.. isolates and MDR isolates obtained
400 during the study period at medical facilities.
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Table 1. Isolation of *P. aeruginosa* with or without multidrug resistance in medical facilities

Isolates		2007	2008	2009
P. aeruginosa				
	Total numbers (No.) [a]	228 449	233 301	223 232
	No. of isolates per 1 000 beds/year	723.0	738.4	706.5
TDR isolates	Total No.	14 340	14 043	13 009
	No. of isolates per 1 000 beds/year	45.4	44.4	41.2
	Ratio (%) [b]	6.3	6.0	5.8
	Patient No. [c]	6 789	6 474	6 231
	No. of patients per 1 000 beds/year	21.5	20.5	19.7
MDR isolates	Total No.	7 688	6 540	5 683
	No. of isolates per 1 000 beds/year	24.4	20.7	18.0
	Ratio (%)	3.4	2.8	2.5
	Patient No.	2 779	2 481	2 246
	No. of patients per 1 000 beds/year	8.8	7.9	7.1

[a]: Numbers of *P. aeruginosa* isolated from the 771 medical facilities that responded to the questionnaire survey.

[b]: Ratios of the numbers of TDR or MDR *P. aeruginosa* to the total numbers of *P. aeruginosa* isolates (%).

[c]: Numbers of patients from whom TDR or MDR *P. aeruginosa* were isolated.

421 Table 2. Isolation of *Acinetobacter* spp.. with or without multidrug resistance in medical facilities

Isolates		2007	2008	2009
Acinetobacter spp..				
	Total numbers (No.) [a)]	32 073	31 330	30 609
	No. of isolates per 1 000 beds/year	101.5	99.2	96.9
	Patient No. [b)]	20 782	20 394	19 625
	No. of patients per 1 000 beds/year	65.8	64.5	62.1
MDR isolates				
	Total No.	84	143	331
	No. of isolates per 1 000 beds/year	0.3	0.5	1.1
	Rate (%)[c)]	0.3	0.5	1.1
	Patient No.	51	81	97
	No. of patients per 1 000 beds/year	0.2	0.3	0.3

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423 [a)]: Numbers of *Acinetobacter* spp.. isolated from the 771 medical facilities that responded to the questionnaire survey.

424 [b)]: Numbers of patients from whom MDR *Acinetobacter* spp.. were isolated.

425 [c)]: Ratio of numbers of MDR *Acinetobacter* spp.. to the total numbers of *Acinetobacter* spp.. isolated (%)

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444 Table 3 Isolation of *Acinetobacter* spp.. With or without multidrug resistance in medical facilities by spp.

Isolates		2007	2008	2009
(No. of medical facilities where species-level identification was performed)		(558)	(571)	(577)
A. baumannii	Total numbers (No.) [a)]	21 116	20 371	20 307
	No. of isolates per 1 000 beds/year	87.1	82.1	81.2
A. lwoffii	Total No.	3 011	3 134	2 838
	No. of isolates per 1 000 beds/year	12.4	12.6	11.4
A. calcoaceticus	Total No.	1 124	1 059	1 144
	No. of isolates per 1 000 beds/year	4.6	4.3	4.6
Other *Acinetobacter* spp..	Total No.	4 125	4 469	4 136
	No. of isolates per 1 000 beds/year	17.0	18.0	16.5
(No. of medical facilities where MDR strains were isolated)		(34)	(33)	(45)
MDR *A. baumannii*	Total No.	35	96	292
	No. of isolates per 1 000 beds/year	0.1	0.4	1.2
	Ratio (%) [b)]	0.2	0.5	1.4
MDR *A. lwoffii*	Total No.	21	12	6
	No. of isolates per 1 000 beds/year	0.1	0.05	0.02
	Ratio (%)	0.7	0.4	0.2
MDR *A. calcoaceticus*	Total No.	3	1	0
	No. of isolates per 1 000 beds/year	0.01	0.004	0
	Ratio (%)	0.3	0.1	0
Other MDR spp..	Total No.	11	18	20
	No. of isolates per 1 000 beds/year	0.05	0.07	0.08
	Ratio (%)	0.3	0.4	0.5

