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Potency of Carbapenems for the Prevention of Carbapenem-Resistant Mutants of *Pseudomonas aeruginosa*

The High Potency of a New Carbapenem Doripenem

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Abstract The potencies of the carbapenems; doripenem (DRPM), meropenem (MEPM) and imipenem (IPM) in preventing the emergence of carbapenem-resistant mutants were examined in *Pseudomonas aeruginosa* strains. The carbapenems predominantly selected carbapenem-resistant mutants or carbapenem mutants with reduced susceptibilities that were specifically resistant to carbapenems and had arisen as a result of the reduced level of expression of the outer membrane protein with a molecular weight of about 48,000 (OprD). The potency of carbapenems in preventing the growth of the mutants differed for DRPM, MEPM and IPM. The isolation frequency of the mutant was examined on agar plates containing each of the carbapenems at a concentration of 1/2 or 1/4 MIC of each carbapenem for that mutant. Mutants were not selected on agar containing DRPM at a frequency of greater than 10^{-9} per cell per generation, whereas mutants of each strain were selected on agar containing MEPM or IPM at frequencies of 10^{-7} to 10^{-9} per cell per generation. The drug concentrations and the drug concentration range for the selective increase of carbapenem resistant mutants in the broth culture containing each carbapenem differed for each carbapenem. DRPM exhibited both the lowest drug concentration and the narrowest range of drug concentration for selection of the carbapenem-resistant mutants. The results shown in this report indicated that DRPM exhibited the greatest ability to prevent the emergence of the mutant.

Keywords carbapenem, doripenem, carbapenem-resistant mutant, outer membrane protein, prevention of mutation

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen for immunocompromised patients. The organism has both an intrinsic and acquired resistance to many antibiotics [1, 2], and treatment of infection by this organism is difficult. Carbapenems have a high potency against a broad spectrum of organisms and are one of the most active groups of β-lactam antibiotics against *P. aeruginosa*.

There is a group of carbapenem resistant *P. aeruginosa* strains that is specifically resistant to carbapenems and which does not show cross resistance to other β-lactams or other antibiotics. This type of imipenem (IPM)-resistant *P. aeruginosa* mutant was first isolated during clinical trials of IPM for the treatment of serious infections caused by *P. aeruginosa* [3]. The IPM-resistant mutants of *P. aeruginosa* were found to lack a 45 KD to 49 KD protein [3~6] in the outer membrane protein D2 (OprD), which forms a channel specific to IPM and its structural analogues [7].

In this study, we investigated the potency of doripenem (DRPM) [8~12], meropenem (MEPM) and IPM in preventing the emergence of carbapenem-resistant mutants under laboratory conditions.

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Materials and Methods

Bacteria, and Media

One hundred and forty-four clinical *P. aeruginosa* isolates were examined for carbapenem resistance. Of the 144 strains, 100 strains were isolated from Gunma University Hospital, 30 strains from Isesaki City Hospital, Isesaki City, Japan, and 14 strains from Gunma Chuo-hospital, Maebashi, Japan. Mueller-Hinton (MH) (Nissui, Tokyo, Japan) broth and MH agar or antibiotic medium 3 (AB3) (Difco, Detroit, Mich.) and AB3 agar were used to test the MICs for each antibiotic. Agar plates were prepared by adding 1.5% agar to broth medium. All bacterial strains were grown at 37°C. MICs obtained using MH medium and AB3 medium were not essentially different (data not shown). Mutation frequencies and the kinetics of bacterial growth were examined for each medium. As a result, AB3 medium was used throughout this study for its capacity to maintain a stable pH during course of bacterial growth.

Determination of MICs

MIC was determined by the agar dilution method. Overnight cultures of the strains grown in broth were diluted 100 times with fresh broth. One loopful of each dilution was plated on agar plates containing two-fold serially-diluted antibiotics. The plates were incubated for 18 hours at 37°C.

Antibiotics

Antibiotics used in this study were as follows: DRPM (a new carbapenem that had been developed by Shionogi Pharmaceuticals, Osaka) [8~12], MEPM (Sumitomo Pharmaceuticals, Osaka), IPM (Banyu Pharmaceuticals, Tokyo), piperacillin (PIPC, Toyama Chemicals, Tokyo), ceftazidime (CAZ, Tanabe Pharmaceuticals, Osaka) and gentamicin (GM, Schering-Plough K.K., Osaka) and ofloxacin (OFLX, Daiichi Seiyaku Co., Ltd., Tokyo).

Isolation Frequency of Mutant

The frequency of isolation of the mutant was estimated using the following equation for mutation frequency [13]: Mutation frequency per cell per generation=$[\Pi-\Pi 0/1n(P/P0)]$ where $\Pi 0$ and $P0$ are, respectively, the proportion of mutant cells and the total number of cells in the culture at the start of the experiment, and Π is the proportion of mutant cells after incubation when the number of total cells has risen to P.

Analysis of Outer Membrane Proteins (OMPs)

The OMPs of all strains were analyzed by SDS-PAGE of the sarkosyl insoluble fraction of membrane-preparation as described by Spratt [14]. Throughout this study, the OMPs of the carbapenem-resistant mutants of the representative strains obtained in each experiment were examined to confirm whether the mutants lacked, or had reduced amount of, OMP of molecular weight 45,000~49,000 (data not shown).

Results

Distribution of Carbapenem MICs among *P. aeruginosa* Isolates

The carbapenem resistance levels (MICs) of the 144 strains of *P. aeruginosa* were examined. There was a bi-modal distribution of the MICs for each drug in these strains (data not shown). However, the MICs showed a continuous distribution, and there was no complete break between the bi-modal distributions. The MICs of DRPM for these strains ranged from 0.1 to 1.6 μg/ml, and from 1.6 to 12.5 μg/ml. The MICs to MEPM ranged from 0.1 to 1.6 μg/ml, and from 1.6 to 50 μg/ml. The MICs of IPM for these strains ranged from 0.1 to 6.3, and from 6.3 to 100 μg/ml. The MIC_{90} values of DRPM, MEPM, and IPM were 3.2, 6.3, and 25 μg/ml, respectively. Ten carbapenem susceptible strains were chosen for further study. The susceptibilities to carbapenems and other antimicrobial agents by the ten strains are shown in Table 1.

Appearance of Colonies of Carbapenem-resistant Mutants within the Zone of Growth Inhibition

Figure 1 shows a typical result of the growth inhibition of the *P. aeruginosa* GP17 strain. The MICs of DRPM, MEPM and IPM for the parent strain GP17 were 0.1, 0.1 and 1.6 μg/ml, respectively.

In experiments with IPM and MEPM, there were several colonies within the clear zone of growth inhibition, or on the border between the clear zone of growth inhibition and the zone of cell growth. However, there were no colonies within the clear zone of growth inhibition when DRPM was used. The colonies that grew within the clear zone of growth inhibition in the MEPM or IPM experiments, or on the border in the MEPM experiment showed MIC values of 1.6 μg/ml to DRPM, 3.2 μg/ml to MEPM and 25 μg/ml to IPM. The MICs of PIPC, CAZ, GM and OFLX for the mutant strains were the same as those of parent strain. SDS-PAGE gel analysis of the OMPs of the representative mutant strain showed a marked reduction in a specific OMP with a molecular weight of about 48,000 (data not shown). These results indicated that the mutant resulted from the reduced expression of OprD and they exhibited reduced

Table 1 Drug susceptibilities of the *P. aeruginosa* strain and the carbapenem-resistant mutants

Strain No.	Strain[a]	MIC (μg/ml)						
		DRPM	MEPM	IPM/CS	PIPC	CAZ	GM	OFLX
GP 2	wild type	0.2	0.4	1.6	100	25	1.6	1.6
	carbapenem-resistant mutant	3.2	6.3	12.5	100	25	1.6	1.6
GP 3	wild type	0.2	0.4	1.6	12.5	3.2	3.2	1.6
	carbapenem-resistant mutant	3.2	6.3	25	12.5	3.2	3.2	1.6
GP 5	wild type	0.8	3.2	3.2	12.5	6.3	50	50
	carbapenem-resistant mutant	6.3	12.5	12.5	12.5	6.3	50	50
GP10	wild type	0.2	0.2	1.6	3.2	3.2	1.6	1.6
	carbapenem-resistant mutant	1.6	3.2	12.5	3.2	3.2	1.6	1.6
GP13	wild type	0.1	0.1	1.6	6.3	3.2	1.6	1.6
	carbapenem-resistant mutant	1.6	3.2	12.5	6.3	3.2	1.6	1.6
GP14	wild type	0.2	0.2	3.2	6.3	3.2	3.2	3.2
	carbapenem-resistant mutant	1.6	3.2	25	6.3	3.2	3.2	3.2
GP17	wild type	0.1	0.1	1.6	6.3	3.2	3.2	1.6
	carbapenem-resistant mutant	1.6	3.2	25	6.3	3.2	3.2	1.6
GP33	wild type	0.2	0.8	0.8	25	12.5	3.2	12.5
	carbapenem-resistant mutant	6.3	25	12.5	25	12.5	3.2	12.5
GP59	wild type	0.1	0.4	1.6	12.5	6.3	1.6	6.3
	carbapenem-resistant mutant	3.2	12.5	12.5	12.5	6.3	1.6	6.3
GP66	wild type	0.2	0.8	1.6	12.5	6.3	1.6	6.3
	carbapenem-resistant mutant	3.2	12.5	12.5	12.5	6.3	1.6	6.3

[a] Throughout this study, the drug susceptibilities of the mutant strains isolated in the presence of each selective drug of DRPM, MEPM and IPM were examined. The phenotype of drug susceptibilities of the mutants derived from a given strain were not essentially different in each experiment in this study. The drug susceptibilities of the representative mutant strains are shown in this table.

susceptibility or resistance to DRPM, MEPM, or IPM compared to the parent strain [3, 6, 7]. These results also suggested that both MEPM and IPM could specifically select for the carbapenem reduced susceptible mutants or carbapenem-resistant mutants of *P. aeruginosa* strains, and that DRPM could prevent growth of the mutants at a concentration that would inhibit cell growth. Throughout this study, the term "carbapenem-resistance" was used to where a mutant had reduced susceptibility to carbapenems compared to its parent strain, and not as any indication of absolute MIC.

