ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1996, p. 349–353 Vol. 40, No. 2
0066-4804/96/$04.00+0

Multifocal Outbreaks of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* Resistant to Broad-Spectrum β-Lactams, including Carbapenems

KAZUYOSHI SENDA,[1,2,3] YOSHICHIKA ARAKAWA,[1]* KAZUMITSU NAKASHIMA,[2,3] HIDEO ITO,[1] SATOSHI ICHIYAMA,[2,3] KAORU SHIMOKATA,[3] NOBUO KATO,[1] AND MICHIO OHTA[1]

Department of Bacteriology,[1] Department of Clinical Laboratory Medicine,[2] and First Department of Internal Medicine,[3] Nagoya University School of Medicine, Nagoya 466, Japan

Received 12 September 1995/Returned for modification 29 September 1995/Accepted 28 November 1995

A total of 3,700 *Pseudomonas aeruginosa* isolates were collected from 17 general hospitals in Japan from 1992 to 1994. Of these isolates, 132 carbapenem-resistant strains were subjected to DNA hybridization analysis with the metallo-β-lactamase gene (bla_{IMP})-specific probe. Fifteen strains carrying the metallo-β-lactamase gene were identified in five hospitals in different geographical areas. Three strains of *P. aeruginosa* demonstrated high-level imipenem resistance (MIC, ≥128 μg/ml), two strains exhibited low-level imipenem resistance (MIC, ≤4 μg/ml), and the rest of the strains were in between. These results revealed that the acquisition of a metallo-β-lactamase gene alone does not necessarily confer elevated resistance to carbapenems. In several strains, the metallo-β-lactamase gene was carried by large plasmids, and carbapenem resistance was transferred from *P. aeruginosa* to *Escherichia coli* by electroporation in association with the acquisition of the large plasmid. Southern hybridization analysis and genomic DNA fingerprinting profiles revealed different genetic backgrounds for these 15 isolates, although considerable similarity was observed for the strains isolated from the same hospital. These findings suggest that the metallo-β-lactamase-producing *P. aeruginosa* strains are not confined to a unique clonal lineage but proliferated multifocally by plasmid-mediated dissemination of the metallo-β-lactamase gene in strains of different genetic backgrounds. Thus, further proliferation of metallo-β-lactamase-producing strains with resistance to various β-lactams may well be inevitable in the future, which emphasizes the need for early recognition of metallo-β-lactamase-producing strains, rigorous infection control, and restricted clinical use of broad-spectrum β-lactams including carbapenems.

Pseudomonas aeruginosa is a clinically troublesome gram-negative pathogen that causes a wide range of opportunistic infections and nosocomial outbreaks. People whose specific or nonspecific defense systems have been impaired particularly tend to suffer from serious or fatal infections caused by this organism (12, 15). *P. aeruginosa* isolates generally demonstrate resistance to various antimicrobial agents. Their resistance to multiple antimicrobial agents is often associated with the production of specific enzymes, such as an inducible AmpC-type cephalosporinase (14); in addition, outer membrane permeability and active efflux systems can play important roles in limiting the access of antimicrobial agents to inner cell targets (13, 18, 19). Carbapenems are potent agents for chemotherapy of infectious diseases caused by *P. aeruginosa*, since the β-lactamases produced by this organism are generally ineffectual against carbapenems. However, a group of β-lactamases that hydrolyze carbapenems as well as other broad-spectrum β-lactams has been found in *Stenotrophomonas* (*Xanthomonas*) *maltophilia* (23), in several strains of *Aeromonas hydrophila* (27), and in *Bacteroides fragilis* (22, 34). These carbapenem-hydrolyzing enzymes were identified as metallo-β-lactamases belonging to Ambler's class B (1) or to the group 3 classified by Bush et al. (6). These enzymes are hardly blocked by suicide β-lactamase inhibitors, such as clavulanate, sulbactam, and tazobactam (7, 20). Recently, a novel metallo-β-lactamase, IMP-1, was identified from a clinical isolate of *Serratia marcescens* TN9106 in our laboratory (10, 21). Moreover, we found by Southern hybridization analysis that the genes responsible for carbapenem resistance in *P. aeruginosa* isolates were quite similar to the bla_{IMP} gene found in *S. marcescens* TN9106 (25, 26). Therefore, this study was undertaken to determine the distribution of *P. aeruginosa* with a sequence homologous to the gene for the IMP-1-type metallo-β-lactamase in Japan.