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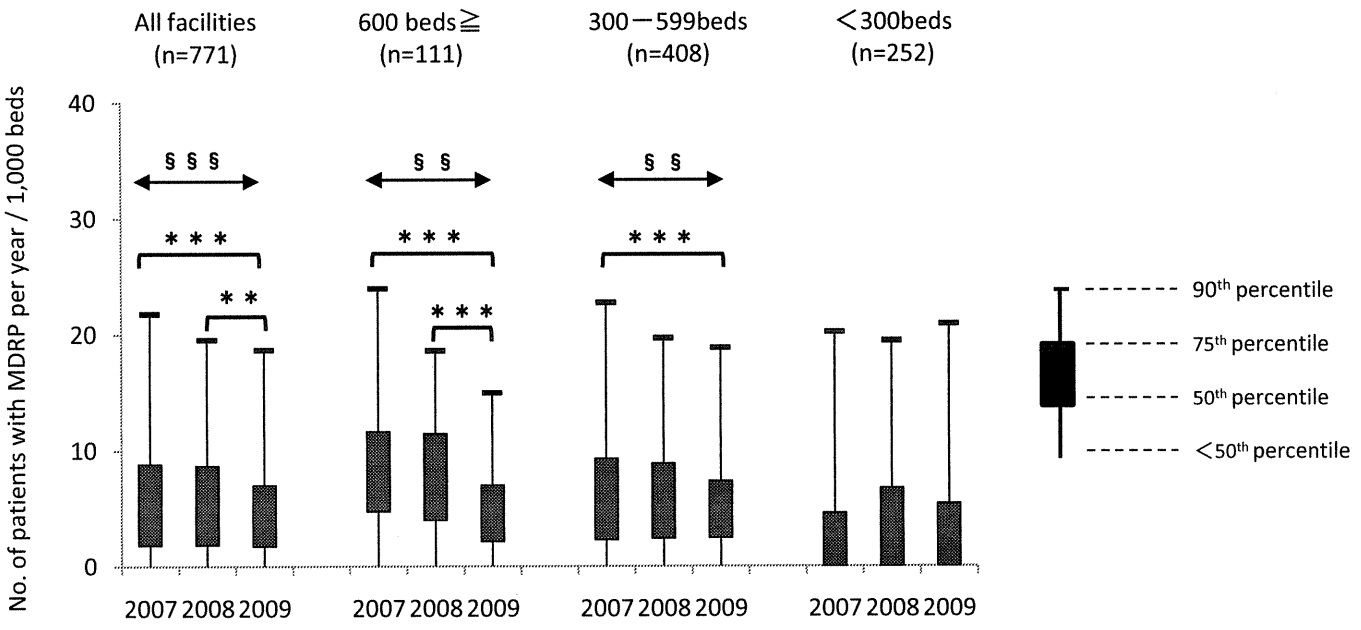
446 [a)]: Numbers of *Acinetobacter* spp.. isolated from the 771 medical facilities that responded to the questionnaire survey.

447 [b)]: Ratio of the numbers of MDR *Acinetobacter* spp.. to the total numbers of *Acinetobacter* spp.. isolated (%).

448

Figure 1


All facilities
(n=771)
600 beds≧
(n=111)
300－599beds
(n=408)
＜300beds
(n=252)
No. of patients with MDRP per year / 1,000 beds
40
30
20
10
0
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2007 2008 2009
2007 2008 2009
2007 2008 2009
2007 2008 2009
90th percentile
75th percentile
50th percentile
＜50th percentile