Plating of Viable Cells of Each Strain on Selective Agar Plates Containing Different Concentrations of Each Carbapenem and the Carbapenem-resistance Level

To examine the presence of carbapenem-resistant mutants in the bacterial cell population that could arise during cell growth and to show quantitatively the results of Fig. 1, the number of cells of broth culture from each strain that grew

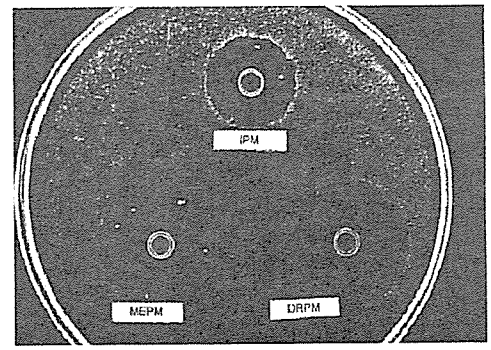

Fig. 1. Presence of carbapenem-resistant mutants within zones of growth inhibition of *P. aeruginosa* around cup containing carbapenem on agar plate.

0.1 ml of overnight culture of *P. aeruginosa* strain was spread on AB3 agar plate. Three cups were placed on the agar plate. 0.2 ml of 0.8 μg/ml of each carbapenem was put into each cup and the plate was incubated at 37°C for 18 hours. Colonies that grew in the clear zone of growth inhibition were purified on a drug free agar plate and resistance levels to carbapenems were examined by agar dilution methods.

on selective agar plates containing different concentrations of each carbapenem was determined (*i.e.*, population analysis) [15]. The results are shown as a survival curve in Fig. 2.

Almost all inoculated cells from each strain grew on the selective agar plates containing a drug concentration under the strain specific MIC of each carbapenem. The number of bacterial cells of each strain that grew on the selective agar plate containing the strain specific MIC of each carbapenem was around 10^2 to 10^4 cell per ml.

A number of colonies from each strain were grown on selective agar plates containing drug concentrations above the strain specific MIC of each drug. The drug concentration for MEPM ranging from the strain specific MIC to the drug concentration required for the complete inhibition of cell growth in each strain was greater than the drug concentration ranges of DRPM and IPM. The drug concentration range corresponded to the relatively wide zone of incomplete inhibition of cell growth by meropenem between the zone of complete inhibition and the zone of no inhibition of cell growth, as shown in Fig. 1.

Ten colonies of each strain grown on selective agar plates containing half of the concentration of each drug required for the complete inhibition of cell growth in each strain were examined for their drug resistance levels. One or two of the colonies that grew on the DRPM plates of GP2, GP33 and GP59 showed a carbapenem resistance (MIC) that was twice the DRPM concentration for the complete inhibition of cell growth, while the other colonies from each strain had the same level of carbapenem susceptibility as that of the parent strain. The ten colonies grown on the MEPM plates showed carbapenem resistance (MIC) that was the same level as the MEPM concentration for the complete inhibition of cell growth. The ten colonies grown on IPM showed carbapenem resistance (MIC) that was the same or twice the level of the IPM concentration for complete inhibition of cell growth.

The mutants selected with a given carbapenem showed resistance only to carbapenems and were susceptible to other antibiotics (Table 1). The SDS-PAGE gel of OMPs from a representative mutant from each strain showed a marked reduction in the specific OMP of around 48,000 molecular weight (data not shown).

Isolation Frequency of Carbapenem-resistant Mutants of *P. aeruginosa* Strains Grown in Broth without Carbapenem

The results of the population analysis shown in Fig. 2 implied that the isolation frequencies of carbapenem-resistant mutants would be different for the selective drugs DRPM, MEPM, and IPM when mutants are selected at a given concentration of each drug for each strain. The drug concentration for the selective agar plates was half the MIC of DRPM and MEPM, and 1/2 or 1/4 the MIC of IPM for the mutant of each strain.

Carbapenem-resistant mutants of the strains were not selected on selective agar plates containing DRPM, however, they were selected on selective agar plates containing MEPM or IPM at a frequency of around 10^{-7} to 10^{-9} per cell per generation (Table 2).

The carbapenem-resistant mutants tested from a given parent strain that had been selected with DRPM, MEPM, or IPM had the same resistance phenotype to carbapenems, and susceptibility to other antibiotics as the parent strain (Table 1).

OMPs of *P. aeruginosa* Strains

The OMPs of the representative strains and the carbapenem-resistant mutants shown in Table 2 were examined (Fig. 3). The OMPs of the GP3 and GP33 parent strains and the carbapenem-resistant mutants isolated from the selective agar plates containing either DRPM, MEPM, or IPM are shown in Figs. 3A and 3B, respectively. The OMPs of the representative mutant of each strain of the GP2, GP17 and GP66 strains are shown in Figs. 3C, D, and E, respectively. Marked reductions in the concentration of a specific outer membrane protein with a molecular weight of around 48,000 were observed for each of the mutants.

Increased Carbapenem-resistance Level (MIC) of *P. aeruginosa* Strain after Incubation with Carbapenem

If the carbapenem-resistant mutants arise during growth of the strain in broth culture, and the mutant strain can be increased selectively in the presence of carbapenem, the MIC of the carbapenem for the broth culture will increase beyond that seen for the parent strain.

The maximum resistance levels (MIC) of DRPM were lower than those of MEPM or IPM (the highest for the three carbapenems) for all strains tested (Fig. 4). The drug concentration ranges in broth cultures that gave rise to the maximum-resistance levels to each carbapenem in each strain were different for each of the carbapenems. The maximum resistance level to DRPM for each strain was obtained with broth cultures containing drug concentrations ranging from 1/4 or 1/2 MIC to MIC or twice MIC (Fig. 4). The maximum resistance level to MEPM for each strain was obtained with broth cultures containing drug concentrations ranging from 1/4 or 1/2 MIC to greater than MIC,with levels of four, eight or 16 times the MIC for each of the strains being observed (Fig. 4). The maximum-resistance level to IPM for each strain was obtained with

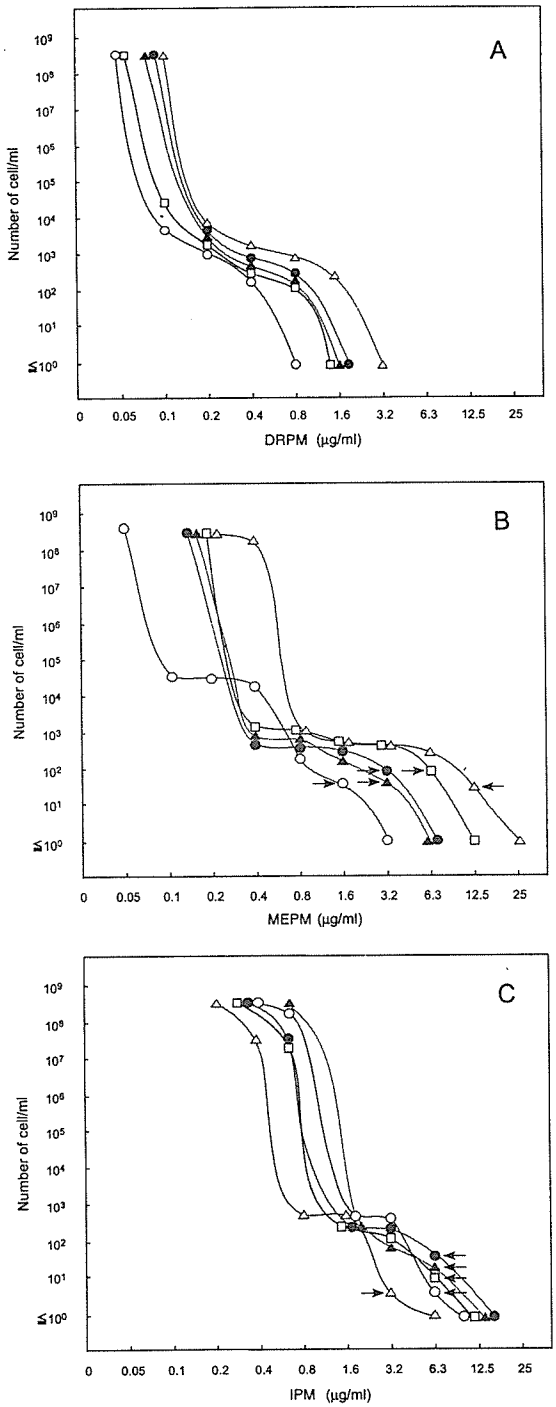

Fig. 2. Isolation of carbapenem-resistant mutant of *P. aeruginosa*.

Five representative strains are shown. Population analysis of each *P. aeruginosa* strain was carried out. Viable titers and antibiotic resistance levels (population analysis) of bacterial cells from each strain were determined by plating 0.1 ml diluted cultures on an AB3 agar plate containing different concentrations of each carbapenem. After overnight incubation of the plates at 37°C, the number of colonies was counted. Ten colonies from each strain that were picked from colonies grown on an agar plate containing one quarter or half of the drug concentration which completely inhibited cell growth of each strain were purified on drug free agar plates three times, and then examined for resistance levels to each carbapenem. The arrow shows that all colonies examined have stable resistance levels to each carbapenem. Viable cell number of each strain grown on selective agar plates containing different concentrations of DRPM (A), MEPM (B), and IPM (C), respectively, were counted. Symbols, ○, GP17; △, GP33; □, GP59; ●, GP2; ▲, GP3.

broth cultures containing drug concentrations ranging from 1/2 MIC to MIC, or two to four times the MIC for each of the strains. The drug concentrations of DRPM in the broth culture of each strain needed to select for carbapenem-resistant mutants showed a narrower drug concentration range than those of MEPM.

The range of DRPM concentrations required in broth culture to prevent growth of the mutants at the concentrations below the DPRM MIC for the carbapenem-resistant mutants of each strain were greater than those of MEPM, and equal to or greater than those of IPM.