(This work was presented in part at the 19th International Congress of Chemotherapy, Montréal, Québec, Canada, 16 to 21 July 1995 [25], and the Fifth International Symposium on *Pseudomonas*, Tsukuba, Japan, 21 to 25 August 1995 [26].)

MATERIALS AND METHODS

Bacterial strains. A total of 3,700 clinical isolates of *P. aeruginosa* were collected from 17 general hospitals in different geographical areas in Japan from 1992 to 1994, and strains were stocked for a clinical surveillance study by the Working Group on Antimicrobial Resistance Survey in Japan. For the first study, 99 strains collected in 1992 which showed high-level resistance to both imipenem and ceftazidime (MIC, ≥32 μg/ml) were subjected to DNA hybridization analysis with a bla_{IMP}-specific probe. For the second study, 33 strains collected in 1993 and 1994 which showed high-level ceftazidime resistance (MIC, ≥128 μg/ml) were probed, since all the bla_{IMP}-positive strains detected in the first study belonged to this group.

Antibiotics. Antibiotics used in this study were provided by the following sources: ampicillin, Meiji-seika Kaisha, Ltd., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; cefsulodin, Takeda Chemical Industries, Ltd., Osaka, Japan; cefmenoxime, cefmetazole, and panipenem, Sankyo Co., Ltd., Tokyo, Japan; sulbactam-cefoperazone, Pfizer Pharmaceutical Inc., Tokyo, Japan; cefotaxime, Fabwerke Hoechst AG, Frankfurt, Germany; ceftazidime, Japan Glaxo Co., Tokyo, Japan; cephaloridine, moxalactam, and tobramycin, Shionogi and Co., Ltd., Osaka, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; meropenem, Sumitomo Pharmaceutical Ltd., Osaka, Japan; piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan; chloramphenicol, tetracycline, and trimethoprim, Sigma Chemical Co., St. Louis, Mo.; amikacin, Bristol-Meyers Squibb K. K., Tokyo, Japan; gentamicin, Schering-Plough K. K., Osaka, Japan;

* Corresponding author. Mailing address: Department of Bacteriology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan. Phone: 81 52 741 2111, ext. 2052. Fax: 81 52 731 9479.

ciprofloxacin, Bayer Yakuhin, Ltd., Osaka, Japan; norfloxacin, Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan; ofloxacin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; and rifampin, Nippon Ciba-Geigy Co., Ltd., Hyogo, Japan.

DNA manipulation and Southern hybridization analysis. Restriction endonucleases and DNA-labeling kits were supplied by Nippon Gene Co., Ltd. (Toyama, Japan). Plasmid pHIP29 carrying the DNA probe of bla_{IMP} was constructed as described previously (10). *Escherichia coli* JM109 carrying pHIP29 were cultured in Luria-Bertani (LB) broth supplemented with 30 μg of chloramphenicol per ml. The hybridization probe, the 0.5-kb *Hin*dIII-*Hin*dIII insert of pHIP29 containing an intergenic region of bla_{IMP}, was excised from a low-melting-point agarose gel (agarose L; Nippon Gene Co., Ltd.) after *Hin*dIII digestion and agarose gel electrophoresis. The gel block containing the DNA probes was melted, diluted, and labeled with [α-^{32}P]dCTP directly by using Klenow fragment in the labeling buffer of the DNA-labeling kit. Total DNA preparations were adsorbed onto nitrocellulose membrane filters (Schleicher and Schuell, Dassel, Germany). Plasmid DNA was prepared by the method of Kado and Liu (11) and blotted onto nylon membranes (Hybond-N; Amersham, Little Chalfont, Buckinghamshire, United Kingdom) after agarose gel electrophoresis by the method of Southern (30). DNA hybridization was performed under the high-stringency conditions that a sequence similarity higher than 60% would detect (3, 10).

Susceptibility testing. Antimicrobial susceptibility testing was performed by the agar dilution method according to the National Committee for Clinical Laboratory Standards document M7-A3 (17). Mueller-Hinton II agar and Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, Md.) were used for susceptibility testing.