Figure 2

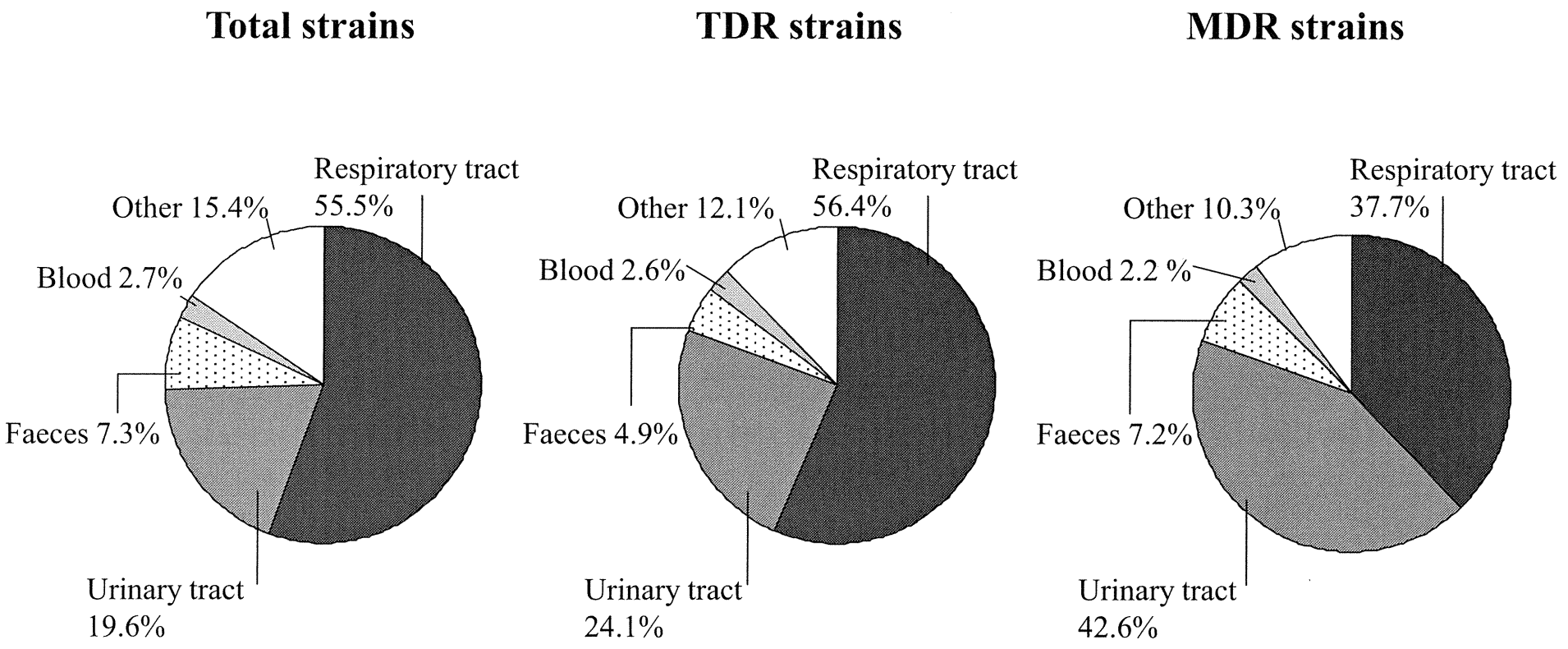
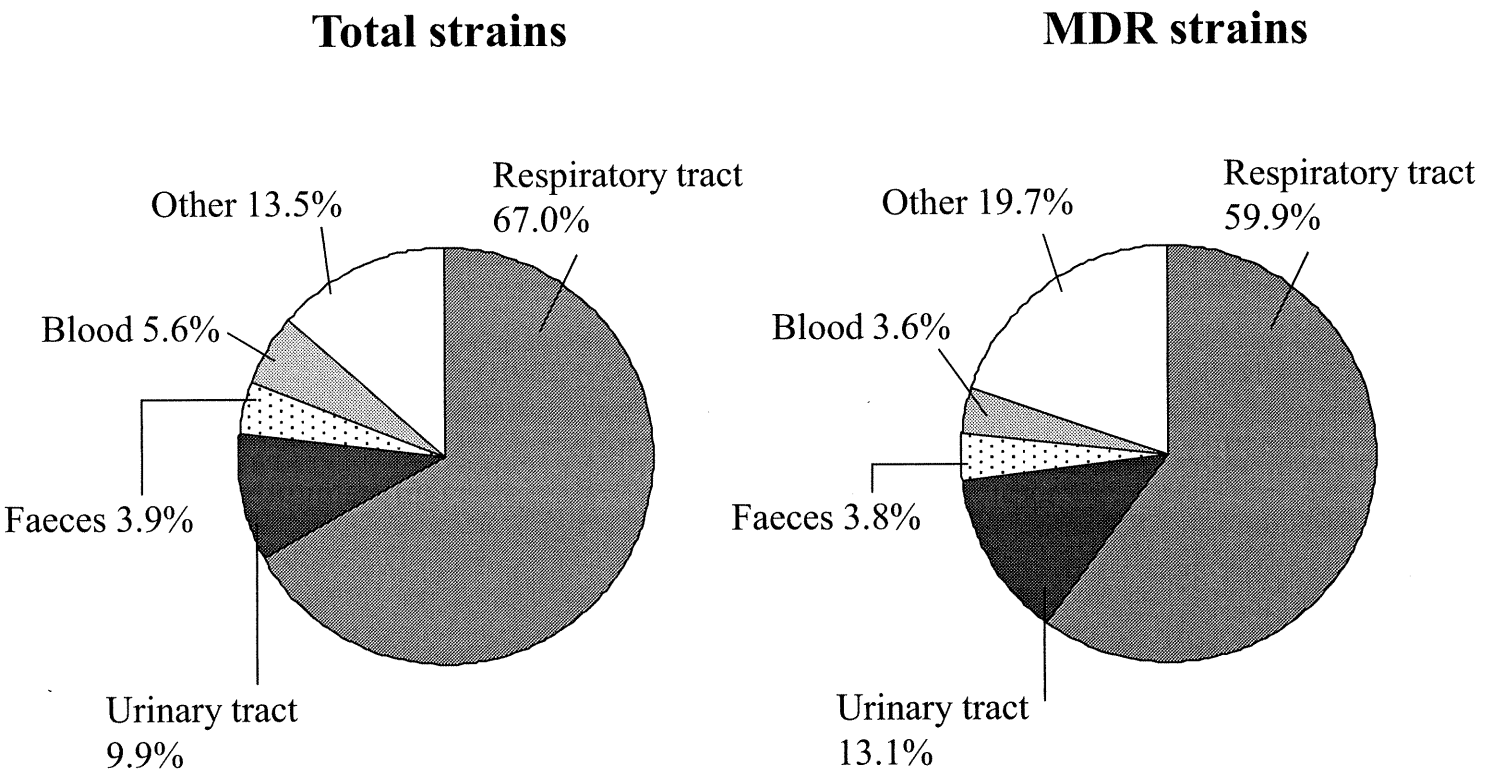