Discussion

The mutants of the *P. aeruginosa* strain selected on agar

Table 2 Isolation frequency of carbapenem-resistant mutants and drug susceptibilities of the mutant strains

Strain No.	Drug	Carbapenem susceptibility of parent MIC (μg/ml)	Carbapenem susceptibility of mutant MIC (μg/ml)	Drug concentration in selective agar plate[a] (μg/ml)	Isolation frequency of carbapenem-resistant mutant (per cell per generation)
GP2	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	6.3	3.2	1.74×10^{-8}
	IPM	1.6	12.5	6.3	4.81×10^{-8}
GP3	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	6.3	3.2	7.95×10^{-7}
	IPM	1.6	25	6.3	1.60×10^{-8}
GP5	DRPM	0.8	6.3	3.2	$<2\times10^{-9}$
	MEPM	3.2	12.5	6.3	4.57×10^{-8}
	IPM	3.2	12.5	6.3	1.45×10^{-8}
GP10	DRPM	0.2	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.2	3.2	1.6	1.61×10^{-8}
	IPM	1.6	12.5	6.3	2.90×10^{-9}
GP13	DRPM	0.1	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.1	3.2	1.6	1.09×10^{-7}
	IPM	1.6	12.5	6.3	4.63×10^{-8}
GP14	DRPM	0.2	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.2	3.2	1.6	8.47×10^{-9}
	IPM	3.2	25	6.3	2.90×10^{-9}
GP17	DRPM	0.1	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.1	3.2	1.6	8.36×10^{-8}
	IPM	1.6	25	6.3	3.07×10^{-8}
GP33	DRPM	0.2	6.3	3.2	$<2\times10^{-9}$
	MEPM	0.8	25	12.5	1.41×10^{-8}
	IPM	0.8	12.5	3.2	2.17×10^{-8}
GP59	DRPM	0.1	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	12.5	6.3	2.57×10^{-8}
	IPM	1.6	12.5	6.3	1.22×10^{-8}
GP66	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.8	12.5	6.3	4.96×10^{-8}
	IPM	1.6	12.5	6.3	4.51×10^{-9}

[a] To determine the drug concentrations for selective agar plates, preliminary experiments were performed several times with different concentrations of each carbapenem for selective agar plates for each strain, and the isolation frequency of the mutant and the carbapenem-resistance levels were examined, and the most competent concentration of each carbapenem for each strain to specifically select the carbapenem-resistant mutant was determined. The data obtained in the experiment shown in Fig. 3 were also used to determine the drug concentration.

plates containing each of the carbapenems exhibited resistance or reduced susceptibility to carbapenems, and showed the same susceptibility to other antibiotics as the parent strain. The mutant strains exhibited a marked reduction in the concentration of a specific outer membrane protein with a molecular weight of about 48,000. These data indicated that the carbapenems predominantly selected carbapenem-resistant mutants that lacked or had a reduced expression of D2 porin (OprD) [3~7], although there was also a possibility that a mutant with increased expression of MexAB-OprM would be present in the culture [16~19].

The anti-pseudomonas activities of carbapenems are affected by carbapenem-specific porin OprD [3~7] and MexAB-OprM expression [16~22], which have influx and efflux functions, respectively. The loss or reduced expression of OprD of the *P. aeruginosa* strain increases the MICs of the carbapenems [3~7]. Previous studies suggested that the activities of MRPM and DRPM, but

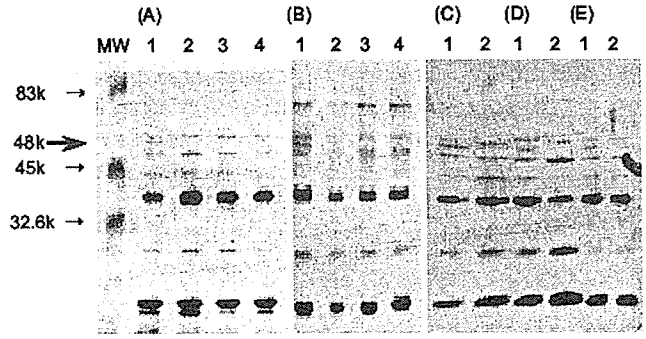

Fig. 3. SDS-PAGE of outer membrane proteins from carbapenem susceptible *P. aeruginosa* strains and the carbapenem-resistant mutants.

(A) *P. aeruginosa* GP3 and the mutants. lane 1, carbapenem susceptible GP3; lane 2 to lane 4, carbapenem-resistant mutants of GP3 isolated from selective agar plate containing DRPM, MEPM and IPM, respectively. (B) *P. aeruginosa* GP33 and the mutants. lane 1, carbapenem susceptible GP33; lane 2 to lane 4, carbapenem-resistant mutants of GP33 isolated from selective agar plate containing IPM, MEPM, and DRPM, respectively. (C) lane 1, carbapenem susceptible *P. aeruginosa* GP2; lane 2, carbapenem-resistant GP2 isolated from agar plate containing DRPM. (D) lane 1, carbapenem susceptible *P. aeruginosa* GP66; lane 2 carbapenem-resistant GP66 isolated from agar plate containing MEPM. (E) lane 1, carbapenem susceptible *P. aeruginosa* GP17; lane 2 carbapenem-resistant GP17 isolated from agar plate containing IPM; MW, molecular weight marker: bold arrow indicates outer membrane protein with molecular weight around 48,000. The carbapenem resistant mutant isolated from the selective agar plate containing doripenem was derived from the broth culture containing carbapenem (doripenem) to selectively increase the mutant strain as shown in following experiment described in Fig. 4.

not IPM, are also decreased by the increased production of the efflux protein MexAB-OprM [16, 17, 19, 21], although this mechanism rarely confers outright nor does it confer high level resistance. It confers almost the same level of resistance as is seen in the altered OprD-derivative [19, 21]. This type of mutant is predominantly selected by carbenicillin or CAZ [19]. A multiple resistance mechanism requiring both a lack of OprD for influx and increased expression of MexAB-OprM for efflux would be required to confer a relatively high resistance to MEPM and DRPM for *P. aeruginosa* [17, 19, 22~24]. This type of mutant has been isolated by selection of a MEPM-resistant mutant from a OprD deficient derivative on agar containing MEPM [23]. This type of double mutant would not be selected readily during carbapenem therapy [19, 21, 23, 24].

The MIC_{90} values of DRPM for *P. aeruginosa* strains were one dilution lower than those of MEPM and eight dilutions lower than IPM, indicating that the MICs of IPM were the highest of the three carbapenems [9, 12, 19]. DRPM resembled MEPM, and was more effective than IPM with respect to the MICs for *P. aeruginosa* strains. However, the potency of selection for the mutant cells or the potency of growth prevention of the mutant cells was different for DRPM, MEPM and IPM. A combination of population analysis to determine the presence of the carbapenem-resistant mutant in the culture of each strain (Fig. 1 and Fig. 2), the calculation of the isolation frequency of the carbapenem-resistant mutant on agar containing each carbapenem (Table 2), and experiments to selectively increase the carbapenem-resistant mutant in broth culture containing different concentrations of each carbapenem (Fig. 4) resulted in our drawing the following conclusions: IPM and MEPM selected the carbapenem-resistant mutant at the drug concentration that inhibited growth of the parent strain at a frequency of around 10^{-7}~10^{-8} per cell of the culture. However, DRPM could not specifically select the mutant strain at a drug concentration capable of inhibiting growth of the parent strain. In contrast to MEPM and IPM, the mutant could not be selected on agar at concentrations of four to 16 times the MIC of DRPM for the parent strain, which were 1/2 MIC for the mutant strains. The maximum drug concentrations of DRPM, MEPM, and IPM in the broth culture that allowed selective increase in carbapenem-resistant mutant in the broth culture were 1/8 to 1/16 MIC of DRPM, 1/2 to 1/4 MIC of MEPM and 1/2 to 1/16 MIC of IPM for the mutant strains, respectively. These data indicated that DRPM has the greatest potency in preventing the emergence of the carbapenem-resistant mutants. The present study suggested that the clinical evaluation of DRPM for the prevention of the emergence of the mutant strain may be worthwhile.

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Strain No.	Selective drug in broth culture	Drug susceptibility (MIC (μg/ml))		MIC for culture with selective drug / MIC for culture without selective drug	Range of drug concentration (ratio to MIC for wild type strain) which gave rise to the maximum resistance level
		Culture without selective drug (wild type strain)	Culture with selective drug[a]		1/4 1/2 1 2 4 8 16 32
GP3	Doripenem	0.2	3.2	16	*
	Meropenem	0.4	6.3	16	*
	Imipenem	1.6	25	16	*
GP13	Doripenem	0.1	1.6	16	*
	Meropenem	0.1	3.2	32	*
	Imipenem	1.6	12.5	8	*
GP14	Doripenem	0.2	1.6	8	*
	Meropenem	0.2	3.2	16	*
	Imipenem	3.2	25	8	*
GP17	Doripenem	0.1	1.6	16	*
	Meropenem	0.1	3.2	32	*
	Imipenem	1.6	25	16	*
GP33	Doripenem	0.2	6.3	32	*
	Meropenem	0.8	25	32	*
	Imipenem	0.8	12.5	16	*
GP59	Doripenem	0.1	3.2	32	*
	Meropenem	0.4	12.5	32	*
	Imipenem	1.6	12.5	8	*

Fig. 4. Increased carbapenem resistance levels of *P. aeruginosa* strains after incubation with carbapenem.

Overnight culture of each strain was diluted appropriately. Approximately 10^6 bacterial cells of each strain were inoculated into 1 ml of AB3 broth containing each different concentrations of each carbapenem and were incubated at 37°C for 18 hours with gentle shaking. After incubation, the resistance levels (MICs) to the same carbapenem used in broth culture were examined against each strain using agar dilution methods with around 10^4 inoculated cells. In this experiment, approximately 10^4 CFU or 10^6 CFU were inoculated onto each of a series of agar plates containing various antibiotic concentrations. The resistance levels of each strain in the broth culture containing each of the drugs were not essentially different from the different inoculum sizes on the drug plates. The results obtained by inoculating approximately 10^4 cells, are shown in here. [a]The MIC of each carbapenem in culture with the selective drug indicated the maximum level in the increased MIC for the cultures. Asterisk (*) in figure indicates drug resistance level (MIC) of each carbapenem for the strain with maximum resistance (*i.e.*, carbapenem-resistant mutant) where the MIC was indicated by ratio of MIC against a culture with selective drug to MIC against the culture without the selective drug (*i.e.*, MIC against the wild type strain).