Transfer of imipenem resistance. Conjugal transfer of imipenem resistance from *P. aeruginosa* MNA1428 and MNA1455 to *Escherichia coli* CSH2 (imipenem and ceftazidime MICs were both lower than 0.5 μg/ml) was performed as described elsewhere (10). Colonies of transconjugants were replica plated on agar supplemented with 2.0 μg of imipenem per ml to confirm transfer of imipenem resistance. Transfer of imipenem resistance by transformation was also performed. Plasmid DNA was prepared from *P. aeruginosa* MNA1428 and MNA1455 and introduced into *E. coli* HB101 (imipenem and ceftazidime MICs were both lower than 0.5 μg/ml) by electroporation (28). Transformants were isolated on LB agar supplemented with 8 μg of ceftazidime per ml. Plasmid DNA extracts from *P. aeruginosa* MNA1428 and MNA1455 were also incorporated into imipenem-susceptible *P. aeruginosa* MTB977 and MKA1143 by electroporation. Transformants were isolated on LB agar supplemented with 8 μg of imipenem per ml.

Chromosomal DNA analysis by PFGE. Chromosomal DNA was prepared from each strain for analysis by pulsed-field gel electrophoresis (PFGE) by the method of Smith and Cantor (29) and the modification of Ichiyama et al. (9), digested with *Spe*I for 18 h, and electrophoresed with a contour-clamped homogenous electric field system (Pulsaphor Plus; Pharmacia LKB Biotechnology, Uppsala, Sweden), as described previously (9). Electrophoresis was carried out at 180 V for 22 h, with pulse times ranging from 10 to 45 s.

RESULTS

Clinical associations of strains tested. Of the 3,700 strains collected, 1,533 strains (41.4%) were isolated from lower respiratory tracts, and 783 strains (21.2%) were isolated from urine specimens. Of the 3,700 strains, 679 strains (18.4%) demonstrated imipenem resistance (MIC, ≥16 μg/ml) according to NCCLS document M7-A3 (17), and 255 strains (6.9%) also exhibited resistance to ceftazidime (MIC, ≥32 μg/ml). The susceptibilities of each clinical isolate to imipenem and ceftazidime are plotted in Fig. 1. Most strains were isolated from patients given various combinations of antimicrobial agents including antipseudomonal cephems (cefsulodin or ceftazidime) and/or imipenem prior to identification.

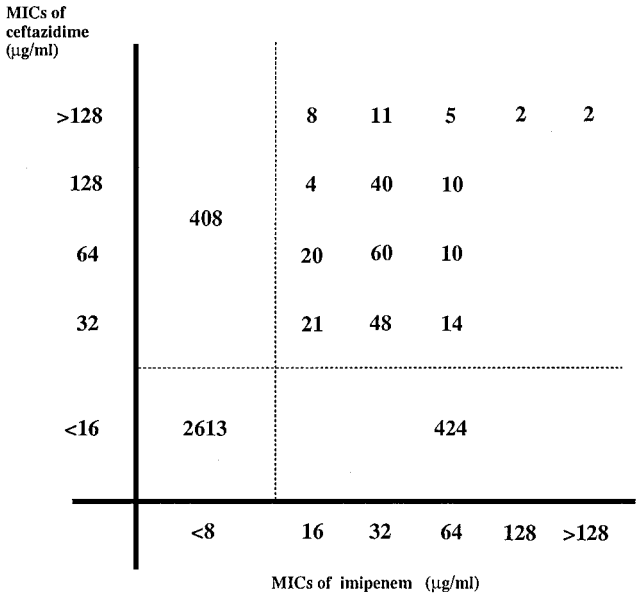

FIG. 1. Susceptibility plots of 3,700 clinical isolates. The susceptibilities to imipenem and ceftazidime for 3,700 strains isolated from 17 hospitals in different geographical areas in Japan are plotted. The broken lines indicate the breakpoints of resistance for both agents that were recommended by NCCLS document M7-A3 (17).