Total strains
Respiratory tract 55.5%
Other 15.4%
Blood 2.7%
Faeces 7.3%
Urinary tract 19.6%
TDR strains
Respiratory tract 56.4%
Other 12.1%
Blood 2.6%
Faeces 4.9%
Urinary tract 24.1%
MDR strains
Respiratory tract 37.7%
Other 10.3%
Blood 2.2 %
Faeces 7.2%
Urinary tract 42.6%

Figure 3


Total strains
Respiratory tract 67.0%
Other 13.5%
Blood 5.6%
Faeces 3.9%
Urinary tract 9.9%
MDR strains
Respiratory tract 59.9%
Other 19.7%
Blood 3.6%
Faeces 3.8%
Urinary tract 13.1%

Structural Biology and Crystallization Communications

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

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PDB Reference: AmpC^{D}, 2zj9, r2zj9sf.

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Expression and purification

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

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

2.2. Crystallization

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

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

Values in parentheses are for the highest resolution shell.

Data collection	
Resolution (Å)	50.0–1.70 (1.76–1.70)
Wavelength (Å)	1.07
Unit-cell parameters (Å, °)	a = 47.1, b = 47.4, c = 81.5, α = 82.6, β = 80.9, γ = 65.4
Space group	*P*1
Redundancy	2.2 (1.7)
Completeness (%)	94.3 (82.8)
R_{merge}†	0.062 (0.267)
No. of observed reflections	141726 (9810)
No. of unique reflections	65343 (5771)
$\langle I/\sigma(I)\rangle$	18.1 (2.6)
Refinement statistics	
σ cutoff	0
Resolution (Å)	39.2–1.70 (1.74–1.70)
No. of reflections used	62030 (3855)
B factors (Å^2)	
Average	16.9
Protein	15.6
Water	28.6
No. of non-H atoms‡	
Protein	5796
Water	611
R.m.s.d. from ideal§	
Bond lengths (Å)	0.012
Angles (°)	1.4
$R_{working}$¶	0.163 (0.228)
R_{free}††	0.206 (0.306)

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

2.3. Data collection and refinement

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

3. Results and discussion

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

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

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

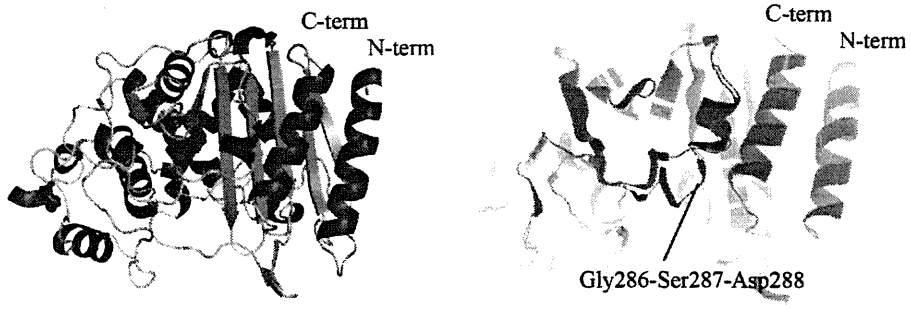

Figure 1
(*a*) Overall structure of AmpC^{D} β-lactamase. Only molecule *B* is depicted. α-Helices, β-strands and loops are shown in red, green and cyan, respectively. (*b*) Slabbed view of overall structure superposition of native AmpC β-lactamase (red; PDB code 1ke4) and AmpC^{D} β-lactamase (blue).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