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Organization of Tn*2610* Containing Two Transposition Modules

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Transposon Tn*2610*, found in a conjugative plasmid from an *Escherichia coli* isolate recovered at a hospital in Chiba, Japan, in 1975, was completely sequenced. Tn*2610* is 23,883 bp long and is bracketed by two transposition modules, a Tn*1721*-like module and a Tn*21*-derived module, which correspond, respectively, to the long inverted repeats IRa and IRb previously described for this transposon. Although both *tnpA* genes are intact, only that in the Tn*21*-derived module (IRb) functions in the transposition, while that in the Tn*1721*-derived module (IRa) cannot recognize the 38-bp imperfect repeat at the end of the IRb element. Both *tnpR* and *res* are present in IRa, while the *tnpR* gene of IRb is interrupted by the insertion of an IS*26* insertion element. The intervening region, between the *res* site of the Tn*1721* module and IS*26*, carries multiple integron-associated resistance genes within a Tn*21* backbone, including a region identical to that found in the genome of *Salmonella enterica* serovar Typhimurium DT104. These findings suggest that Tn*2610* originated from Tn*1721* and Tn*21*, with extensive recombination events with other elements which have resulted in a complex mosaic structure.

Tn*2610* is a multidrug resistance transposon that was originally identified in 1983 on a self-transmissible plasmid, pCS200, originating from an *Escherichia coli* strain isolated in 1975 at a hospital in Chiba, Japan (20). Tn*2610* is 24 kb long and is flanked by 3-kb inverted-repeat (IR) sequences. The intervening nonrepeated region was shown to include genes for resistance to ampicillin, streptomycin, and sulfonamide. Preliminary analysis revealed that Tn*2610* carries two copies of the transposition genes *tnpA* and *tnpR* and that these regions form a stable heteroduplex (19).

Several large transposons conferring resistance to more than one antibiotic have been identified. Among these, Tn*21*, Tn*1691*, Tn*2603*, and Tn*2424*, which are classified as class II transposons, seem to be evolutionarily related (11, 13, 18). On the basis of restriction maps and heteroduplex analyses, we originally proposed that the Tn*21*-related transposons had descended from an ancestral mercury resistance transposon, resembling Tn*2613*, by subsequent insertions of antibiotic resistance genes and/or insertion sequences (18). This hypothesis has been supported by sequence data from a large group of these transposons (11). Current knowledge on Tn*2610* suggests that it may also have evolved from an ancestral mercury resistance transposon via a series of recombination events resulting in a complex configuration.

To confirm this hypothesis, we determined the complete sequence of Tn*2610* and compared its structure to that of other known elements.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *E. coli* DH5α (*supE44* Δ*lacU169* [φ80 *lacZ* ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), AB2463 (*recA thr leu thi lac gal ara xyl mtl pro his arg str tsx sup*), and P3478Rif, a rifampin-resistant mutant of P3478 (*thy polA*). The Tn*2610*-containing plasmid used in this study was pTKY170, formerly termed pMK1::Tn*2610*#4 (20). Subcloning for DNA sequencing was performed in pUC18 (17). Plasmids pTKY171 and pTKY172 are pAO3 derivatives carrying Tn*1722* and Tn*1722* with a kanamycin-resistant determinant, respectively. Plasmid pAO3 is a small derivative of plasmid ColE1 (21). Plasmid pTKY173 is a pTKY172 derivative defective in the *tnpA* gene. Plasmids pTKY174 and pTKY175 are pACYC184-based plasmids loaded with *tnpA* genes from Tn*2610* and Tn*1722*, respectively. Bacterial cells were routinely cultured at 37°C in Luria-Bertani (LB) medium or on LB agar. For selection with trimethoprim, Mueller-Hinton agar (Difco Laboratories) was used with 0.5% (vol/vol) lysed horse blood. Antibiotics were added at the following concentrations: ampicillin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 25 μg ml^{-1}; trimethoprim, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}.

Construction of plasmids. Plasmid pTKY171 (pAO3::Tn*1722*) was derived from plasmid pAO3::Tn*1721* by deletion of the DNA fragment between the SalI site at nucleotide (nt) 6946 in Tn*1721* (Fig. 1) and the BstEII site in pAO3. The pUC4K-derived kanamycin resistance determinant was inserted into the AvaI site at nt 991 in Tn*1722* (Fig. 1), resulting in pTKY172. Plasmid pTKY173 is an AvaI-generated deletion of pTKY172 which was cleaved at nt 2855 and nt 4562 in Tn*1722* and self-ligated. The DNA fragment between the EcoRI site at nt 13 and the BamHI site at nt 4157 in Tn*2610* from pTKY170 was cloned into BamHI-digested pACYC184, resulting in pTKY174. Plasmid pTKY175 was constructed by cloning of the DNA fragment between nt 13 and nt 5624 in Tn*1722* into EcoRI-digested pACYC184.

Determination of transposition proficiency. The mating-out assay was used to determine transposition frequency (19). The pACYC184-based plasmid loaded with a relevant *tnpA* gene was introduced into AB2463 containing R388, a conjugative plasmid devoid of transposable elements, and pTKY173. The resulting strain was used as a donor to mate with the recipient strain P3478Rif. Donor and recipient strains were mixed in a 1:5 ratio and passed through a Millipore filter, and then the filters were incubated on the agar plates at 37°C for 6 h. Transconjugants receiving R388::Tn*1722* were selected with kanamycin and rifampin. Transconjugants receiving R388 were selected with trimethoprim and rifampin. The transposition frequency was expressed as the ratio of the number of kanamycin-resistant transconjugants to the number of R388 transconjugants.

DNA isolation and restriction mapping. Plasmid DNA for restriction analysis and cloning was isolated by the alkaline lysis method (3). Restriction enzymes (TaKaRa Bio Inc., TOYOBO) were used in accordance with the manufacturer's

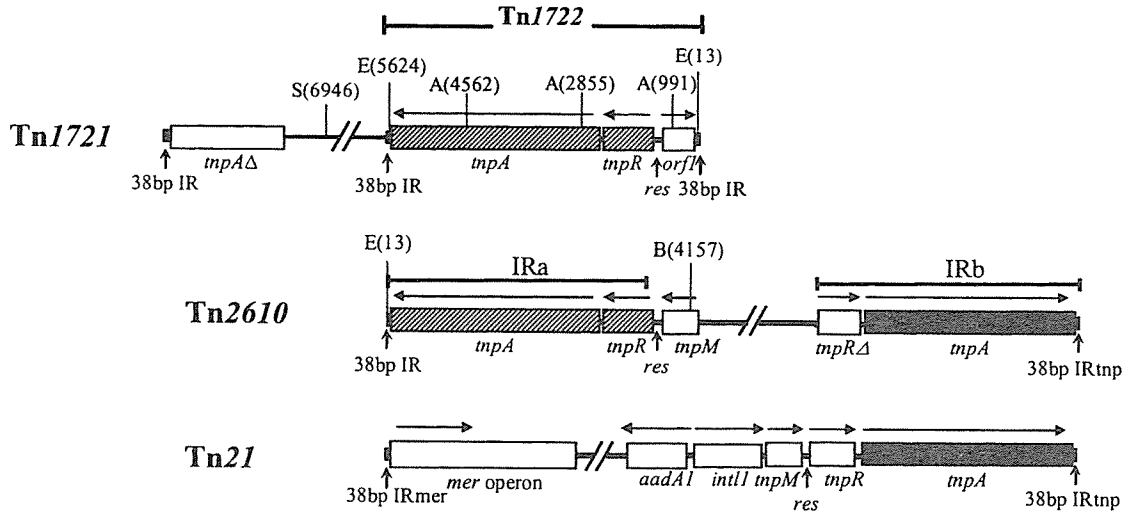

FIG. 1. Structure of the transposition modules forming the backbone of Tn*2610*. Arrows above open bars indicate the transcription orientations of the ORFs. Restriction cleavage sites shown are those used for the construction of plasmid derivatives described in Materials and Methods. The structures and restriction sites are based on GenBank sequences for Tn*2610* (accession no. AB207867) and Tn*1721* (X61367). Abbreviations: A, AvaI; B, BamHI; E, EcoRI; S, SalI.

TABLE 1. Features of ORFs and discrete DNA segments in Tn*2610*

Location[a] (5′–3′)	DNA segment	ORF[b]			% Homology (DNA/aa)	Description of gene or gene product (accession no.)
		No.	Name	Size[c]		
1–38	IR					38-bp IR in 1Ra of Tn*2610*
34–3000		S01	*tnpA*	988	99.7/99.2	Transposase from Tn*1721* (X61367)
3004–3564		S02	*tnpR*	186	99.8/100	Resolvase from Tn*1721* (X61367)
3627–3755	*res*					*resIII* (3627–3656), *resII* (3661–3703), *resI* (3717–3755)
3740–4324		S03	*tnpM*	194	100/100	TnpM on Tn*21* (AF071413)
4091–5376	5′-CS					Partial 5′-CS of class 1 integron (AF261825)
4091–4114	IRi					IRi of class 1 integron (AF261825)
4293–5306		S04	*intI1*	337	100/100	Integrase of class 1 integron (AY214164)
5873–6079		S05	small ORF	68	100/100	Small ORF from pIP1527 (X03988)
6079–7338		S06	*ereB*	419	100/100	Erythromycin esterase type II (X03988)
7661–9145		S07	orf2	494	100/100	Putative OrfA from SGI1 (AF261825)
9420–10073		S08	*groEL/intI1*	217	100/100	GroEl/integrase fusion protein from SGI1
9777–10209	5′-CS					Partial 5′-CS of class 1 integron (AF261825)
10154–10209	*attI*					*attI* of class 1 integron (AF261825)
10279–11145		S09	*pse-1*	288	100/100	PSE-1 β-lactamase from SGI (AF261825)
11275–12054		S10	*aadA2*	259	100/100	Streptomycin resistance protein (AF164956)
12056–12114	59 bp					59-bp element of class 1 integron (AF261825)
12114–14134	3′-CS					3′-CS of class 1 integron (AF261825)
12218–12565		S11	*qacEΔ1*			Quaternary ammonium compound and disinfectant resistance partial protein on SGI
12559–13398		S12	*sul1*	279	100/100	Sulfonamide resistance protein on SGI
13526–14026		S13	*orf5*	166	100/100	Putative acetyltransferase in Tn*21* (AF071413)
14050–14134		S14	*orf6Δ*			Hypothetical partial protein (AF261825)
14135–14160	IR					Terminal IR of IS*1326* (AY123253.3)
14202–14987		S15	*istB*	261	100/100	Unknown function of IS*1326* (AY123253.3)
14974–16497		S16	*istA*	507	100/100	Possible transposase of IS*1326* (AY123253.3)
16579–16590	IR					Terminal IR of IS*1353* (AY123523)
16620–18164		S17	OrfAB	516	100/100	OrfAB from IS*1353* (AY123523)
18180–18192	IR					Terminal IR of IS*1353* (AY123253.3)
18195–18280	IR					Terminal IR of IS*1326* (AY123253.3)
18215–19075		S18	*tniBΔ1*			Truncated TniB from In2 (U42226)
19078–19512		S19	*tniA*			Partial TniA of In2 (U42226)
19513–19527	IR					IR of IS*26* (AY123523)
19575–20282		S20	*tnpA*	235	100/100	Transposase of IS*26* (AY123523)
20321–20333	IR					IR of IS*26* (AY123523)
20334–20881		S21	*tnpR*			Truncated resolvase of Tn*21* (AF071413)
20884–23850		S22	*tnpA*	988	100/100	Transposase of Tn*21* (AF071413)
23846–23883	IR					38-bp IR in IRb of Tn*2610*

[a] Nucleotide position in the sequence deposited under accession no. AB207867.
[b] Named ORFs are based on those previously characterized.
[c] Expressed as the number of amino acid residues.