Dot blot analysis and susceptibility testing. The bla_{IMP}-specific DNA probes hybridized to 15 strains of 132 tested strains from 3,700 clinical isolates. Clinical associations of the 15 isolates are given in Table 1. Two apparent outbreaks were identified in Nagasaki and Kumamoto; 8 strains were isolated from a hospital in Nagasaki, and 3 strains were from a hospital in Kumamoto. Potential outbreaks were also suspected in Sapporo and Tokyo. All patients infected by these 15 strains were

TABLE 1. Clinical associations of *P. aeruginosa* strains carrying the metallo-β-lactamase gene

Strain	Hospital	Ward	Source	Collection date (yr. mo)
MNA1428	Nagasaki	Internal Medicine	Sputum sample	1992.6
MNA1455	Nagasaki	Surgery	Surgical wound	1992.2
MNA14102	Nagasaki	Urology	Urine sample	1992.1
MNA14141	Nagasaki	Gynecology	Urine sample	1992.8
MNA14115	Nagasaki	Internal Medicine	Urine sample	1992.10
MNA14109	Nagasaki	Urology	Urine sample	1992.6
MNA1443	Nagasaki	Surgery	Bile sample	1993.10
MNA1417	Nagasaki	Urology	Urine sample	1993.9
MKU1560	Kumamoto	Internal Medicine	Bile sample	1993.11
MKU1550	Kumamoto	Surgery	Bile sample	1993.10
MKU1563	Kumamoto	Internal Medicine	Bile sample	1993.11
MTA815	Tokyo A	Pediatrics	Sputum sample	1993.10
MTA854	Tokyo A	Internal Medicine	Sputum sample	1993.12
MTB9131	Tokyo B	Internal Medicine	Blood sample	1992.9
MSA137	Sapporo	Dermatology	Sputum sample	1993.12

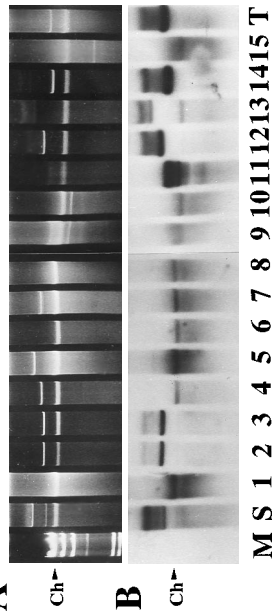

FIG. 2. Plasmid profiles and the bla_{IMP} probing patterns for 15 clinical isolates. (A) Plasmid profiles for the metallo-β-lactamase-producing *P. aeruginosa* strains. Plasmids were prepared from 15 clinical isolates producing metallo-β-lactamase and subjected to agarose gel electrophoresis. (B) Results of Southern hybridization analysis. The 0.5-kb *Hin*dIII fragment found in the coding region of the bla_{IMP} gene (10) was used as the DNA probe. Plasmid profiles for the 15 strains appear to be diverse, although a little similarity is observed among eight strains isolated from a hospital in Nagasaki (lanes 1 through 8). Lanes: M, *Hin*dIII-digested DNA markers; S, *S. marcescens* AK9373 (2, 10), which carries the bla_{IMP} metallo-β-lactamase gene; 1, strain MNA1428; 2, MNA1455; 3, MNA14102; 4, MNA14115; 5, MNA14141; 6, MNA14109; 7, MNA1443; 8, MNA1417; 9, MKU1560; 10, MKU1550; 11, MKU1563; 12, MTA815; 13, MTA854; 14, MTB9131; 15, MSA137; T, a transformant of *E. coli* HB101 that harbors a large plasmid containing the metallo-β-lactamase gene. A hybridization signal corresponding to the large plasmid transferred to *E. coli* HB101 was noted. Chromosomal DNA positions (Ch) are indicated by the arrowheads. The clinical association of each strain is given in Table 1.

[1]inpatients. Sapporo is located in the northernmost district of Japan, while Nagasaki and Kumamoto are located in the south district. The distance between Sapporo and Nagasaki is about 1,500 km. The results of susceptibility testing of 23 antimicrobial agents with the 15 bla_{IMP}-positive strains are shown in Table 2. All 15 strains demonstrated high-level resistance to all broad-spectrum cephems including ceftazidime (MIC, >128 μg/ml), though susceptibilities to carbapenems appeared to be diverse (Table 2). In particular, MNA1455 and MNA14102 demonstrated very high-level resistance to all antimicrobial agents, including quinolones and aminoglycosides.