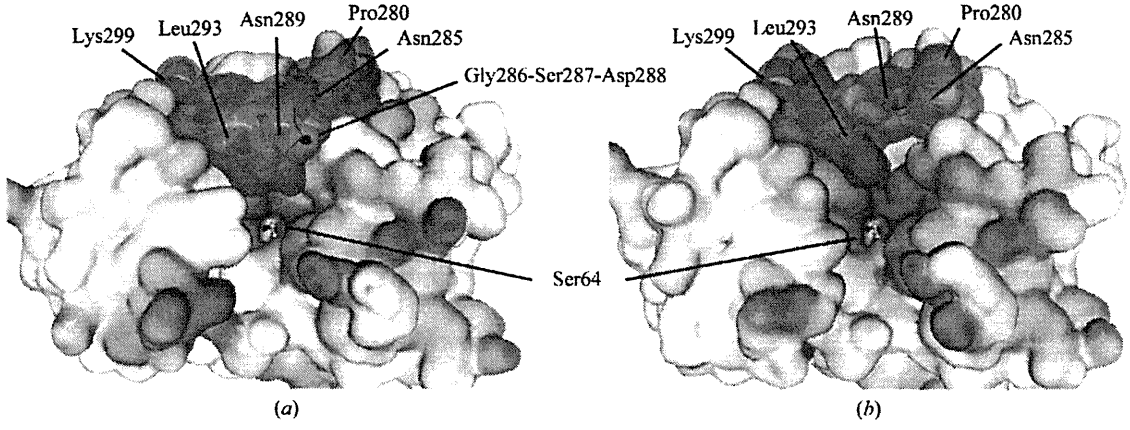

Figure 2
Electrostatic potential representations of (*a*) native AmpC β-lactamase and (*b*) AmpC^{D} β-lactamase. The catalytic residue Ser64 is represented as a ball-and-stick model (the O$^{\gamma}$ atom of Ser64 is coloured red). The Pro280–Lys299 region in native AmpC β-lactamase and AmpC^{D} β-lactamase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

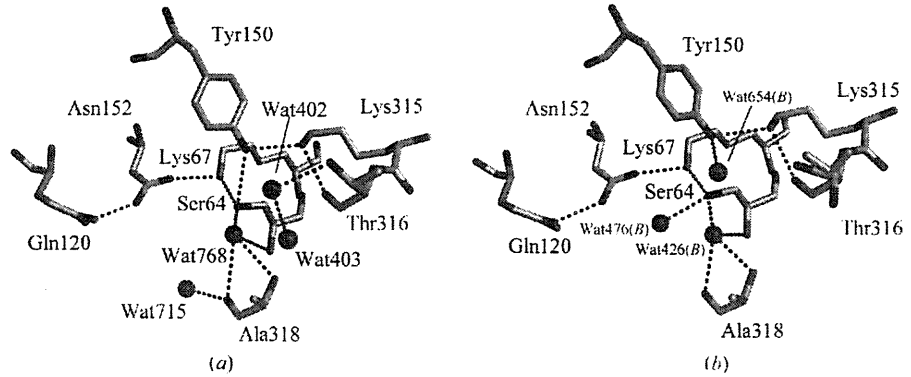

Figure 3
Substrate-binding site of (*a*) native AmpC β-lactamase determined to 1.72 Å resolution and (*b*) AmpC^{D} β-lactamase. In each structure, only molecule *B* is depicted. C, O and N atoms of the enzyme are shown in grey, red and blue, respectively. Water molecules cited in the text are labelled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

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

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

In the structure of native AmpC (Fig. 3*a*), four water molecules (Wat402, Wat403, Wat715 and Wat768) were observed in the substrate-binding site, where Wat402 was presumed to be the deacylating water (Powers & Shoichet, 2002) which hydrogen bonds to Thr316 $O^{\gamma 1}$ and Wat403. Wat715 is hydrogen bonded to the main-chain carbonyl O atom of Ala318 and Wat768 interacts with Ser64 and Ala318. In the substrate-binding site of AmpC^D, three water molecules [Wat426(*B*), Wat476(*B*) and Wat654(*B*) in molecule *B*] were located in the active site (Fig. 3*b*). As in native AmpC, a water molecule [Wat426(*B*) in the AmpC^D structure] is bound in the site which stabilizes the tetrahedral intermediate of the lactamase reaction (Usher *et al.*, 1998; Murphy & Pratt, 1988) and is hydrogen bonded to the main-chain N atoms of Ser64 and Ala318, Ser64 O^{γ} and the main-chain carbonyl O atom of Ala318.

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

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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*