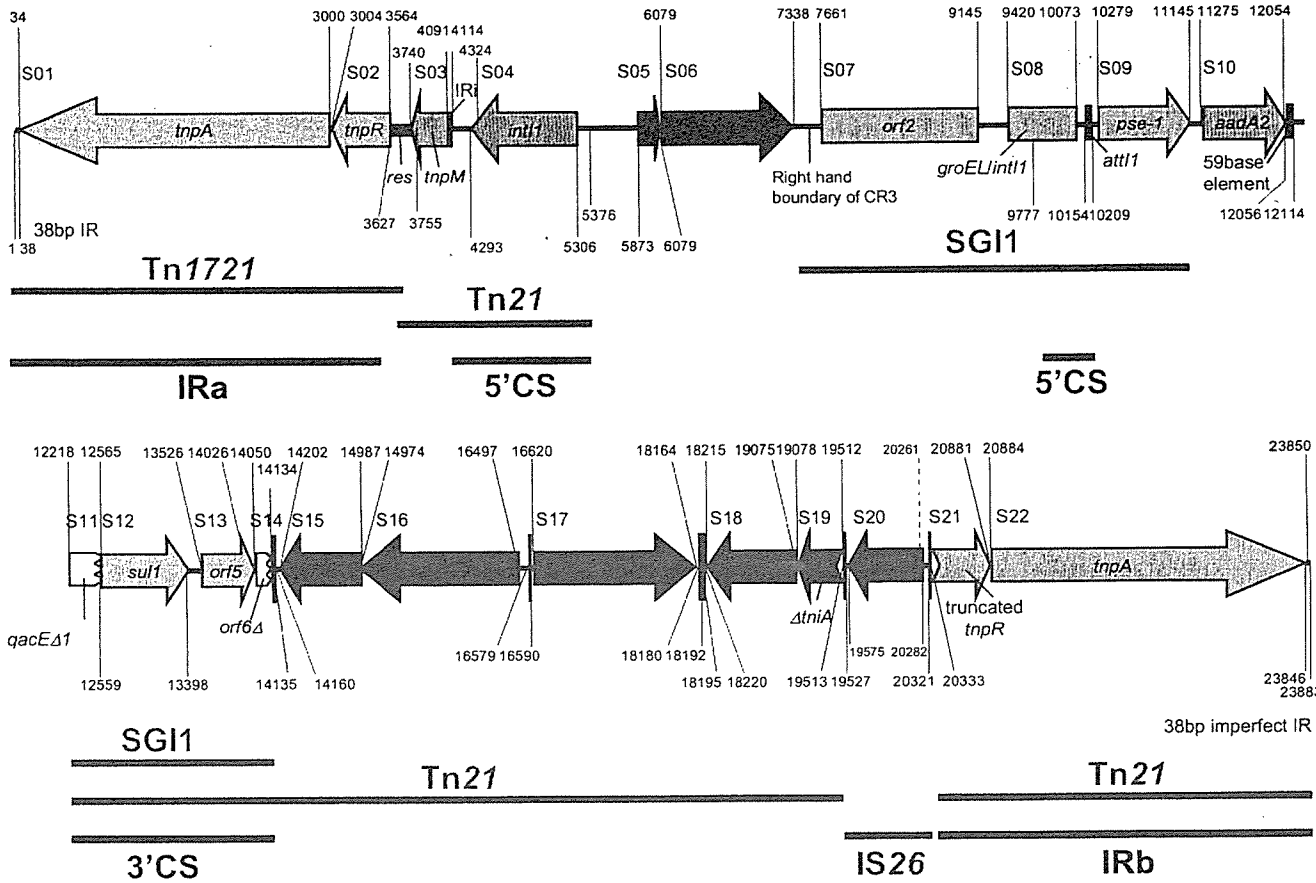

FIG. 2. Genetic organization of Tn*2610* based on complete nucleotide sequence analysis. Labeled lines represent the regions in which sequences exhibit significant homology to extant sequences on various genetic elements. The accession numbers of the sequences used for comparative analysis are given in Table 1.

instructions. DNA fragments were separated by electrophoresis on 1% (wt/vol) agarose gels, and individual fragments were isolated from the gels using a QIAEX II gel extraction kit (QIAGEN). HindIII-digested lambda phage DNA fragments and HinfII-digested pBR322 plasmid DNA fragments were used as size markers.

Sequence analysis. Sequencing was performed in the facility at QIAGEN, Japan, on an ABI PRISM model 3100 sequencer. DNA sequences were assembled using the GENETYX version 10.1 software package. PCR was used to amplify the pTKY170 fragments to confirm the boundaries between the cloned fragments predicted by mapping and to obtain sequences. The sequence obtained was used to query the GenBank database in order to identify putative genes by using the BLAST program via the World Wide Web interface of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih/BLAST).

Nucleotide sequence accession number. The 23,883-bp sequence of Tn*2610* has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AB207867.

RESULTS AND DISCUSSION

General features of the Tn*2610* sequence. To complete the sequence of Tn*2610*, plasmid pTKY170 (formerly named pMK1::Tn*2610*#4 [20]), containing a complete copy of the transposon, was initially subjected to restriction analysis, and relevant fragments were subcloned for sequencing. The complete sequence of the 23,883-bp region bracketed by the previously characterized inverted repeats IRa (formerly named IR-R) and IRb (formerly named IR-L), which are defined by 5-bp direct repeats at their outer ends, was determined. A total of 22 open reading frames (ORFs) were identified and are listed in Table 1. The putative products encoded by these ORFs either were identical to or exhibited significant homology to protein sequences available in GenBank. The nature and positions of relevant features (even in the noncoding regions) are also shown in Table 1. Figure 2 is a linear map of Tn*2610* showing the transposon structure.

Tn*2610* contains two modules for transposition. Analysis of the sequence showed that Tn*2610* is composed of two transposition modules, a Tn*1721*-like module and a Tn*21*-derived module, which correspond, respectively, to IRa and IRb (Fig. 2). Within the Tn*1721*-like (IRa) module, the transposase gene *tnpA*, the resolvase gene *tnpR*, and the *res* site show strong homology to the corresponding regions of Tn*1721* (Table 1; Fig. 2 and 3) (1). A 38-bp sequence was identified at one end of IRa, differing in 3 bases from that in Tn*1721* (Fig. 3A). The 129-bp *res* site of IRa is identical to that of Tn*1721* as far as *resI*, where recombination has occurred with the *res* site of Tn*21* (Fig. 3B) (16).

In contrast, IRb is a Tn*21* remnant including a partially deleted *tnpR* gene, *tnpA*, and a 38-bp IR identical to those in

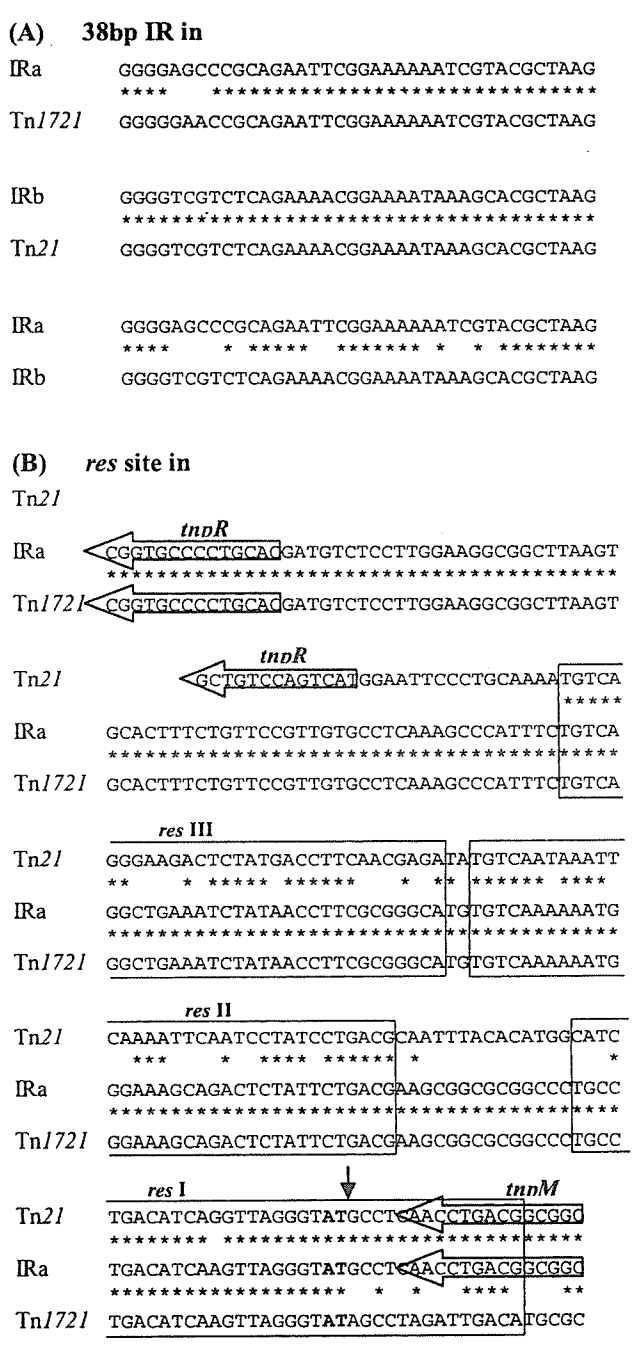

FIG. 3. Comparison of the 38-bp inverted repeats (A) and *res* sites (B). The sequences of the 38-bp inverted repeats and *res* sites in Tn*2610* IRa (this study), Tn*1721* (accession no. X61367), and Tn*21* (accession no. AF071413) are aligned, with asterisks indicating identical bases. The *res* subsites (9) are boxed, and the AT site at which resolvase-mediated recombination takes place is boldfaced. The recombination crossover point to generate the hybrid *res* is indicated by a vertical arrow. *tnpR* and *tnpM*, regions adjacent to each end of the *res* sites.