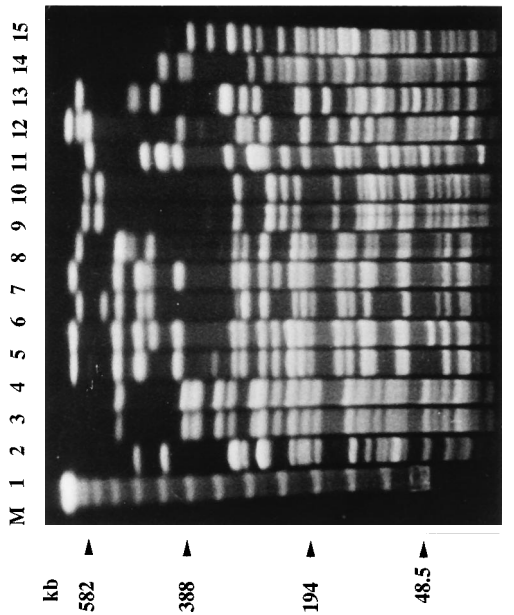

FIG. 3. DNA fingerprinting profiles for the 15 clinical isolates. Total DNA that contains both genomic and plasmid DNAs was prepared for each clinical isolate, digested with *Spe*I, and then subjected to pulsed-field gel electrophoresis (see text). Lanes: M, DNA molecular size markers; 1, strain MNA1428; 2, MNA1455; 3, MNA14102; 4, MNA14115; 5, MNA14141; 6, MNA14109; 7, MNA1443; 8, MNA1417; 9, MKU1560; 10, MKU1550; 11, MKU1563; 12, MTA815; 13, MTA854; 14, MTB9131; 15, MSA137.

TABLE 2. Susceptibilities of *P. aeruginosa* strains carrying the metallo-β-lactamase gene

Strain	MIC (μg/ml) of antimicrobial agent[a]																					
	IPM	PPM	MPM	ABPC	PIPC	CER	CMZ	CMX	CPZ-SBT	CTX	MOX	CFS	CAZ	AZT	NFLX	OFLX	CPFX	GM	TOB	AMK	TC	TMP	CP
MNA1428	128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	4	8	2	>128	>128	>128	64	>128	>128
MNA1455	>128	>128	>128	>128	128	>128	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	128	32	>128	>128	64	>128	>128
MNA14102	>128	>128	>128	>128	128	>128	>128	>128	>128	>128	>128	>128	>128	64	128	128	64	>128	>128	>128	64	>128	>128
MNA14115	32	64	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	16	64	64	32	>128	128	32	>128	>128	32
MNA14141	8	32	32	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	64	128	128	128	>128	32	64	>128	>128
MNA14109	8	32	8	>128	128	>128	>128	>128	>128	>128	>128	>128	>128	32	32	128	32	>128	>128	128	64	>128	32
MNA1443	8	32	64	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	>128	128	64	4	8	1	128	>128	>128
MNA1417	4	16	8	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	16	16	128	32	>128	128	32	64	32	32
MKU1560	2	4	16	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	0.5	0.5	0.06	8	16	1	8	32	32
MKU1550	16	16	>128	>128	128	>128	>128	>128	>128	>128	>128	>128	>128	64	1	4	1	32	32	4	32	>128	128
MKU1563	64	>128	>128	>128	32	>128	>128	>128	>128	>128	>128	>128	>128	64	1	4	2	4	4	8	64	>128	>128
MTA815	8	32	8	>128	32	>128	>128	>128	>128	>128	>128	>128	>128	4	0.5	2	0.25	>128	64	>128	64	>128	64
MTA854	16	32	64	>128	32	>128	>128	>128	>128	>128	>128	>128	>128	4	8	4	0.25	>128	16	128	64	>128	>128
MTB9131	32	>128	128	>128	32	>128	>128	>128	>128	>128	>128	>128	>128	32	2	8	1	>128	>128	32	128	>128	>128
MSA137	32	64	128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	64	1	2	0.5	>128	>128	16	>128	>128	128

[a] Abbreviations: IPM, imipenem; PPM, panipenem; MPM, meropenem; ABPC, ampicillin; PIPC, piperacillin; CER, cephaloridine; CMZ, cefmetazole; CMX, cefmenoxime; CPZ-SBT, cefoperazone-sulbactam; CTX, cefotaxime; MOX, moxalactam; CFS, cefsulodin; CAZ, ceftazidime; AZT, aztreonam; NFLX; norfloxacin; OFLX, ofloxacin; CPFX, ciprofloxacin; GM, gentamicin; TOB, tobramycin; AMK, amikacin; TC, tetracycline; TMP, trimethoprim; CP, chloramphenicol.