Tn*21* (Fig. 2). The *tnpR* gene is interrupted by the insertion of an IS*26* insertion element, leading to the loss of 12 bp including the ATG start codon of the gene and the *res* site. Taking all these findings together, we conclude that Tn*2610* is bracketed by 38-bp imperfect IRs (10-bp differences) (Fig. 3) at both ends and carries two intact *tnpA* genes, one intact *tnpR* gene, and one *res* site as a transposition module.

TABLE 2. Complementation of a Tn*1722*Δ*tnpA* mutant

Complementing plasmid (*tnpA*)	Transposition frequency[a]
pTKY175 (Tn*1722*)	2.3×10^{-2}
pTKY174 (Tn*2610* IRa)	3.0×10^{-2}
pACYC184	$<10^{-4}$

[a] Determined as described in Materials and Methods.

Our earlier analysis indicated that *tnpA* in IRb is functional, while *tnpA* in IRa is not functional, in the transposition of Tn*2610* (19). Although the IRa module shows strong homology to that of Tn*1721*, the corresponding genes are not identical. The *tnpA* gene in IRa differs from the Tn*1721* sequence at nine positions, leading to the alteration of 7 amino acid residues. Furthermore, a 3-base difference is found in the 38-bp IR sequences between IRa and Tn*1721* (Fig. 3A). Therefore, the inability of *tnpA* in IRa to promote the transposition of Tn*2610* may be due to a mutation. To determine whether the *tnpA* gene of IRa is active, the ability of the product to promote the transposition of Tn*1721* was examined by complementation analysis of a Tn*1722 tnpA*-defective mutant as described in Materials and Methods. As shown in Table 2, the *tnpA* gene in IRa complemented the *tnpA* defect in Tn*1722*, suggesting that it is active even though it cannot promote the transposition of Tn*2610*. The Tn*1721*-like *tnpA* product of IRa probably cannot recognize the 38-bp element at the end of IRb, while the Tn*21*-like *tnpA* product of IRb recognizes both 38-bp elements, even though they are imperfect. This hypothesis is supported by a report showing that the Tn*21 tnpA* products can act on the IR of Tn*501* (which is identical to the IR of Tn*1721*) but the Tn*501 tnpA* product cannot promote the transposition of Tn*21* (8).

The intervening nonrepeated region of Tn*2610*. Analysis of the intervening nonrepeated region from nt 3514 to nt 20333 reveals the presence of discrete DNA regions carried between the two transposition modules in Tn*2610* (Fig. 2).

The Tn*1721*-like transposition module merges (at the *res* site) with a Tn*21*-derived sequence (nt 3565 to 5376) including *tnpM* and the 5′ conserved segment (5′-CS) of the class 1 integron (the insertion site of the integron IRi into *tnpM* is identical to that in Tn*21*). The *intI1* gene, in this case, is not preceded by an *attI1* site (14) with inserted gene cassettes but by a segment containing the *ereB* gene (2) with part of CR3 (15). The right-hand boundary of CR3 merges with a long region (nt 7339 to 14134) identical to a part of *Salmonella* genomic island I (SGI1) (5) of *Salmonella enterica* serovar Typhimurium phage type DT104 except for the presence of an additional *aadA2* cassette in Tn*2610*. This region includes *orf2* (5, 11), the remnant of the 5′-CS of a class 1 integron containing an *intI1-groEL* hybrid, two gene cassettes (bla_{PSE-1} and *aadA2*), and the 3′-CS of a class 1 integron including *qacE*Δ*1*, *sul1*, *orf5*, and *orf6*Δ (Fig. 2), which is also identical to that found in In2 carried on Tn*21* (11, 15). The homology with In2 continues down to the *tniA* gene, which is interrupted by the

IS*26* insertion element (at nt 20333) located between this region and IRb (Fig. 2).

This complex mosaic structure is likely derived by multiple recombination events which involved Tn*21*-like and SGI1-like sequences, as well as other sequences.

Concluding remarks. The present study has shown that Tn*2610* is a composite transposon comprising two transposition modules, Tn*1721*-like IRa and Tn*21*-derived IRb, surrounding a central region containing the drug resistance genes *ereB*, *pse-1*, *aadA2*, and *sul1*. It is proposed that the ancestors of Tn*1721* and Tn*21* were independently inserted into a plasmid or genome via transposition events catalyzed by their own transposition modules, leading to the backbone of Tn*2610*. Later, genes could have been lost by deletion during or after the acquisition of the regions that include the integrons. This seems plausible, since a transposon carrying *mphB* with an organization very similar to that of the transposition modules in Tn*2610* has been found in *E. coli* (12).

SGI1 has been identified in DT104, whose prevalence increased dramatically in the 1990s (4, 5, 7, 10). DT104 isolates have been reported to be resistant to a core group of antibiotics including ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (commonly abbreviated ACSSuT). Furthermore, a number of variants of SGI1 that are associated with different resistance phenotypes (e.g., ACSSuS plus trimethoprim, SSu, ASu, and ASSuT) have been identified, suggesting that the multidrug resistance region of SGI1 was subject to recombination events that generated variants (4, 6). Since Tn*2610* was found in a plasmid from a strain isolated before SGI1-containing strains, it could also have been involved in the generation of SGI1 structures.

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Evaluation of antimicrobial activity of β-lactam antibiotics by Etest against clinical isolates from 100 medical centers in Japan (2004)

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Abstract

This antimicrobial resistance surveillance study was performed in 100 medical centers. The susceptibility of 9347 strains including *Escherichia coli* (997 strains), *Klebsiella* spp. (997 strains), *Enterobacter* spp. (988 strains), *Citrobacter* spp. (834 strains), indole-positive *Proteae* spp. (855 strains), Serratia spp. (925 strains), *Acinetobacter* spp. (902 strains), *Pseudomonas aeruginosa* (996 strains), oxacillin-susceptible *Staphylococcus aureus* (992 strains), and coagulase-negative staphylococci (861 strains) to 7 β-lactam antibiotics, cefepime, cefpirome, ceftazidime, cefoperazone/sulbactam, imipenem and piperacillin (for Gram negatives), or oxacillin (for Gram positives) was tested. No strain resistant to these β-lactams except for ceftazidime was found in oxacillin-susceptible *S. aureus* and coagulase-negative staphylococci. *E. coli* (16.5%) clinical isolates were resistant to piperacillin, whereas 1.5% or less (cefpirome = 1.5%) was resistant to other β-lactams. *Klebsiella* spp. strains were more susceptible to imipenem (99.7%), cefepime (98.4%), and cefpirome (97.3%). Isolates of *Enterobacter* spp., *Citrobacter* spp., indole-positive *Proteae*, and *Serratia* spp. were susceptible to imipenem, cefepime, and cefpirome, as well. *Acinetobacter* spp. strains were most susceptible to cefoperazone/sulbactam (0.8% resistance), imipenem (3.2%), ceftazidime (6.0%), and cefepime (7.0%) than other β-lactam antibiotics tested. Isolates of *P. aeruginosa* were more susceptible to ceftazidime (9.9% resistance), cefoperazone/sulbactam (14.9%), and cefepime (11.2%) than piperacillin (15.5%), cefpirome (19.1%), and imipenem (19.3%). The percentage of imipenem-resistant *P. aeruginosa* is around 20% in clinical isolates in Japan.

Keywords: β-Lactams; Etest; Susceptibility; Drug resistance

1. Introduction

β-Lactam antibiotics are widely used in the clinical field. β-Lactamases are the major resistance mechanism toward these antibiotics in Gram-negative bacteria (Jacoby and Munoz-Price, 2005). Other resistance mechanisms for β-lactams are the decrease of target sensitivity because of modification of diffusion barriers and active efflux pumps (Nakae, 1995; Nikaido, 2001).

Plasmid-encoded class B β-lactamases, metallo-β-lactamases (MBLs), are classified into 3 main molecular groups: IMP-type, VIM-type, and SPM-type enzymes (Walsh et al., 2005). Gram-negative bacilli producing IMP-type and VIM-type MBLs have been increasingly reported in Europe, Asia, North America, and South America. IMP-1 was first described in Japan and is the predominant MBL. IMP-type MBLs have been found in clinical isolates such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Acinetobacter baumannii*, *Achromobacter xylosoxidans*, *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter youngae*, *Klebsiella pneumoniae*, or *Shigella flexneri* (Walsh et al., 2005). Metallo-β-lactamase–producing *P. aeruginosa* and *Providencia rettgeri* isolates were detected in our previous surveillance program in 2002 (Ishii et al., 2005; Kimura et al., 2005a; Shiroto et al., 2005). Kimura et al. (2005a) reported that only 1.9% of *P. aeruginosa* produced MBL.