Genetic backgrounds of 15 metallo-β-lactamase producers. Plasmid profiles for the metallo-β-lactamase-producing strains appeared to be diverse, although some similarities were found among the strains isolated from the same hospital. Several strains harbored an apparent large plasmid(s), while others did not (Fig. 2A). In strains MNA1455, MNA14102, MKU1563, MTA815, MTA854, and MTB9131, the bla_{IMP}-specific DNA probes clearly hybridized to a large plasmid(s) (Fig. 2B). In the other strains, the same DNA probes also hybridized to the chromosomal DNA position, but resistance to carbapenem was transferred from these strains to *E. coli* and *P. aeruginosa* by electroporation in association with the acquisition of the large plasmid.

In hybridization of the bla_{IMP}-specific probe to *Hin*dIII-digested total DNA, the 0.5-kb *Hin*dIII fragment was generally observed among the 15 isolates, as was previously shown for the bla_{IMP} gene found in *S. marcescens* TN9106 (21). On the other hand, hybridization patterns after *Bam*HI or *Eco*RI digestion were more diverse (data not shown), suggesting different organization in the regions flanking the metallo-β-lactamase gene.

The DNA fingerprinting patterns of *Spe*I-digested total DNA preparations by PFGE for the 15 isolates (Fig. 3) were roughly classified into groups corresponding to the hospitals from which these strains were isolated. Moreover, the strains isolated from the same hospital tended to exhibit similar patterns, and the genetic similarity analysis with the computer-assisted, automated Dendron system (24) of these strains revealed clonal relatedness (data not shown).

Transfer of imipenem resistance. Conjugation failed to transfer imipenem resistance from *P. aeruginosa* MNA1428 and MNA1455 to *E. coli* CSH2, but transformation of *E. coli* HB101 by electroporation with plasmid DNA prepared from MNA1428 and MNA1455 was successful. These transformants showed resistance to 16 μg of ceftazidime per ml and 2.0 μg of imipenem per ml. Hybridization of bla_{IMP}-specific probe to a plasmid transferred from MNA1455 to HB101 is shown in Fig. 2B. Imipenem resistance of *P. aeruginosa* MNA1428 and MNA1455 was also transferred to imipenem-susceptible *P. aeruginosa* MTB977 and MKA1143 by electrotransformation. The MICs of imipenem for these strains with and without the plasmid were 32 and 4.0 μg/ml, respectively.

DISCUSSION

In this study, plasmid profiles, hybridization studies, and genomic fingerprinting analyses suggested that the metallo-β-lactamase-producing *P. aeruginosa* strains have heterogeneous genetic backgrounds, although the gene responsible for carbapenem resistance in each isolate seems identical or highly related to the bla_{IMP} gene found in *S. marcescens* TN9106 (21). We recently reported that the metallo-β-lactamase gene (bla_{IMP}) cassette of *S. marcescens* AK9373 (10) was mediated by a novel integron-like element carried by a transferable large plasmid (2). Hence, it was speculated that the bla_{IMP} gene cassette might be easily transposed into other plasmids or chromosomes. Actually, the GTTRRRY sequence, which was found in association with integrase-dependent recombination (8), was detected in the flanking regions of the bla_{IMP} genes of both *S. marcescens* TN9106 and *S. marcescens* AK9373 (2). Moreover, our unpublished observation obtained by PCR analyses suggested that several metallo-β-lactamase gene-positive *P. aeruginosa* isolates also had the integrase gene and/or the *aac(6′)-Ib* gene similar to those found in *S. marcescens* AK9373. From these findings, it is possible that the bla_{IMP}-like genes of *P. aeruginosa* have been translocated into various plasmids or chromosomes with the help of an integron or similar element and disseminated among the *P. aeruginosa* strains that possess different genetic backgrounds and that these strains have been proliferating multifocally in Japan.