A surveillance program by the Japan Antimicrobial Resistance Study Group was carried out from 1997 to 2002 (Ishii et al., 2005; Lewis et al., 1999; Yamaguchi et al., 1999). The present study was designed to provide up-to-date β-lactam antibiotic susceptibility in clinical isolates including *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp. (*Proteus*

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Table 1
Specimens used in this study

	S. aureus	CNS	*E. coli*	*Klebsiella* spp.	*C. freundii*	*Enterobacter* spp.	Indole-positive proteae	*Serratia* spp.	*Acinetobacter* spp.	*P. aeruginosa*
Urinary tract	45	163	550	240	275	167	376	197	95	188
Urine	15	94	389	149	191	117	235	127	59	118
Urinary catheter	6	18	100	71	68	36	116	61	27	61
Others	24	51	61	20	16	14	25	9	9	9
Pulmonary tract	372	120	89	387	101	401	80	445	522	491
Sputum	180	32	56	259	64	247	48	272	343	336
BALF	11	1	3	10	1	15	2	9	8	12
Intratracheal sputum	17	6	8	39	3	35	11	63	57	68
Pharyngeal mucus	84	28	15	52	22	72	14	52	82	38
Others	80	53	7	27	11	32	5	49	32	37
Gastrointestinal tract	16	11	110	118	290	114	169	37	26	33
Gastric or duodenal secretion	–	1	6	16	6	8	5	5	2	3
Feces	12	6	81	64	243	65	124	16	11	16
Others	4	4	23	38	41	41	40	16	13	14
Skin	282	133	75	77	72	111	106	86	70	106
Skin or decubitus	112	55	21	17	16	29	30	26	26	37
Abscess	170	78	54	60	56	82	76	60	44	69
Blood and fluids	91	221	130	113	62	118	63	75	84	73
Blood	66	174	100	69	17	52	22	38	68	35
Spinal fluid	–	4	–	–	–	–	–	1	–	–
Others	25	43	30	44	45	66	41	36	16	38
Additional Specimens	127	89	4	11	7	15	10	31	39	54
Ophthalmic secretion	19	34	1	2	–	6	6	9	7	6
Ear secretion	108	55	3	9	7	9	4	22	32	48
Unspecified	59	124	39	51	27	62	50	55	65	51

CNS = coagulase-negative staphylococci; BALF = bronchoalveolar lavage fluid.

vulgaris, *Providencia* spp., and *Morganella morganii*), *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, oxacillin-susceptible *Staphylococcus aureus*, and oxacillin-susceptible coagulase-negative staphylococci in Japan in 2004. One hundred hospitals participated in this surveillance program during 2004. Participating centers covered all regions in Japan. These nationwide surveillance data include the newest information on the problem of drug-resistant bacteria.

2. Materials and methods

2.1. Bacterial isolates

The collection and subsequent testing of clinical isolates by the 100 participant centers began in July and concluded in September 2004. Each participant center had an average of 726 beds. Forty-seven and 31 participating centers use MicroScan WalkAway system (Dade Behring, Tokyo, Japan) and Vitek system (bioMérieux, Tokyo, Japan) to identify the organisms, respectively. Fourteen centers used other systems such as Phoenix system (Nippon Becton Dickinson, Tokyo, Japan), Raisus system (Nissui Pharmaceutical, Tokyo, Japan) or Api sires (bioMérieux), Enterotube system (Nippon Becton Dickinson), and so on. We could not confirm the system used in 8 participating centers. Each laboratory was instructed to comprise its respective collection of consecutive bacterial strains of up to 10 isolates from a single patient each of 10 designated species groups as stated in a prevalence format. These 10 organism groups were *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp., *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, oxacillin-susceptible *S. aureus* (minimal inhibitory concentration [MIC], ≤ 2 μg/mL), and oxacillin-susceptible coagulase-negative staphylococci (MIC, ≤ 0.25 μg/mL). The combined overall collection of bacterial strains by the 100 centers totaled 9347 strains including 997 *E. coli*, 997 *Klebsiella* spp., 988 *Enterobacter* spp., 834 *Citrobacter* spp., 855 indole-positive *Proteus* spp., 925 *Serratia* spp., 902 *Acinetobacter* spp., 996 *P. aeruginosa*, 992 oxacillin-susceptible *S. aureus*, and 861 oxacillin-susceptible coagulase-negative staphylococci. The specimens from which the strains in this study were isolated are listed on Table 1. Although compliance was complete, 1 *S. aureus* strain was omitted from the analysis because the documented oxacillin-resistant *S. aureus* criteria were redefined by the Clinical and Laboratory Standards Institute (CLSI, 2005; formerly the National Committee for Clinical Laboratory Standards) during the protocol period. In addition, 6 *Proteae* isolates were also omitted from analysis because these strains were identified as *Proteus mirabilis*, an indole-negative *Proteae* by Phoenix system (Nippon Becton Dickinson) in the Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Tokyo, Japan.

Table 2
Antimicrobial activity of 7 tested β-lactams against clinical isolates (2004)

Organism (%) (no. tested)	Antibiotics	MIC (μg/mL)		MIC (μg/mL) (range)	Category	
		50%	90%		Susceptible	Resistance
S. aureus (992)	Oxacillin	0.38	0.75	0.023 to 2	100.0	0.0
	Ceftazidime	12	16	4 to >256	20.0	2.2
	Cefepime	4	4	0.5 to 24	99.7	0.0
	Cefpirome	1	2	0.064 to 4	100.0	0.0
	Cefoperazone/sulbactam (2:1)	3	4	0.38 to 16	100.0	0.0
	Imipenem	0.032	0.047	<0.016 to 0.38	100.0	0.0
Coagulase-negative staphylococci (861)	Oxacillin	0.19	0.25	<0.016 to 0.25	100.0	0.0
	Ceftazidime	6	12	0.5 to >256	83.0	2.7
	Cefepime	1	2	0.064 to >256	99.0	0.5
	Cefpirome	0.5	1	0.032 to >256	99.5	0.3
	Cefoperazone/sulbactam (2:1)	1.5	4	0.25 to >256	99.7	0.3
	Imipenem	0.023	0.047	<0.016 to >256	99.4	0.5
E. coli (997)	Piperacillin	2	>256	0.023 to >256	72.4	16.5
	Ceftazidime	0.19	0.5	<0.016 to >256	98.3	1.0
	Cefepime	0.047	0.125	<0.016 to >256	98.8	0.9
	Cefpirome	0.064	0.19	<0.016 to >256	97.9	1.5
	Cefoperazone/sulbactam (2:1)	0.25	2	<0.016 to >256	98.0	0.7
	Imipenem	0.25	0.5	<0.016 to 6	99.9	0.0
Klebsiella spp. (997)	Piperacillin	8	>256	0.5 to >256	82.2	11.2
	Ceftazidime	0.19	0.75	<0.016 to >256	98.5	1.1
	Cefepime	0.064	0.25	<0.016 to >256	98.4	0.8
	Cefpirome	0.064	0.38	<0.016 to >256	97.3	1.4
	Cefoperazone/sulbactam (2:1)	0.38	2	0.047 to >256	95.6	3.9
	Imipenem	0.25	0.5	0.032 to 16	99.7	0.2
C. freundii (834)	Piperacillin	3	>256	0.5 to >256	76.1	19.2
	Ceftazidime	0.5	>256	0.064 to >256	81.8	16.7
	Cefepime	0.064	2	<0.016 to >256	97.4	1.6
	Cefpirome	0.064	4	0.023 to >256	96.2	2.0
	Cefoperazone/sulbactam (2:1)	0.75	32	0.064 to >256	85.9	5.9
	Imipenem	0.5	1.5	0.032 to >256	99.8	0.1
Enterobacter spp. (988)	Piperacillin	3	>256	0.125 to >256	78.2	14.5
	Ceftazidime	0.25	96	0.023 to >256	80.2	16.8
	Cefepime	0.064	1.5	<0.016 to >256	96.9	1.7
	Cefpirome	0.094	4	0.023 to >256	93.3	3.4
	Cefoperazone/sulbactam (2:1)	0.5	32	<0.016 to >256	88.2	7.1
	Imipenem	0.5	1.5	0.016 to >256	99.2	0.1
Indole-positive *Proteae* (855)	Piperacillin	0.75	24	0.047 to >256	89.4	6.0
	Ceftazidime	0.094	1	<0.016 to >256	95.8	2.0
	Cefepime	0.064	0.25	<0.016 to >256	98.6	1.2
	Cefpirome	0.094	0.5	<0.016 to >256	97.4	1.9
	Cefoperazone/sulbactam (2:1)	1	4	0.064 to >256	98.4	0.7
	Imipenem	2	3	<0.016 to >256	98.0	0.7
Serratia spp. (925) (cefoperazone/sulbactam, 924)	Piperacillin	3	96	0.023 to >256	80.5	9.8
	Ceftazidime	0.25	1.5	0.023 to >256	96.2	3.5
	Cefepime	0.125	1	0.023 to >256	96.0	2.6
	Cefpirome	0.094	0.75	<0.016 to >256	96.3	3.0
	Cefoperazone/sulbactam (2:1)	2	32	0.023 to >256	86.6	5.8
	Imipenem	0.5	1	0.047 to >256	98.3	1.3
Acinetobacter spp. (902)	Piperacillin	16	>256	0.016 to >256	85.4	13.3
	Ceftazidime	4	12	0.094 to >256	89.7	6.0
	Cefepime	3	16	0.032 to >256	85.9	7.0
	Cefpirome	2	24	0.047 to >256	85.7	8.6
	Cefoperazone/sulbactam (2:1)	2	6	0.023 to >256	98.2	0.8
	Imipenem	0.38	0.75	0.032 to >256	96.0	3.2
P. aeruginosa (996)	Piperacillin	8	>256	<0.016 to >256	84.0	15.5
	Ceftazidime	2	24	0.38 to >256	85.8	9.9
	Cefepime	4	32	0.064 to >256	73.7	11.2
	Cefpirome	6	128	0.064 to >256	65.7	19.1
	Cefoperazone/sulbactam (2:1)	8	128	0.19 to >256	72.9	14.9
	Imipenem	2	24	0.19 to >256	71.5	19.3

2.2. Antimicrobial susceptibility testing

Susceptibility testing of each isolate was determined by using Etest (AB Biodisk, Solna, Sweden) after the protocol described previously (Ishii et al., 2005; Lewis et al., 1999; Yamaguchi et al., 1999). Bacteria were cultured on a 90-mm-diameter Mueller–Hinton agar (Nippon Becton Dickinson) for 16 h at 35 °C. Isolated colonies were resuspended in sterile saline to obtain a turbidity of no. 0.5 McFarland. Each cell suspension was spread on a 135-mm-diameter Mueller–Hinton agar plate (Nippon Becton Dickinson) with a cotton swab, and the Etest strips were placed on the plates according to the manufacturer's instructions. The following strips were used: oxacillin (for Gram-positive bacteria), piperacillin (for Gram-negative bacteria), ceftazidime, cefepime, cefpirome, cefoperazone/sulbactam, and imipenem. Results were recorded after 16 to 20 h of incubation at 35 °C except for *S. aureus* and coagulase-negative staphylococci, for which incubation was for 24 h. MIC values were interpreted as the point of intersection of the inhibition ellipse with the Etest strips edge. All clinical laboratories used the same lot of Etest strips, Mueller–Hinton agar plates, and reference strains. Clinical and Laboratory Standards Institute does not have criteria (susceptible, intermediate, or resistant) regarding cefpirome and cefoperazone/sulbactam. As the expedient breakpoints, the same values for cefepime were used as criteria for cefpirome and the value for cefoperazone alone was used as criteria for cefoperazone/sulbactam. All 100 hospitals provided their results to the Department of Microbiology and Infectious Diseases, Toho University School of Medicine, for analysis. If uncertain data were found in the provided results, including identification and susceptibility testing, all tests were repeated. Identification and determination of MIC values were done using the Phoenix system (BD Diagnostic System, Sparks, MD) at Toho University School of Medicine.