All of the bla_{IMP} hybridization-positive 15 clinical isolates were resistant to penicillins and cephems, but the levels of resistance to carbapenems were diverse. For example, MKU1560 and MNA1417 exhibited low-level carbapenem resistance (MIC, ≤4 μg/ml). In our previous work, cryptic bla_{IMP} genes were also identified in several isolates of *S. marcescens* (10). On the other hand, in *P. aeruginosa*, imipenem proved to be capable of penetrating cells via an outer membrane protein, such as D2 porin, and the absence of these channels resulted in acquisition of moderate imipenem resistance (5, 33). Actually, several *P. aeruginosa* clinical isolates still demonstrated moderate carbapenem resistance (MIC, 8 μg/ml) without producing metallo-β-lactamase. Thus, the acquisition of a metallo-β-lactamase gene alone does not necessarily confer elevated resistance to carbapenems, and the secondary changes in regulatory systems of metallo-β-lactamase gene expression, outer membrane permeability, active efflux systems in bacterial membrane, and/or multiplication of structure gene may well be implicated in acquisition of high-level carbapenem resistance.

Transferable imipenem resistance has been documented in *P. aeruginosa* isolated from a hospital in Toyama, Japan several years ago (31), although the molecular structure of β-lactamase has not been characterized yet (16). Conjugal transfer of imipenem resistance has also been reported in *Bacteroides fragilis* (4), which produces a different type of metallo-β-lactamase from IMP-1. In those days, isolation of such organisms was regarded as only a local and an exceptional problem in Japan. Recently, however, a metallo-β-lactamase gene identical to the bla_{IMP} gene of *S. marcescens* was also detected from a clinical isolate of *Klebsiella pneumoniae* (32). In this study, we found at least five potential outbreaks of *P. aeruginosa* carrying metallo-β-lactamase genes in hospital from different geographical areas. Thus, the metallo-β-lactamase gene identical or similar to the bla_{IMP} gene may have been spreading in gram-negative rods in Japan. This emphasizes the necessity for early recognition of metallo-β-lactamase-producing isolates, rigorous infection control, and restricted clinical use of broad-spectrum β-lactams including carbapenems.

ACKNOWLEDGMENTS

We are grateful to Hidehiko Saito, First Department of Internal Medicine, Nagoya University School of Medicine, for his encouragement. We thank Keizo Yamaguchi (Head of the Working Group on Antimicrobial Resistance Survey in Japan) and Eizai Co., Ltd., for providing *P. aeruginosa* clinical isolates.

This work was supported by a research fund (grant No. 9014) from the Daiko Foundation.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. Lond. B Biol. Sci. **289:**321–331.
2. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39:** 1612–1615.
3. **Arakawa, Y., M. Ohta, N. Kido, M. Mori, H. Ito, T. Komatsu, and N. Kato.** 1989. Chromosomal β-lactamase of *Klebsiella oxytoca*, a new class A enzyme that hydrolyzes broad-spectrum β-lactam antibiotics. Antimicrob. Agents Chemother. **33:**63–70.
4. **Bandoh, K., K. Watanabe, Y. Muto, Y. Tanaka, N. Kato, and K. Ueno.** 1992. Conjugal transfer of imipenem resistance in *Bacteroides fragilis*. J. Antibiot. (Tokyo) **45:**542–547.
5. **Büscher, K. H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob. Agents Chemother. **31:**703–708.

6. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
7. **Bush, K., C. Macalintal, B. A. Rasmussen, V. J. Lee, and Y. Yang.** 1993. Kinetic interactions of tazobactam with β-lactamases from all major structural classes. Antimicrob. Agents Chemother. **37:**851–858.
8. **Hall, R. M., D. E. Brooker, and H. W. Stokes.** 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. Mol. Microbiol. **5:**1941–1959.
9. **Ichiyama, S., M. Ohta, K. Shimokata, N. Kato, and J. Takeuchi.** 1991. Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **29:**2690–2695.
10. **Ito, H., Y. Arakawa, S. Ohsuka, R. Wacharotayankun, N. Kato, and M. Ohta.** 1995. Plasmid-mediated dissemination of the metallo-β-lactamase gene bla_{IMP} among clinically isolated strains of *Serratia marcescens*. Antimicrob. Agents Chemother. **39:**824–829.
11. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
12. **Koch, C., and N. Høiby.** 1993. Pathogenesis of cystic fibrosis. Lancet **341:** 1065–1069.
13. **Livermore, D. M.** 1992. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2046–2048.
14. **Lodge, J. M., S. D. Minchin, L. J. V. Piddock, and S. J. W. Busby.** 1990. Cloning, sequence and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal ampC β-lactamase. Biochem. J. **272:**627–631.
15. **Lowbury, E. J. L., B. T. Thom, B. T. Lilly, J. R. Babb, and K. Whittall.** 1970. Sources of infection with *Pseudomonas aeruginosa* in patients with tracheostomy. J. Med. Microbiol. **3:**39–56.
16. **Minami, S., H. Araki, T. Yasuda, M. Akama, S. Iyobe, and S. Mitsuhashi.** 1993. High-level imipenem resistance associated with imipenem-hydrolysing beta-lactamase production and outer membrane alteration in a clinical isolate of *Pseudomonas aeruginosa*. Int. J. Exp. Clin. Chemother. **6:**21–28.
17. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically, 2nd ed. Approved standard. NCCLS document M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
18. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
19. **Nikaido, H., K. Nikaido, and S. Harayama.** 1991. Identification and characterization of porins in *Pseudomonas aeruginosa*. J. Biol. Chem. **266:**770–779.
20. **Ohsuka, S., Y. Arakawa, T. Horii, H. Ito, and M. Ohta.** 1995. Effect of pH on activities of novel β-lactamases and β-lactamase inhibitors against these β-lactamases. Antimicrob. Agents Chemother. **39:**1856–1858.
21. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
22. **Rasmussen, B. A., Y. Gluzman, and F. P. Tally.** 1990. Cloning and sequencing of the class B β-lactamase gene (*ccrA*) from *Bacteroides fragilis* TAL3636. Antimicrob. Agents Chemother. **34:**1590–1592.
23. **Saino, Y., F. Kobayashi, M. Inoue, and S. Mitsuhashi.** 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. **22:**564–570.
24. **Schmid, J., E. Voss, and D. R. Soll.** 1990. Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. J. Clin. Microbiol. **28:**1236–1243.
25. **Senda, K., Y. Arakawa, S. Ichiyama, H. Ito, K. Nakashima, K. Shimokata, and M. Ohta.** 1995. Multifocal proliferation of carbapenem resistant *Pseudomonas aeruginosa* producing plasmid-mediated metallo-β-lactamase. Can. J. Infect. Dis. **6:**335C. (Abstract 1242.)
26. **Senda, K., Y. Arakawa, S. Ichiyama, H. Ito, K. Nakashima, K. Shimokata, and M. Ohta.** 1995. Multifocal outbreaks of metallo-β-lactamase producing *Pseudomonas aeruginosa* that shows consistent resistance to broad-spectrum β-lactams including carbapenems, abstr. L-3, p. 170. *In* Program and book of abstracts of *Pseudomonas* 1995. Fifth International Symposium on *Pseudomonas*: Biotechnology and Molecular Biology.
27. **Shannon, K., A. King, and I. Phillips.** 1986. β-Lactamases with high activity against imipenem and Sch 34343 from *Aeromonas hydrophila*. J. Antimicrob. Chemother. **17:**45–50.
28. **Sheen, J.** 1987. High-efficiency transformation by electroporation, p. 1.8.4–1.8.6. *In* F. E. Ausubel, R. Brent, R. E. Kingston, D. D. Moor, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
29. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. **155:**449–467.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
31. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**147–151.
32. **Yamaguchi, H., M. Nukaga, and T. Sawai.** 1994. Appearance of an R plasmid mediated metallo-β-lactamase in gram-negative enteric bacteria. DNA Data Base of Japan (DDBJ) entry name KPNRD4 and accession no. D29636.
33. **Yoneyama, H., and T. Nakae.** 1993. Mechanism of efficient elimination of porin D2 in outer membrane of imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**2385–2390.
34. **Yotsuji, A., S. Minami, M. Inoue, and S. Mitsuhashi.** 1983. Properties of novel β-lactamase produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **24:**925–929.