2.3. Quality control

For quality control (QC) of the Etest strips, the following reference strains were used: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853 (CLSI, 2005). Clinical and Laboratory Standards Institute does not have the accuracy value of minimal inhibitory concentration regarding cefpirome and cefoperazone/sulbactam. In this study, an expedient range was determined near (±1 tube) the middle of the concentration for cefepime and cefoperazone/sulbactam, as done in our previous reports (Ishii et al., 2005). The laboratories were required to test a set of all organisms in a replicate manner.

3. Results

3.1. Quality assurance

Validity of generated data was assured by employing appropriate QC and quality assurance measures. Values obtained for the challenge set of strains resulted in 143 of the 6787 values falling out of the appropriate susceptibility category (2.1%). Of these 2.1%, 0.3% (n = 20 strains) were very major (false-susceptible) errors and 0.5% (n = 34 strains) were major (false-resistant) errors. Overall, this equates to 97.9% of MIC categoric results being acceptable.

3.2. Activity against staphylococci

Because the CLSI recommends that oxacillin-resistant staphylococci be considered as resistant to all β-lactam antibiotics, only oxacillin-susceptible strains were collected in this study. Of all tested, 992 isolates of *S. aureus* and 861 isolates of oxacillin-susceptible coagulase-negative staphylococci strains were susceptible to cefepime, cefpirome, cefoperazone/sulbactam, and imipenem (Table 2). However, 22 *S. aureus* (2.2%) and 23 oxacillin-susceptible coagulase-negative staphylococci (2.7%) were resistant to ceftazidime. The rank order of activity for all tested agents using MIC_{90} values was imipenem > oxacillin > cefpirome > cefoperazone/sulbactam > cefepime > ceftazidime.

3.3. Activity against E. coli and Klebsiella spp.

A total of 997 *E. coli* and 997 *Klebsiella* spp. isolates were tested. Generally, all agents tested except piperacillin (16.5% resistant) were highly active against *E. coli* and *Klebsiella* spp. (Table 2). No imipenem-resistant strains of *E. coli* and *Klebsiella* spp. were observed in this study.

3.4. Activity against other Enterobacteriaceae

Enterobacter spp. and *Citrobacter freundii* showed lower rates of susceptibility to piperacillin (76.1–78.2%), ceftazidime (80.1–81.8%), and cefoperazone/sulbactam (85.9–88.2%) compared with the other tested β-lactams (Table 2). Susceptibility rates for cefepime (96.9–97.4%) and imipenem (99.2–99.8%) were superior to cefpirome (93.3–96.2%). For the indole-positive *Proteus* spp., susceptibility rates of piperacillin (89.4%) and imipenem (98.0%) were lower than for other β-lactam antibiotics. *Serratia* spp. showed lower rates of susceptibility to piperacillin (80.5%) and cefoperazone/sulbactam (86.6%) compared with the other tested β-lactams (96.0–98.3%).

3.5. Activity against nonfermentative Gram-negative bacilli

For *Acinetobacter* spp., cefoperazone/sulbactam was the most active antibiotic (98.2% susceptible), followed by imipenem (96.0%), ceftazidime (89.7%), cefepime (85.9%), and cefpirome (85.7%). Piperacillin (85.4%) showed a lower susceptibility rate than the other tested β-lactams (Table 2).

The resistant rates of *P. aeruginosa* strains showed the highest value to all tested antibiotics except piperacillin and ceftazidime. The breakpoint of piperacillin for *P. aeruginosa* is 128 μg/mL. This value is higher compared with its breakpoint for other organisms.

4. Discussion

Etest is known for being a simple and reproducible method (Bolmstrom, 1993). With Etest, it is easy to maintain quality compared with other methods such as the disk diffusion method or the broth microdilution method. The same lot of Etest strips, QC strains, and Mueller–Hinton agar plates was used to determine the MIC values in all hospitals for this study. The QC assurance kept almost categoric accuracy. However, we should also point out that the results for cefpirome within QC ranges for *E. coli* ATCC 25922 were only 87.6%. In 2002, we also observed the same phenomena (Ishii et al., 2005). Maybe, it is easy for the inoculum effect of *E. coli* or for the condition of incubation to influence cefpirome in the Etest strip compared with other antibiotics.

All centers participating in this surveillance were not small-sized hospitals (average number of beds = 726), so results reflect large hospital data.

Imipenem kept antibiotic activity against Gram-positive and Gram-negative bacteria except for indole-positive *Proteus* spp., *Acinetobacter* spp., and *P. aeruginosa* (Table 2) compared with previous studies (Ishii et al., 2005; Lewis et al., 1999; Yamaguchi et al., 1999). Against *Acinetobacter* spp., the combination of cefoperazone and sulbactam had the most potent antimicrobial effect. Sulbactam is one of the effective antibiotics against carbapenem-resistant *A. baumannii* (Go et al., 1994). In 2002, the resistance ratio of *Acinetobacter* spp. to cefepime or cefpirome was 7.6% and 11.6%, respectively (Ishii et al., 2005). The present surveillance data show that the resistance ratio for cefepime and cefpirome are 7.0% and 8.6%, respectively. Multidrug-resistant *Acinetobacter* spp. isolates are becoming a problem in Europe (Marque et al., 2005). Fortunately, these data suggested that expanded-spectrum cephalosporin-resistant *Acinetobacter* spp. is not increasing in Japan until now.

Extended-spectrum β-lactamase (ESBL)–producing Enterobacteriaceae is well known as expanded-spectrum cephalosporin-resistant strains (Bradford, 2001; Jacoby and Munoz-Price, 2005). In this study, 4.5% (45 strains) of *E. coli* and 4.9% (49 strains) of *K. pneumoniae* show an MIC value of 2 μg/mL or more to ceftazidime, which suggests that they are ESBL producers according to the document of CLSI (2005). Among *Klebsiella* spp., 30 *K. pneumoniae* and 19 *Klebsiella oxytoca* isolates were possible ESBL producers according to the screening test. These *Klebsiella* spp. isolates were collected in 18 hospitals, and *E. coli* was isolated from 21 hospitals. Extended-spectrum β-lactamase producers were confirmed by the disk method. Because some *K. oxytoca* strains produce K1 enzyme, which behaves like an ESBL, and it is impossible to separate the K1 β-lactamase from an ESBL by a phenotypic test, all *K. oxytoca* isolates were omitted from the confirmatory test. Thirteen *E. coli* strains (1.3%) and 3 *K. pneumoniae* strains (0.3%) were confirmed as ESBL producers. These values are lower than those reported in the European Union, the United States, or Korea (Jacoby and Munoz-Price, 2005). One reason could be the different detection method for ESBL producers. In Japan, ceftazidime-hydrolyzing ESBL producers are uncommon in the clinical field (Kimura et al., 2004). On the other hand, cefotaxime-hydrolyzing ESBL producers, CTX-M–type β-lactamases, are dominant in clinical isolates or animals (Kojima et al., 2005; Yagi et al., 2000). Surveillance of ESBL producers should be preformed not only using ceftazidime but also other β-lactam antibiotics such as cefotaxime, cefpodoxime, ceftriaxone, or aztreonam as recommended by the document from CLSI (2005).

Class B β-lactamase–producing *P. aeruginosa* is resistant to most β-lactams except for monobactams (Jacoby and Munoz-Price, 2005; Walsh et al., 2005). Class B β-lactamases or metallo-enzymes require zinc for its enzymatic activity (Walsh et al., 2005). If zinc ions are removed by chelators such as ethylenediaminetetraacetic acid, 2-mercaptopropionic acid, or dipicolinic acid, class B enzymes will also lose their enzymatic activity (Arakawa et al., 2000). Fifty-four strains of *P. aeruginosa* (5.4%) were confirmed as MBL producers in this surveillance program by using imipenem and ceftazidime disk in the presence/absence of dipicolinic acid (Kimura et al., 2005b). *P. aeruginosa* (1.9%) produced MBL in 2002 (Kimura et al., 2005a). The present data suggest that MBL-producing *P. aeruginosa* is increasing in Japan. On the other hand, imipenem-resistant *P. aeruginosa* was present in 19.3% (192 isolates) of the isolates in this study. So, this result suggests that class B β-lactamase is not the main mechanism for carbapenem resistance in *P. aeruginosa*. Except for 7 strains of clonal isolates from 1 hospital, all imipenem-resistant *P. aeruginosa* isolated came from ubiquitous outbreaks.

Multidrug-resistant *P. aeruginosa* is a serious problem in the world (Tacconelli et al., 2002). They are resistant to carbapenems, fluoroquinolones, and aminoglycoside at the same time. We determined additional antibiotic susceptibility for imipenem-resistant *P. aeruginosa* by using the Phoenix system. Ninety-three isolates show resistance to levofloxacin and 15 isolates to amikacin (data not shown). All amikacin-resistant strains also showed resistance to levofloxacin, so the incidence of multidrug-resistant *P. aeruginosa* is 1.5% (15 strains). Because no effective and safe antibiotic for this kind of strains is available, continued surveillance for multidrug-resistant *P. aeruginosa* is necessary.

In conclusion, imipenem-resistant *P. aeruginosa* is increasing compared with previous data. The results of this study suggest that the participation of OprD is a major resistance mechanism against imipenem in *P. aeruginosa*. In contrast, susceptibility of *P. aeruginosa* to piperacillin has improved compared with previous data. Overall, cefepime is maintaining its antibiotic activity against Gram-positive and Gram-negative bacteria. Continuous surveillance is necessary to evaluate commercial antibiotics currently in use.

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